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Differential regulation of chemotaxis: Role of Gβγ in chemokine receptor-induced cell migration

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ABSTRACT

CC and CXC chemokine receptor signalling networks are regulated in different ways. Here we show that intracellular calcium release and cell migration occur independent of Gβγ activation in response to CCL3, whereas CXCL11 induced migration of activated T-lymphocytes depends on Gβγ activation. Treatment of a range of cell types with gallein, a pharmacological inhibitor of Gβγ signalling, did not result in a reduction in CCL3 induced cellular migration, but resulted in enhanced calcium mobilisation following chemokine stimulation. Inhibition of PI3 kinase (PI3K) and AKT, which are activated downstream of Gβγ, equally had no effect on calcium release and a minor effect on cell migration. Similarly, inhibition of ERK1/2 did not prevent CCL3 induced migration. Interestingly, Gβγ as well as PI3K activation is necessary for CXCL11 induced migration of activated T-cells. These data not only confirm a role for Gβγ signalling in CXCL11 induced migration, but also demonstrate that targeting Gβγ as a therapeutic target to prevent migration in inflammatory disease may not be beneficial, at least not for CCL3 induced migration. This highlights the distinct differences in the mechanisms on how CC- and CXC-receptors activate cellular migration.

1. Introduction

Infiltration and attraction of leukocytes into areas of inflammation are, in part, instigated by the response of immune cells to a chemotactic gradient of chemokines localised in these areas. Chemoattractant chemokines are central to the process of cellular attraction to sites of inflammation, and signal by activation through a family of GPCRs referred to as chemokine receptors [1]. All the CC-motif chemokine receptors signal predominantly through the Gαi family of heterotrimeric G proteins. Activation of Gαi typically results in inhibition of cAMP production and the release of intracellular calcium from intracellular stores [2,3]. The Gβγ subunit of the heterotrimeric G protein complex can interact with and modulate the activity of many downstream effectors such as PLCβ, PI3K, adenylyl cyclase, as well as members of the GRK family [4].

The immediate cellular consequence of receptor activation is the release of intracellular calcium, which acts as a secondary messenger resulting in the activation of store operated calcium channels. The release of calcium into the cytosol is implicated in playing a role in cell migration [5]. Additional studies have shown a role for calcium mobilisation in chemotaxis in leukocytes [6], SMC migration [7], and the metastasis of breast cancer [8]. We have recently demonstrated that calcium independent chemotaxis in response to CCL3 is possible in THP-1 cells [9]. These results may be an indication that chemotaxis through chemokine receptors may be governed by receptor specific activation of signalling networks.

Following activation of CCR5 signalling, activity is regulated by agonist-dependent processes which involve GRK-dependent phosphorylation, β-arrestin-mediated desensitisation and receptor internalisation [10]. Once dissociated from the receptor, Gβγ assumes a conformation that allows it to bind one or more of the seven known GRKs [11] and, in doing so, directs phosphorylation at residues on the active GPCR intracellular loops and carboxyl terminus, resulting in desensitisation of the receptor and preventing further heterotrimer activation [12]. CCR5, as well as the related receptor CCR1, is activated by CCL3 and leads to cell migration [13–15]. Both receptors are expressed on the same cell types, e.g. THP-1 cells, and therefore it is not always possible to clarify precisely which of the different receptors interact with the signalling network.

Abbreviations: CHO, Chinese hamster ovary; CCL3, Chemokine ligand 3; CCR5, Chemokine receptor 5; DMEM, Dulbecco's modified Eagle's medium; EDTA, Ethylenediaminetetraacetic acid; ERK, Extracellular signal-regulated kinases; FCS, Foetal calf serum; GPCR, G protein coupled receptor; GRK, G-protein coupled receptor kinase; PBS, Phosphate buffered saline; PI3K, Phosphatidylinositol 3-kinases; PLCβ, Phospholipase Cβ; S.E.M., Standard error of the mean.

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Gallein, a synthetic derivate of the naturally occurring dye haematoxylin, acts as a small molecule modulator of Gβγ function [16]. Recent studies have shown that gallein inhibits Gβγ-dependent chemotaxis and inflammation events in differentiated HL60 cells and in vivo in neutrophils [17,18]. Collectively, gallein has been shown to block fMLP-dependent PI3K-γ activation, Rac1 activation, superoxide production, and neutrophil migration, as well as, prevent Gβγ interactions with GRK2, and sodium channels [18–20].

Previous findings have shown that treatment of breast cancer cells with the structurally related, similarly efficacious Gβγ inhibitor, M119K, resulted in inhibition of CXCR4 induced cell migration [21]. We therefore hypothesised that blockade of Gβγ signalling would represent a mechanism to reduce CC-chemokine activated cell migration as well. Using chemotaxis and calcium flux assays to determine CCL3 and CXCL11-induced cell migration, we investigated the role of Gβγ signalling and its downstream effectors in several cell types.

2. Materials and methods

2.1. Cells and materials

Culture conditions for CHO.CCR5, HEK.CCR5, THP-1 and HeLa-RC49 have previously been described [9,22–25]. Briefly, CHO.CCR5 cells were transfected with pcDNA3 encoding CCR5 and selected for stable expression in 10% FCS/DMEM/glutamine (2 mM) in the presence of G418. HeLa-RC49 cells expressing CCR5 were obtained from D. Kabat and described previously [26]. HEK.CCR5 cells stably express CCR5 and were a generous gift from British Biotech (Oxford, UK). CHO.CCR5 and HeLa-RC49 cells only express CCR5 and can therefore act as a model to test the effects of CCR5 activation without the interference of CCR1. The overexpression systems of CHO.CCR5 and HeLa-RC49 cells are necessary to test the pharmacological details of CCR5 activation.

Blood was sampled from healthy normal subjects according to a protocol approved by a local ethics committee (reference number 2008042). Peripheral blood mononuclear cells (PBMCs) were subsequently isolated as previously described by Sabroe et al. [27]. Lymphocytes were separated from monocytes by allowing the latter to adhere to a tissue culture flask for 2 h at 37 °C and were activated by culture in the presence of IL-2 (200 mg/mL) and concanavalin A (30 mg/mL) for at least 10 days.

The chemokine used for CCR5/CCR1 activation was CCL3 (D26A), generously donated by Lloyd Czaplewski of British Biotech and has been described before [25,28,29]. Wortmannin was purchased from Sigma (Poole, UK). PD98059, Y27632, gallein and LY294002 were purchased from Tocris (Germany), AS605240 was obtained from Stratech Scientific [30], and CXCL11 was from Peprotech (UK). Where indicated, cells were pre-treated with 10, 20, or 30 μM gallein, or shown concentrations of wortmannin, PD98059, Y27632, LY294002 or AS605240 for 30 min before induction of calcium flux or chemotaxis with CCL3 or CXCL11. The phospho-AKT InstantOne™ ELISA was purchased from eBiosciences (Hatfield, UK) and used according to the manufacturer's guidelines.

2.2. Analysis of intracellular calcium ion concentration

Cells were loaded with Fura-2 as described previously [25,28]. Inhibitors were present during the incubation period. Following incubation with inhibitors, cells were washed with calcium flux buffer and were resuspended at 2×10^6 cells/mL in calcium flux buffer. Chemokine-induced intracellular calcium mobilisation was determined as described by Grynkiewicz [31] using a BMG Labtech Fluostar OPTIMA fluorometer. In all experiments chemokine was added after 15 s of incubation in the fluorometer. Calcium mobilisation was monitored for a further 60 s following chemokine challenge.

2.3. Chemotaxis assays

Cells were harvested and washed twice with pre-warmed, sterile PBS, then resuspended in serum-free RPMI 1640 containing 0.1% BSA at a concentration of 6.25×10^7 cells/mL. 20 μL of resuspended cell media was loaded into the upper compartment of a microchemotaxis chamber (Receptor Technologies, Adderbury, UK) and chemoattractants were loaded in a final volume of 31 μL at indicated concentrations in the lower compartment. The two compartments were separated by a polyvinylpyrollidone-free polycarbonate filter with 5 μm pores. Chemotaxis was performed and analysed as previously described [9].

2.4. AKT activation ELISA

Briefly, THP-1 cells were pre-incubated with gallein for 30 min prior to stimulation with 200 nM of CCL3. Following 10 min of stimulation cells were lysed and the ELISA was performed as per manufacturer's instructions. Absorbance was measured at 450 nm using a BMG Fluostar Optima. Results are expressed as the percentage difference compared to CCL3 treated controls.

2.5. Analysis of data

Data were analysed using GraphPad Prism 5 (GraphPad Software). Concentration/response curves for CCL3 in calcium flux assays were fitted well by models assuming a Hill coefficient of 1. Statistical analyses were performed using a one-way ANOVA with a Bonferroni multiple comparison test as post-hoc test with a p value <0.05 deemed significant. In all figures, data represent the mean ± S.E.M. of at least three independent experiments.

3. Results

3.1. Gallein does not inhibit CCR5 mediated calcium release

We investigated the role of Gβγ subunit activation for signal transduction and chemotaxis in different cell types. Inhibition of Gβγ signalling with gallein did not reduce CCR5-dependent calcium mobilisation following CCL3 stimulation at any concentration. Previous studies suggest that pre-incubation with gallein at 10 μM completely abrogates any signalling via the Gβγ subunits [18]. In our hands, pre-incubation of CHO.CCR5, HeLa-RC49 or THP-1 cells with 10 μM gallein showed no reduction at all in calcium release following 100 nM CCL3 stimulation (Fig. 1a). Similar results were achieved in HEK.CCR5 cells (data not shown). Additionally, no difference in the kinetics of calcium mobilisation was detected under these conditions (Fig. 1b). These data demonstrate that signalling via the Gβγ subunit of CCR5 remains unperturbed by treatment with 10 μM gallein.

In order to rule out cell type specific potency issues, we examined the effect of increasing gallein concentrations on CCR5-mediated calcium release. All cell types were pre-incubated with two or three fold the concentration described to be effective by other groups [16–18]. Cells were stimulated with increasing concentrations of CCL3. Following gallein treatment at 20 μM or 30 μM, stimulation with 300 nM CCL3 resulted, not in a depletion of calcium release, but in 2.30 (±0.156) and 1.86 (±0.110) fold increases in calcium mobilisation in CHO.CCR5 and 1.23 (±0.136) and 2.27 (±0.237) fold increases in HeLa-RC49 (Fig. 1c–e). To confirm the effect of gallein on a more relevant model of inflammation, activated T-lymphocytes, isolated from healthy volunteers, were pretreated with 10 μM gallein before challenging with either CXCL11 or CCL3. Gallein pretreatment did not inhibit or potentiate CCL3 mediated calcium mobilisation in these cells, however CXCL11 calcium mobilisation was slightly increased, in line with our observations for other cell types.