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Diverse signalling by different chemokines through the chemokine receptor CCR5

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Abbreviations:
CHO cells, Chinese hamster ovary cells
ECL, enhanced chemiluminescence
FCS, foetal calf serum
FITC, fluorescein isothiocyanate
GPCR, G protein-coupled receptor
HIV, human immunodeficiency virus
MCP, monocyte chemoattractant protein
MIP, macrophage inflammatory protein
PBS, phosphate-buffered saline
RANTES, regulated on activation, normal T cell expressed and secreted

ABSTRACT

We have investigated the signalling properties of the chemokine receptor, CCR5, using several assays for agonism: stimulation of changes in intracellular Ca^{2+} or CCR5 internalisation in CHO cells expressing CCR5 or stimulation of [^{35}S]GTPγS binding in membranes of CHO cells expressing CCR5.

Four isoforms of the chemokine CCL3 with different amino termini (CCL3, CCL3(2–70), CCL3(5–70), CCL3L1) were tested in these assays in order to probe structure/activity relationships. Each isoform exhibited agonism. The pattern of agonism (potency, maximal effect) was different in the three assays, although the rank order was the same with CCL3L1 being the most potent and efficacious. The data show that the amino terminus of the chemokine is important for signalling. A proline at position 2 (CCL3L1) provides for high potency and efficacy but the isoform with a serine at position 2 (CCL3(2–70)) is as efficacious in some assays showing that the proline is not the only determinant of high efficacy.

We also increased the sensitivity of CCR5 signalling by treating cells with sodium butyrate, thus increasing the receptor/G protein ratio. This allowed the detection of a change in intracellular Ca^{2+} after treatment with CCL7 and Met-RANTES showing that these ligands possess measurable but low efficacy.

This study therefore shows that sodium butyrate treatment increases the sensitivity of signalling assays and enables the detection of efficacy in ligands previously considered as antagonists. The use of different assay systems, therefore, provides different estimates of efficacy for some ligands at this receptor.

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1. Introduction

The G protein-coupled chemokine receptor, CCR5, was originally characterised as a receptor responding functionally to the CC-chemokines CCL3 (MIP-1α), CCL4 (MIP-1β) and CCL5 (RANTES) [1]. CCR5 was subsequently described as the primary co-receptor for macrophage tropic human immunodeficiency virus type 1 (HIV-1) [2–5].

Chemokine interaction with CCR5 initiates several events. Receptor associates with G proteins, leading to activation of signalling processes, e.g. changes in Ca^{2+} followed by receptor phosphorylation by G protein-coupled receptor kinases (GRKs) [6]. This results in association of β-arrestin with the receptor [7,8] and desensitisation via uncoupling of receptor and G protein. A number of CC-chemokines (CCL3, CCL4, CCL5, CCL7, CCL8 and CCL13) bind to CCR5 with different affinities and abilities to activate the receptor [9]. These chemokines can be divided in two subgroups based on amino acid sequence identity [10,11]. CCL3, CCL4 and CCL5 form one subgroup and are full agonists, whereas CCL7, CCL8 and CCL13 form a second subgroup, which share ~60% amino acid identity within the group and ~30% identity with CCL3, CCL4 and CCL5. CCL7 has been reported to bind to CCR5 but cannot activate the receptor in a number of tests [8,9]. CCL8 and CCL13 are partial agonists in some tests, with CCL13 exhibiting a reduced potency. Chemically modified chemokines have been used to study ligand–receptor interaction further. AOP-RANTES [12,13] and AOP-MIP-1α [14] are very potent ligands in several assays. Met-RANTES was described as an antagonist of several signalling processes linked to CCR5 [15]. Subsequently, Met-RANTES was shown to have weak activity in eliciting a Ca^{2+} response in Chinese hamster ovary cells (CHO cells) expressing CCR1, CCR3, and CCR5 [16] but no intrinsic activity on Ca^{2+} transients in eosinophils [17].

Two non-allelic isoforms of CCL3 containing 70 amino acids have been described and termed CCL3 and CCL3L1 (Fig. 1) [18]. These isoforms exhibit >90% homology, differing by only three amino acids. Both forms are transcribed and secreted from mammalian cells [19–22]. The mature form of CCL3 (CCL3(5–70)) lacks the four N-terminal amino acids [21,22]. CCL3L1 has been reported to be much more potent for binding to and activating CCR5 than the naturally occurring CCL3 variants [18,23]. These differences have been attributed to the change of the serine to a proline at position 2. Another CCL3 variant has been tested where the Ala at position 1 is lacking (CCL3(2–70)). This CCL3 variant has been used to create a mutant chemokine, where a D27A mutation reduces self-association to form high-molecular mass aggregates [24]. These CCL3 isoforms (CCL3, CCL3L1, CCL3(5–70), CCL3(2–70), CCL3(2–70)D27A), therefore, provide an interesting group of molecules to probe the properties of CCR5 and the relation of structure and function for the chemokine.

As outlined above, some chemokines, e.g. Met-RANTES, CCL13 exhibit low efficacy in some systems but no efficacy in others. It is important to define the relative efficacy of such compounds and this may require increasing the sensitivity of currently used assay systems. Sodium butyrate has been shown to activate viral promoters and enhance protein expression in stably transfected cells, when a viral promoter is used to express the protein [25]. We showed that sodium butyrate increases cell surface CCR5 in stably transfected CHO cells [8]. Here we used sodium butyrate treatment of cells to increase the number of receptors and enhance the activation of CCR5. The data show that sodium butyrate treatment is a useful tool for investigating signalling events induced by low efficacy agonists.

In the present study, therefore, we employed three assays for agonists at CCR5: stimulation of changes in intracellular Ca^{2+}, stimulation of [^{35}S]GTPγS-binding and receptor internalisation assays [8]. These assays were used to examine the CCL3 isoforms mentioned above. Also a range of chemokines including some low efficacy chemokines (Met-RANTES, CCL13) were examined for their activity in membranes of CHO cells expressing CCR5 treated with sodium butyrate.

2. Materials and methods

2.1. Cells and materials

CHO cells stably expressing CCR5 (CHO·CCR5) or CCR5 and CD4 (CHO·CCR5·CD4) were grown as described previously [7,8]. For treatment with sodium butyrate, cells were incubated for 18 h with 5 mM or 10 mM sodium butyrate prior to assaying.

CCL3, CCL4, CCL5, CCL7, CCL8, CCL13 and MetRANTES were purchased from PeproTech (Rocky Hill, NJ), CCL3(5–70) was from R&D Systems (Abingdon Oxon, UK), the CCL3(2–70) and CCL3(2–70)D27A chemokines were a generous gift from British Biotech (Oxford, UK) and described previously [24]. Secondary antibodies were obtained from Sigma (Poole, UK). Anti-CCR5 antibodies HEK/1/85a/7a have been described previously [7,8], anti-$G\alpha i_{1-3}$ protein antibody was obtained from Santa Cruz (Holy Ditch Farm, UK). All other chemicals were from Sigma (Poole, UK).

2.2. Internalisation assay and flow cytometry analysis

CHO·CCR5 and CHO·CCR5·CD4 cells were incubated with serum-free medium for 2 h at 37 °C, harvested with 2 mM

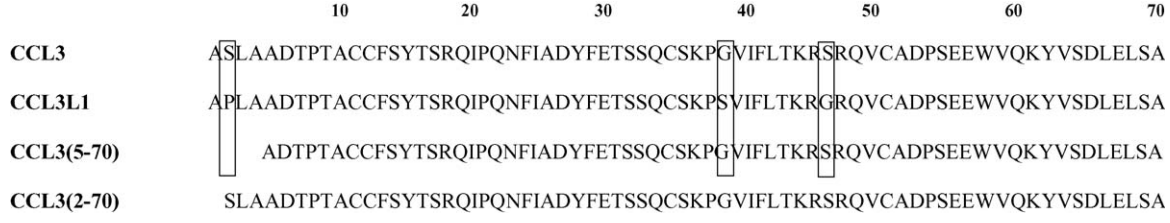

Fig. 1 – Amino acid sequences of the four CCL3 isoforms used. The positions of amino acid residues (2, 39 and 47) where changes occur in the different isoforms are boxed.

EDTA/PBS and then resuspended in medium without serum at 5×10^6 cells/ml. Cells were then incubated with chemokines (50–100 nM) for 1 h at 37 °C, and washed in ice cold PBS containing 1% FCS and 1% NaN_3 for flow cytometry analysis. Cell surface expressed CCR5 was detected by flow cytometry using anti-CCR5 antibody HEK/1/85a/7a and fluorescein isothiocyanate (FITC) 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 FITC-labelled anti-rat IgG. Samples were quantified on a FACScan and data analysed with CellQuest software version 3.1 (Becton Dickinson, San Jose, CA). For each experiment, for example when examining the effects of a chemokine on cell surface CCR5, the relative CCR5 surface expression (%) was calculated as 100 × [mean channel of fluorescence (chemokine treated cells) − mean channel of fluorescence (negative control cells)]/[mean channel of fluorescence (cells treated with medium without serum) − mean channel of fluorescence (negative control cells)]. CHO cells not expressing CCR5, as well as irrelevant monoclonal antibodies were used for negative controls with similar results.

2.3. SDS-polyacrylamide gel electrophoresis (PAGE) and Western blot

Membranes were prepared as described and samples were boiled in 2× sample buffer (75 mM Tris–HCl, pH 6.8, 6% SDS, 10% mercaptoethanol, 10% glycerol). 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, UK).

2.4. Membrane preparation

Membranes were prepared from confluent cells. Cells were harvested in ice-cold HEPES buffer (20 mM HEPES, 6 mM $MgCl_2$, 1 mM EDTA and 1 mM EGTA, pH 7.4) and homogenized with an ultra-turrax (4 × 4 s). The cell debris was collected by centrifugation for 10 min at 1700 × *g*, the resulting supernatant was centrifuged for 1 h at 48,000 × *g* at 4 °C and resuspended in HEPES buffer, aliquoted and stored at −70 °C. Protein levels were determined using the method of [26] with bovine serum albumin as a standard.

2.5. [^{35}S]GTPγS-binding assays

The [^{35}S]GTPγS-binding assays were carried out essentially as described [8]. Cell membranes (30 μg) were incubated in triplicate in [^{35}S]GTPγS-binding buffer containing 20 mM HEPES, 100 mM NaCl, 10 mM $MgCl_2$ and 0.1% BSA, pH 7.4 and 10 μM GDP 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 pre-incubation 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 3 ml of ice-cold PBS using a Brandel cell harvester (Gaithersburg, MD). 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.

2.6. Analysis of intracellular calcium ion concentration

Cells were harvested with 2 mM EDTA/PBS and washed twice in buffer (148 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 10 mM Hepes, 1 mM glucose, 2.5 mM probenecid, 0.1% BSA, pH 7.4) and incubated with 4 μM Fura-2 AM (Molecular Probes, Eugene, Oregon) at 37 °C. After washing cells with buffer, cells were resuspended at 2×10^6 cells ml^{-1} of buffer. Chemokine-induced intracellular calcium mobilisation was determined by spectrofluorometry using a Perkin-Elmer LS50 or a BMG-labtech Fluostar OPTIMA fluorometer. The peak values of intracellular calcium ion concentration following the chemokine challenge were determined as described in [27].

2.7. Data analysis

Data were analysed using GraphPad Prism (GraphPad Software, San Diego, CA). Concentration/response curves for CCL3(5–70), CCL3L1, CCL4, CCL5 in [^{35}S]GTPγS-binding assays were fitted well by models assuming a Hill coefficient of 1 and so data with other chemokines were analysed similarly. Statistical analyses were performed using Student's *t*-test with $P < 0.05$ or using one-way ANOVA with Bonferroni's multiple comparison as a post-test. Data represent the mean ± S.E.M of at least three independent experiments.

3. Results

3.1. Assessment of functional activities of CCL3 isoforms at CCR5

Here we examined the ability of four CCL3 isoforms (CCL3, CCL3L1, CCL3(5–70) and CCL3(2–70)) to activate CCR5 using a [^{35}S]GTPγS-binding assay, an intracellular Ca^{2+} assay and a CCR5 internalisation assay. These activities were assessed using CHO cells stably expressing CCR5 (CHO·CCR5) or co-expressing CCR5 and CD4 (CHO·CCR5·CD4). Expression levels of CCR5 and CD4, respectively, were monitored with flow cytometry [8]. CCL3(2–70)(D27A) was also examined in some tests and found to have similar activity to CCL3(2–70) (data not shown).

To determine ligand-induced internalisation, cells were incubated in serum-free medium and treated with CCL3 isoforms (50 nM) for 1 h and cell surface CCR5 expression was determined using flow cytometry with an anti-CCR5 antibody (Fig. 2). All four isoforms tested caused internalisation of CCR5, but CCL3(2–70), CCL3(5–70) and CCL3L1 lead to more internalisation in CHO·CCR5 and CHO·CCR5·CD4 cells than CCL3

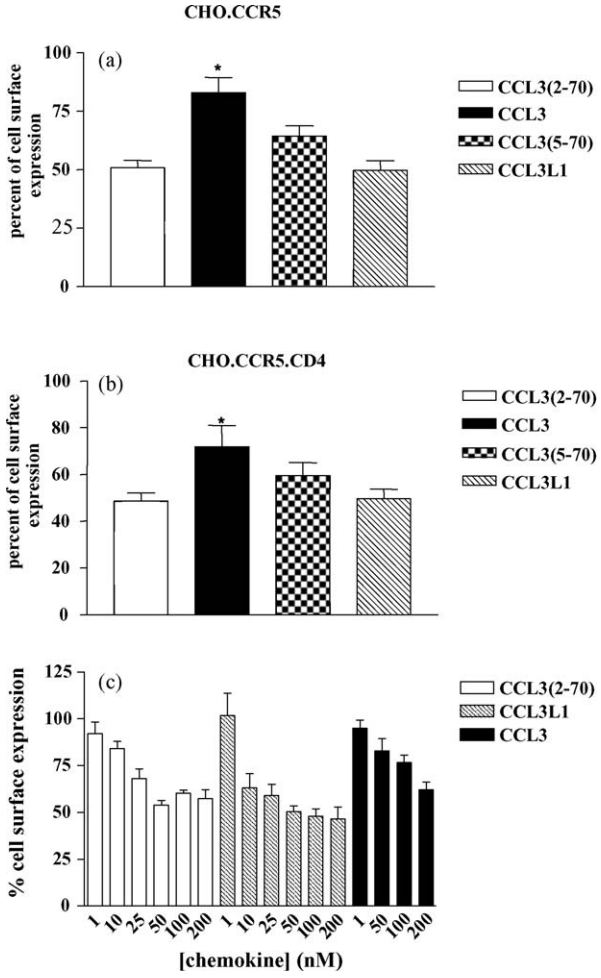

Fig. 2 – Internalisation of CCR5. CHO·CCR5 cells (a) or CHO·CCR5·CD4 cells (b) were incubated with chemokines (50 nM) for 1 h. Internalisation of CCR5 was determined using flow cytometry as described in Section 2. In panel (c), CCR5 internalisation was determined in CHO·CCR5 cells using different concentrations of chemokines as indicated. Data represent the means ± S.E.M. from at least three independent experiments (*P < 0.05 relative to CCL3(2–70)).

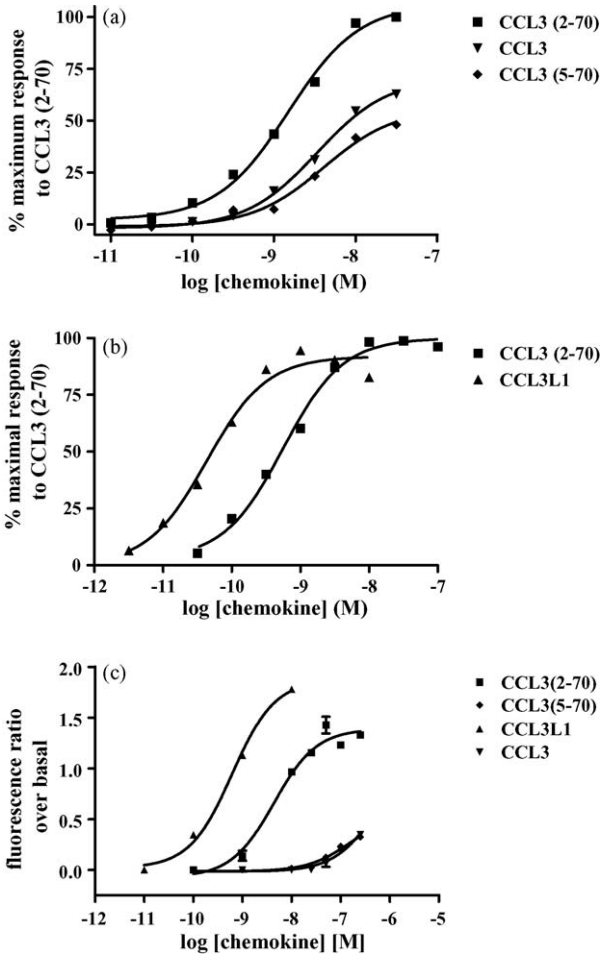

Fig. 3 – Stimulation of [^{35}S]GTPγS binding in membranes of CHO·CCR5 cells and changes in intracellular Ca^{2+} in CHO·CCR5 cells by CCL3 isoforms. [^{35}S]GTPγS binding was determined after 30 min incubation in the presence of increasing concentrations of each agent as described in Section 2. Data shown are the means of triplicate determinations from a representative of at least three separate experiments. Summary data are provided in Table 1. (a) Shows stimulation by CCL3(2–70), CCL3 and CCL3(5–70). (b) Shows stimulation by CCL3(2–70) and CCL3L1. (c) The intracellular calcium ion concentration was determined in CHO·CCR5 cells following stimulation by chemokines as described in Section 2. Data are expressed as fluorescence ratio over basal and are from at least three independent experiments.

($P < 0.05$). When a range of chemokine concentrations was used in these assays, it was found that different chemokines gave similar maximal responses in the internalisation assay but with different potencies.

Membranes of CHO·CCR5 cells were used to determine the activation of heterotrimeric G proteins using a [^{35}S]GTPγS-binding assay. In this assay CCL3(2–70) and CCL3L1 give similar maximal responses and are taken to be full agonists, whereas CCL3 and CCL3(5–70) act as partial agonists ($P < 0.05$) (Fig. 3 and Table 1). CCL3L1 is the most potent isoform ($P < 0.05$), whereas CCL3(5–70) and CCL3 have similar potency to one another ($P > 0.05$) (Fig. 3 and Table 1). CCL3(2–70) is more potent than CCL3L1 and CCL3 ($P < 0.05$) but less potent than CCL3L1 ($P < 0.05$). Similar data were obtained for CCL3(2–70), CCL3L1 and CCL3 using membranes from CHO·CCR5·CD4 cells (data not shown) indicating that CD4 does not affect signalling through CCR5.

The change in intracellular Ca^{2+} in response to the CCL3 isoforms was determined in CHO·CCR5 cells (Fig. 3). CCL3L1 is more potent than CCL3(2–70) for stimulating intracellular Ca^{2+} release although both stimulate similar maximal responses, whereas CCL3(5–70) and CCL3 elicit much smaller responses.

3.2. *Increasing sensitivity for detecting low efficacy agonists at CCR5*

We also examined how the CCR5 signalling system could be made more sensitive for the detection of very low efficacy

Table 1 – Stimulation of [^{35}S]GTPγS binding

Ligand	pEC50 ± S.E.M.	EC_{50} (nM)	Relative efficacy (% of CCL3(2-70))	*n*
CCL3(2-70)	9.17 ± 0.18	0.67	100 ± 4.7	4
CCL3L1	10.40 ± 0.06	0.04	92.7 ± 9.9	4
CCL3	8.46 ± 0.17	3.4	67.9 ± 3.3*	4
CCL3(5-70)	8.54 ± 0.07	2.9	69.7 ± 10.0*	3

Stimulation of [^{35}S]GTPγS binding was determined in membranes from CHO.CCR5 cells as described in Section 2. Data were analysed to provide the EC_{50} and the maximal response, expressed as a percentage of that given by maximally stimulating concentrations of CCL3(2–70). The pEC_{50} of CCL3(2–70) is significantly different from that for CCL3 and CCL3(5–70) ($P < 0.05$), but all three differ from CCL3L1 ($P < 0.05$). The efficacy of CCL3 and CCL3(5–70) are significantly different from that for CCL3(2–70) (*$P < 0.05$). Statistical analysis was done using a one-way ANOVA with Bonferroni's multiple comparison as a post-test.

agonists. We, therefore, used sodium butyrate to increase the level of CCR5 expression in stably transfected CHO cells [8] in order to see whether increasing the expression level leads to an increase of the response to agonists. Under these conditions levels of CD4 are also increased [8].

We treated CCR5-expressing CHO cells with varying concentrations of sodium butyrate and investigated the changes in expression of CCR5 and activation of CCR5 by various ligands. We have already shown that sodium butyrate treatment can increase the number of receptors on the cell surface of CHO·CCR5·CD4 cells [8]. Here we used a CHO·CCR5 cell line which even in an untreated state, expresses CCR5 to a higher level than the CHO·CCR5·CD4 cell line [8]. Treatment of these cells with 5 mM or 10 mM sodium butyrate for 18 h increased receptor number on the cell surface as determined with flow cytometry and Western blot analysis (Fig. 4). Butyrate treatment over the period of the experiment did not affect cell viability, the number of dead cells or the cell shape so that there was no increase in apoptosis. We also estimated levels of inhibitory G proteins using an antibody specific for the α subunit of G_{i1-3}, but we were unable to detect any change in $G\alpha i_{1-3}$ protein levels in treated cells (Fig. 4). Western blot and flow cytometry data suggest at least a two-fold increase in receptor expression in the cells treated with 10 mM sodium butyrate after the 18 h treatment, so that the R/G ratio increases about two fold. After establishing

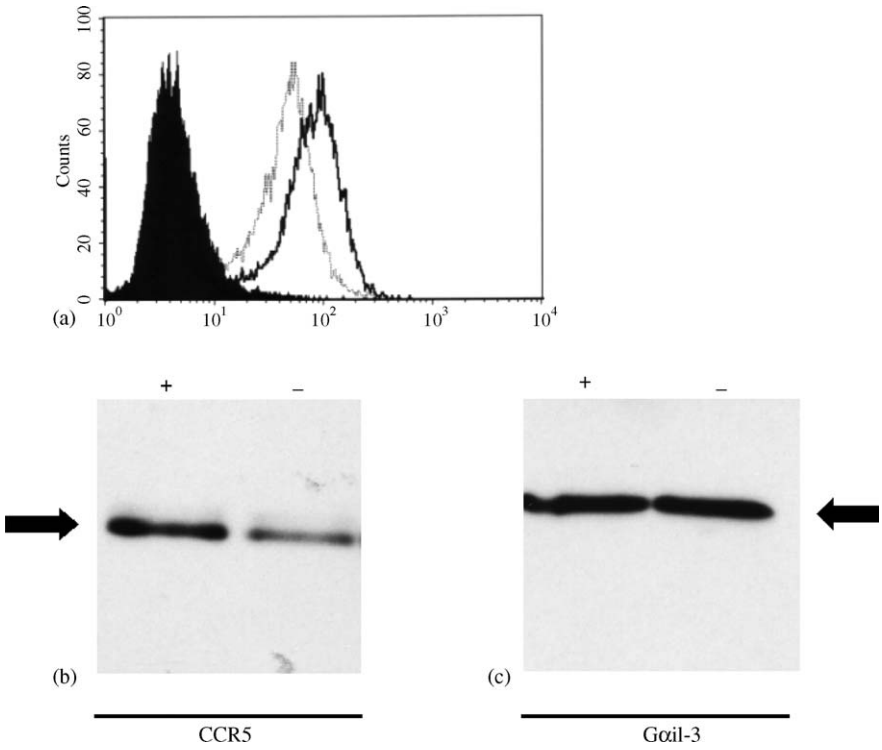

Fig. 4 – Expression levels of CCR5 and $G\alpha i_{(1-3)}$ in CHO.CCR5 cells: effect of treatment with sodium butyrate. (a) CCR5 was detected using flow cytometry in control cells and sodium butyrate-treated cells as described in Section 2; black shading shows stain with an unspecific control antibody, grey line shows control cells, black line shows sodium butyrate-treated cells. (b) CCR5 was determined in membranes from control and sodium butyrate-treated cells using western blots as described in Section 2. (c) Levels of $G\alpha i_{1-3}$ were determined in membranes from control and sodium butyrate-treated cells using western blots as described in Section 2. In (b) and (c) care was taken to load equal amounts of protein in to the control and butyrate-treated lanes. Experiments with different loading levels in both lanes showed that the blots were not saturated. Data shown are representative experiments repeated with similar results at least three times.

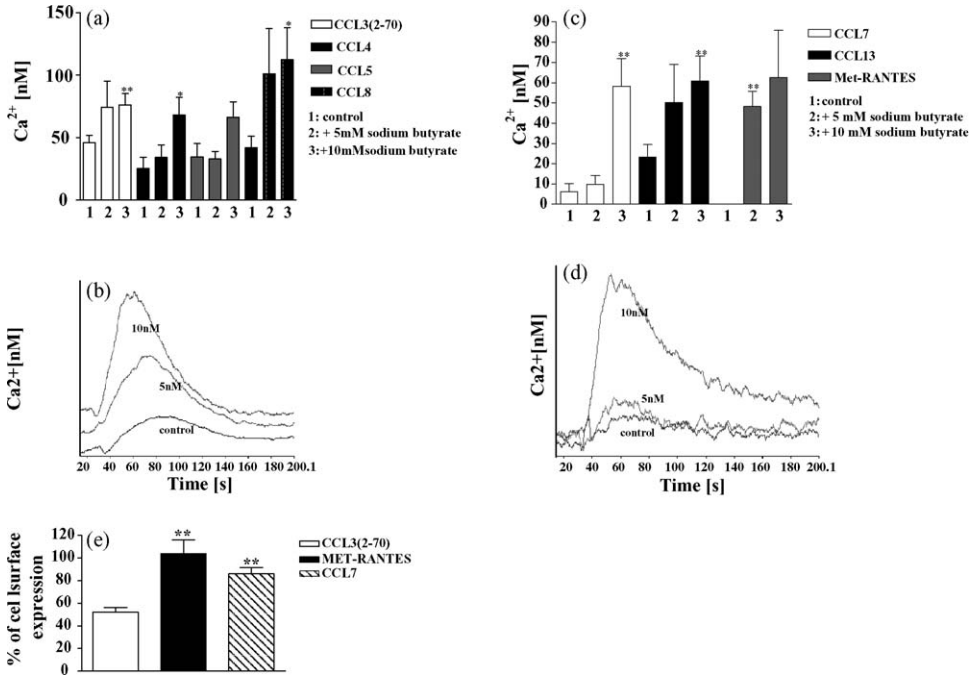

Fig. 5 – Effect of sodium butyrate treatment on chemokine regulation of intracellular calcium ion concentration and chemokine-induced internalisation of CCR5 in CHO·CCR5 cells. (a) and (c) CHO·CCR5 cells were treated with sodium butyrate as described and intracellular calcium ion concentrations were determined after stimulation of CCR5 with the indicated chemokines (10^{-8} M) as described in Section 2. Data are expressed as mean ± S.E.M. and represent at least three independent experiments ($^{*}P < 0.05$; $^{**}P < 0.001$), (b) shows a representative result obtained with CCL3(2–70) in control cells, 5 mM sodium butyrate and 10 mM sodium butyrate-treated cells (c) [see (a)]. (d) Shows a representative calcium trace for CCL13. Control cells show hardly any change in intracellular Ca^{2+}, whereas sodium butyrate-treated cells show an increased signal. (e) Internalisation of CCR5 was induced by treatment of control cells with chemokines as described in Section 2. Data correspond to mean ± S.E.M. from triplicate determinations and are representative of three independent experiments, ($^{**}P < 0.001$; ANOVA with Bonferroni's post-test, Met-RANTES and CCL7 are significantly different from CCL3(2–70) and do not induce internalisation).

the increase in receptor expression levels, we investigated how this altered R/G ratio influences chemokine-induced signalling.

We, therefore, used these treated cells in intracellular Ca^{2+} assays with a range of chemokines. In sodium butyrate-treated cells we detected a higher signal than in untreated cells with all chemokines tested (Fig. 5). Both CCL7 and Met RANTES have been described as antagonists for CCR5 [9,16] with no effect or only a very weak effect on intracellular calcium release. In agreement with these findings, we were unable to see CCL7 or Met-RANTES induced stimulation of Ca^{2+} via CCR5 in untreated cells (Fig. 5, [8]). In sodium butyrate-treated cells, however, we were able to detect a specific signal for CCL7 and Met-RANTES (Fig. 5). These data suggest that sodium butyrate treatment improves the sensitivity of CCR5 signalling in intracellular Ca^{2+} assays.

We also tested CCL7 and Met-RANTES for effects on internalisation of CCR5 in control and sodium butyrate-treated cells. Whereas CCL3(2–70) was able to elicit strong internalisation of CCR5, CCL7 and Met-RANTES did not induce significant internalisation of CCR5 (Fig. 5). In this assay, therefore, these chemokines are acting as antagonists.

We investigated the influence of sodium butyrate treatment on agonist responses in [^{35}S]GTPγS-binding assays. Cells were treated with sodium butyrate for 18 h and membranes were isolated from treated cells and control cells and used in [^{35}S]GTPγS-binding assays. We were able to see a measurable increase in response for CCL13 after treatment with sodium butyrate (Fig. 6), whereas the other chemokines tested did not show an increase in maximal response (Table 2, Fig. 6). Furthermore, we detected an increase in potency after treatment with 10 mM sodium butyrate for CCL3(2–70) (Table 2, Fig. 6) and for 5 mM and 10 mM sodium butyrate treatment for CCL5 (Table 2), whereas we did not see a significant increase in the potency of CCL4 or CCL8. The potency of CCL13 could not be determined accurately as CCL13 is a very weak agonist, so we could not determine the effects of sodium butyrate treatment on the potency of this chemokine. Met-RANTES is able to induce CCR5 activation in this assay even in untreated cells, despite being described as an antagonist for CCR5 in other assays (Fig. 6). We did not observe an increase in potency or response after sodium butyrate treatment for effects of Met-RANTES in [^{35}S]GTPγS-binding assays. CCL7, however, was unable to stimulate [^{35}S]GTPγS binding in membranes from control or sodium butyrate-treated cells (data not shown). In control experiments (data not shown) we examined the effects of butyrate treatment on CHO·CCR5·CD4 cells and found similar effects on

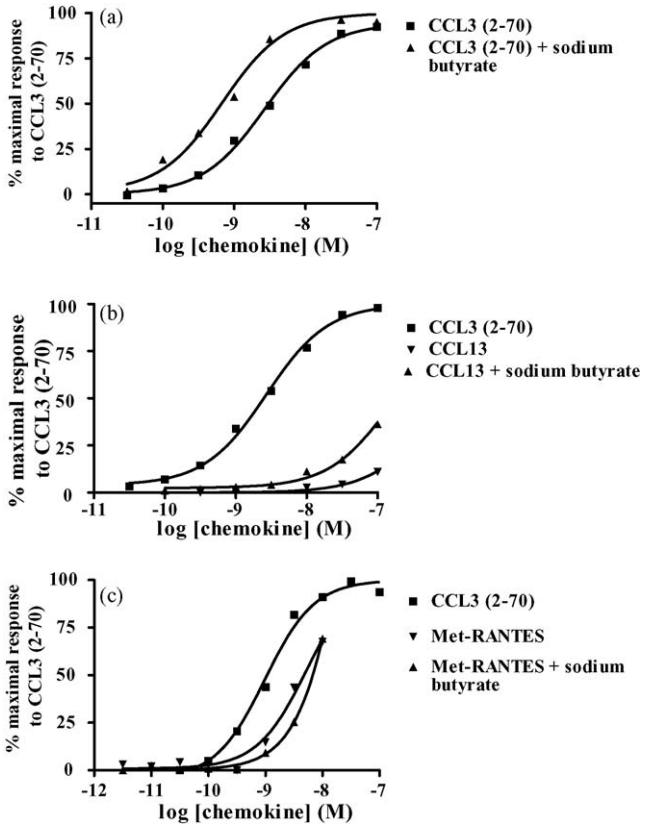

Fig. 6 – Stimulation of [^{35}S]GTPγS binding by chemokines in membranes from control and sodium butyrate-treated CHO·CCR5 cells. [^{35}S]GTPγS binding was determined in the presence of increasing concentrations of each agent as described in Section 2. Data shown are representative graphs of at least three separate experiments performed in triplicate. (a) Stimulation by CCL3(2–70); (b) stimulation by CCL3(2–70) and CCL13; (c) stimulation by CCL3(2–70) and Met-RANTES.

responses to both CCL3 and CCL5 showing that CD4 has no effect on signalling via CCR5 as indicated above.

4. Discussion

In this report we have examined the signalling properties of several chemokines at the chemokine receptor, CCR5, using a range of assays. In the first part of the work we examined four CCL3 isoforms (CCL3, CCL3L1, CCL3(5–70), CCL3(2–70)) whose properties were outlined in the Introduction. These chemokines have different amino terminal sequences and enable the relation between amino terminal structure and chemokine function to be examined. These chemokines were tested in three assays for agonism at CCR5: intracellular Ca^{2+}, [^{35}S]GTPγS-binding and receptor internalisation assays. Overall, the CCL3L1 isoform produces the greatest activation of CCR5 based on efficacy (maximal response) or potency. CCL3(2–70) produces a similar maximal response to CCL3L1 in the different assays although it is not as potent. The CCL3 and CCL3(5–70) forms are less efficacious or less potent than

Table 2 – Stimulation of [^{35}S]GTPγS binding

Chemokine	CHO.CCR5 cells, untreated				CHO.CCR5 cells, treated with 5 mM sodium butyrate				CHO.CCR5 cells, treated with 10 mM sodium butyrate			
	pEC_{50} ± S.E.M.	EC_{50} (nM)	Response in untreated cells (%)	n	pEC_{50} ± S.E.M.	EC_{50} (nM)	Response in untreated cells (%)	n	pEC_{50} ± S.E.M.	EC_{50} (nM)	Response in untreated cells (%)	n
CCL3(2-70)	8.96 ± 0.11	1.1	100	20	9.02 ± 0.12	0.95	107.0 ± 6.4	11	9.43 ± 0.13*	0.37	91.0 ± 5.5	14
CCL4	8.62 ± 0.14	2.4	100	3	8.50 ± 0.03	3.2	109.7 ± 6.5	3	8.79 ± 0.10	1.6	112.1 ± 3.0	3
CCL5	9.02 ± 0.08	0.95	100	7	9.29 ± 0.07*	0.51	94.4 ± 7.1	7	9.41 ± 0.07*	0.39	99.0 ± 12.1	4
CCL8	8.65 ± 0.15	2.2	100	10	8.96 ± 0.14	1.1	136.6 ± 13.3	5	8.78 ± 0.07	1.7	108.4 ± 5.5	7
CCL7	–	–	–	3	–	–	–	3	–	–	–	3

Stimulation of [^{35}S] GTPγS binding was determined in membranes from CHO·CCR5 cells. Control cells and cells treated with sodium butyrate (5 mM or 10 mM) were used as described in Section 2. Data were analysed to provide the EC_{50} and the maximal response, expressed as a % of that given by the same ligand on control cells. *$P < 0.05$ compared with control cells. Statistical analysis was done using an one-way ANOVA with Bonferroni's multiple comparison as a post-test.

the other isoforms tested depending on the assay used. Overall, although there are differences in detail, the rank order of effects of the different chemokines is similar in the different assays.

Some of the differences reported here for the different chemokines could have been a reflection of differences in kinetics for binding to CCR5. We consider this to be unlikely as in internalisation assays we saw no differences in the extent of modulation by CCL3(2–70) for assays up to 24 h. Also in previous work, using CCL3(2–70)D27A, CCL4 and CCL5, the time course of internalisation for these chemokines was of a similar shape but the extent of internalisation differed [8]. This suggests that the kinetics of binding are not limiting the effects of these chemokines and this is likely to hold in the present report. We have also performed CCL5 stimulation of [^{35}S]GTPγS binding for 30 and 240 min and find very similar potencies for the chemokine, again showing that kinetic factors are not limiting the effects.

Some of these forms of CCL3 examined in the present report have been studied in other laboratories. This work has suggested that the absence of a proline residue at position 2 of CCL3 is responsible for its inability to bind with high affinity to the chemokine receptors, CCR5 and D6, whereas murine CCL3, which contains a proline at position 2 binds strongly [28–32]. The naturally occurring non allelic variant of CCL3, CCL3L1 (LD78β) which has a proline residue at position 2 is more potent than the CCL3 isoform and in these studies seems to be the most potent naturally occurring CCR5 ligand [18,33,34]. Based on these studies it has been proposed that the proline residue at position 2 is essential for the increased agonist potency.

The data presented in the present report are in agreement with these findings in that CCL3L1 is more potent and efficacious than CCL3. The observations with CCL3(2–70) suggest, however, that high potency/efficacy can reside in a chemokine that does not contain a proline at position 2. In some tests CCL3(2–70) is as efficacious as CCL3L1 although it may not be as potent. The data show that the amino terminus of chemokines is very important in determining the signalling activity of chemokines and that subtle changes in structure can alter these activities.

In our previous work [8] we showed that in [^{35}S]GTPγS-binding assays, CCL3(2–70)D27A is able to activate CCR5 to the same extent as CCL4, CCL5 and CCL3L1. The present data show that CCL3 and CCL3(5–70) are less efficacious than these chemokines so in vivo CCL4 and CCL5 may produce greater activation of CCR5. It is also of interest that CCL3(2–70)(D27A), which also activates CCR5 better than CCL3 itself was developed as a drug (BB10010) [24]. The present data show that that this chemokine provides similar activation of CCR5 to CCL4 and CCL5.

We also examined the activities of some very low efficacy chemokines using assays where the sensitivity had been increased. This was achieved by treating cells with sodium butyrate [35,36] and this resulted in an increase in receptor expression but no change in inhibitory G proteins, i.e. the R/G ratio had increased. Using intracellular Ca^{2+} assays we observed greater signals in sodium butyrate-treated cells compared to untreated cells following stimulation by CCL3(2–70), CCL4 and CCL5. Modulation of CCR5 levels, for example by different chemokine levels, may alter the ability of cells to respond to chemokines in vivo. Interestingly we also observed that CCL7 and Met-RANTES induced signalling through CCR5 after sodium butyrate treatment. These two chemokines have been previously described as CCR5 antagonists ([9,15,17]), because they do not induce chemotaxis or internalisation of CCR5. Our data however show that CCL7 and Met-RANTES are weak agonists that can promote intracellular calcium changes in CCR5-expressing cells under certain conditions. These data raise the question of whether CCL7 and Met-RANTES are also able to induce chemotaxis in native cells that express a high amount of CCR5 compared to G proteins.

The data also suggest that chemokines may activate signalling pathways differentially in cells. In order to investigate this further we examined stimulation of [^{35}S]GTPγS binding by a range of chemokines in membranes from sodium butyrate-treated or -untreated cells. We did not observe any significant changes in maximal response for the majority of chemokines tested. CCL13 however was able to induce a greater signal in membranes that had been pre-treated with 10 mM sodium butyrate. In untreated cells CCL13 is a very weak agonist, therefore, the response of the receptor for this agonist can be increased using sodium butyrate. Although we did not observe an increase in maximal effect for CCL3(2–70) or CCL5, there was a significant increase in potency for these two chemokines after sodium butyrate treatment. This is consistent with a system where the maximal response is limited by the number of G proteins so that an increase in the receptor/G protein ratio leads to increased agonist potency for full agonists.

We also tested CCL7 and Met-RANTES in [^{35}S]GTPγS-binding assays. These chemokines had previously been described as antagonists, but were able to induce changes in intracellular calcium after sodium butyrate treatment. CCL7 was unable to induce signalling in [^{35}S]GTPγS-binding assays in membranes from control or sodium butyrate-treated cells (data not shown). Met-RANTES was, however, able to activate G proteins in membranes from control and sodium butyrate-treated cells and the sodium butyrate treatment did not modify the response.

The data described here provide new insights into mechanisms of signalling by chemokines via CCR5. An increase in receptor expression can increase the sensitivity of the assays used and therefore allows the identification of very weak agonists. Furthermore chemokines that are normally classified as antagonists can be identified as weak agonists in this system. It is also important to use more than one assay system to characterise chemokines and this is particularly clear based on data from intracellular Ca^{2+} and [^{35}S]GTPγS-binding assays and effects of Met-RANTES and CCL7. The data show that a ligand can act as an antagonist in one system (CCL7 in [^{35}S]GTPγS-binding assays) but as an agonist in a second system (CCL7 in intracellular Ca^{2+} assays).

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