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CCL3 induced migration occurs independently of intracellular calcium release

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ABSTRACT

The molecular signalling pathway of cell migration and whether it can occur independently of the release of intracellular calcium is still not completely understood. Therefore we investigated here the molecular mechanisms of CCL3 induced cell migration and the importance of intracellular calcium for chemotaxis in more detail. We show that CCL3 induced cell migration is dependent on activation of PLC. Several PKC inhibitors block the release of intracellular calcium independently of CCL3 activation and do not affect cell migration. This confirms that the release of intracellular calcium is not necessary for chemotaxis towards CCL3 and that PKC inhibitors should be used with caution in calcium release assays.

1. Introduction

Chemotaxis is mediated by signalling events initiated by binding of chemokines to their cognate receptors and involves re-arrangement of the actin cytoskeleton. However the signal networks that are important for chemokine receptor triggered migration are not yet completely understood. It has been shown that the chemokines CCL3, CCL4 and CCL5 induce migration through the GPCR CCR5 [1]. The chemokine receptor, CCR5, plays an important role as a co-receptor for HIV entry into cells [2–5] and its activation leads to signalling via heterotrimeric G proteins which ends with rapid phosphorylation of the receptor via GRK2 and protein kinase c (PKC) [6–8]. Phosphorylation of the receptor leads to desensitization of the receptor and triggers binding of beta-arrestins and consequently internalisation of the receptor [9–11]. This receptor activation in turn then leads to the migration of cells towards a chemokine gradient, however the mechanisms of migration regulation are not obvious. Recently there have been studies investigating the complex signalling networks initiated by CCR5 [12], not enough detail about how the signal is passed on downstream from the receptor is known.

We therefore used different inhibitors to analyse how chemokine receptors activate cellular migration and to understand whether there calcium flux and chemotaxis are intrinsically linked in the cell signalling network.

Abbreviations: CHO, Chinese hamster ovary; DMEM, Dulbecco's modified eagle's medium; EDTA, ethylenediaminetetraacetic acid; FCS, foetal calf serum; GPCR, G protein coupled receptor; GRK, G-protein coupled receptor kinase; PBS, phosphate buffered saline; PKC, protein kinase c; PLC, phospholipase c; S.E.M, standard error of means; TG, Thapsigargin.

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2. Methods

2.1. Cells and materials

CHO cells expressing CCR5 were obtained as described in [10] and the monocytic cell line THP-1 was obtained from the ATCC. The chemokine used for CCR5/CCR1 activation was CCL3 (D26A), which was generously donated by Lloyd Czaplewski of British Biotech. We have referred to the isoform used in this study in a previous publication as CCL3 (2–70) (D26A) in comparison with the full gene sequence for CCL3 [13] and it is referred to in the text as CCL3. Go6976, U73122, rottlerin, thapsigargin and GF109203X were purchased from Tocris and cells were treated with 10 μM U73122, 50 nM Go6976, 5 μM GF109203X and 4 μM rottlerin for 30 min before induction of calcium flux or chemotaxis. Thapsigargin was used at 1 μM.

2.2. 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, pH 7.4) and incubated with 4 μM Fura-2 (Molecular Probes) at 37 °C for 30 min. Inhibitors were present during the incubation period. After washing cells with buffer, cells were resuspended at 2×10^6 cells/mL of buffer. Chemokine-induced intracellular calcium mobilisation was determined by BMGlabtech Fluostar OPTIMA fluorometer. In all experiments cells were challenged with different concentrations of CCL3 after 15 s incubation in the fluorometer and measurements were taken over a further 60 s. The peak values of intracellular calcium ion concentration following the chemokine challenge were determined as described by Grynkiewicz [14].

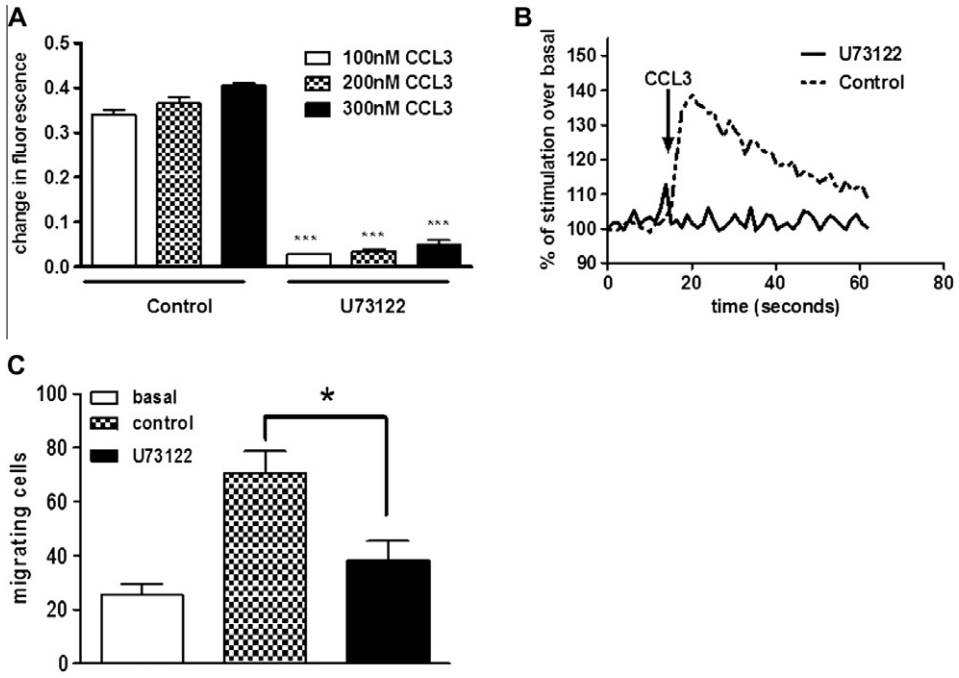

Fig. 1. Phospholipase c activation is essential for the release of calcium and migration of cells. (A) CHO·CCR5 cells were challenged with different concentration of CCL3 after pre-treatment with U73122 or no treatment (control), (B) shows single traces in real time in the presence or absence of U73122 in CHO·CCR5 cells (200 nM CCL3), (C) THP-1 cells were treated with U73122 for 30 min or left untreated (control) before being subjected to a 4 h migration assay towards 1nM CCL3. Basal shows cells migrating through filter without stiumuls. (A) Data are expressed as changes in fluorescence ratio [340 nm/380 nm] where the basal before addition of chemokine is subtracted from peak fluorescence after addition of chemokine. (B) Data in single trace was normalised to stimulation over basal. Data represent mean ± S.E.M. from at least three independent experiments for the bar charts and a representative tracer for the calcium flux, $^{*}p < 0.05$, $^{***}p < 0.001$ in a Student's *t*-test.

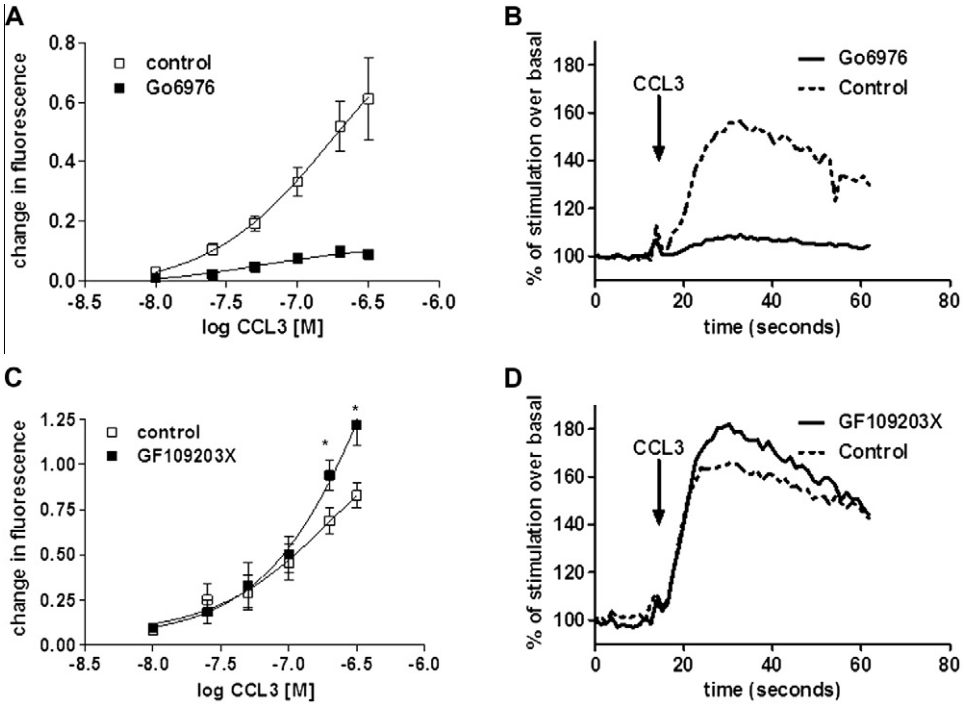

Fig. 2. Use of Go6976 and not GF109203X prevents release of intracellular calcium. (A) CHO·CCR5 cells were treated with Go6976 for 30 min and then challenged with different concentration of CCL3, (B) shows single traces in real time in the presence or absence of Go6976 in CHO.CCR5 cells (200 nM CCL3), (C) CHO.CCR5 cells were treated with GF109203X at 50 μM for 30 min and then challenged with different concentration of CCL3, (D) shows single traces in real time in the presence or absence of GF109203X in CHO.CCR5 cells (200nM CCL3), (A and C) Data are expressed as changes in fluorescence ratio [340 nm/380 nm] where the basal before addition of chemokine is subtracted from peak fluorescence after addition of chemokine. (B and D) Data in single trace was normalised to stimulation over basal. Data represent mean ± S.E.M. from at least three independent experiments.

2.3. Chemotaxis Assays

Cells were harvested and washed twice with PBS, then resuspended in serum-free RPMI 1640 containing 0.1% BSA. Cells were loaded in a total volume of 20 μl into the upper compartment of a microchemotaxis chamber (Receptor Technologies, Adderbury, UK). 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. The chemotaxis chamber was incubated at 37 °C, 100% humidity, and 5% CO_2 for 4 h. The filter was then removed, and the number of cells migrating into each bottom compartment was counted using a hemocytometer. In all experiments, each data point was performed in duplicate.

2.4. Analysis of data

Data were analysed using GraphPad Prism (GraphPad Software). Concentration/response curves for CCL3 in calcium flux assays were fitted well by models assuming a Hill coefficient of 1. Statistical analysis were performed using Student's *t*-test with a p value < 0.05. Data represent the mean ± S.E.M. of at least three independent experiments.

3. Results

We investigated the role of phospholipase c for CCL3 induced calcium release in different cell types. In all cell lines the specific phospholipase c inhibitor, U73122, significantly inhibits the release of intracellular calcium (Fig. 1) as well as migration of cells. Phospholipase c activation usually leads to PKC activation and we therefore investigated whether PKC activation is necessary for the release of calcium as well as cell migration. We generated concentration response curves of CCL3 induced release of intracellular calcium in different cell types. Go6976 inhibits specifically PKCα and β and completely abrogated CCR5 induced calcium release in CHO·CCR5 cells (Fig. 2A and C). The PKC inhibitor GF109203X however, which also blocks PKCα,β and several other PKC isoforms,

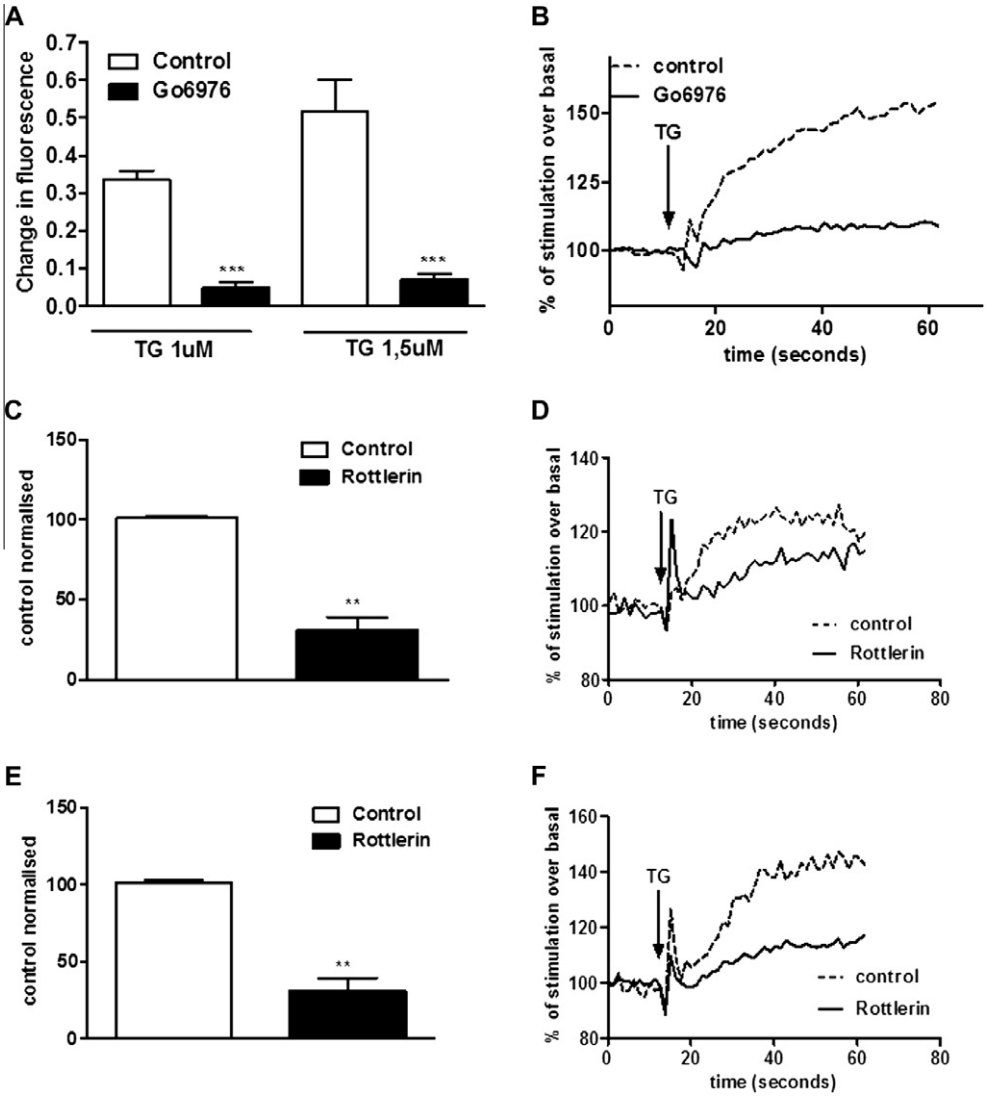

Fig. 3. Protein kinase c inhibitors deplete intracellular calcium stores. (A) THP-1 cells were treated with Go6976 for 30 min and then challenged with 1 or 1.5 μM of thaspigargin (TG). Data are expressed as changes in fluorescence ratio [340 nm/380 nm] where the basal before addition of chemokine is subtracted from peak fluorescence after addition of chemokine. (B) Shows single traces in real time in the presence or absence of Go6976 in THP-1 (1 μM TG), (C) THP-1 cells were treated with rottlerin and then challenged with 1 μM TG. Data is shown as percentage of fluorescence change compared to control cells (D) shows single traces in real time in the presence or absence of rottlerin for THP-1. Data represent mean ± S.E.M. from at least three independent experiments for the bar charts and a representative tracer for the calcium flux, $^{**}p < 0.01$, $^{***}p < 0.001$ in a Student's *t*-test.

does not block the release of intracellular calcium in CHO·CCR5 (Fig. 2C and D) or THP-1 cells (data not shown). Similar observations were made using rottlerin, a PKCδ inhibitor, which blocked calcium release in CHO·CCR5 and THP-1 cells (data not shown). To understand the mechanisms behind these conflicting sets of data, we investigated whether Go6976 and rottlerin have an effect on the intracellular calcium stores in the cells. Indeed, treatment of cells with either Go6976 and rottlerin and subsequent injection of thapsigargin to release calcium from the intracellular stores independently of CCR5 activation, showed, that both Go6976 and rottlerin completely abolish the release of intracellular calcium (Fig. 3). GF109203X has no effect on the calcium stores (data not shown). This therefore proofs that PKC activation is not crucial for CCL3 induced calcium release and furthermore rottlerin and Go6976 should only be used cautiously in calcium assays, since they deplete intracellular calcium stores and abrogate any signal in a receptor independent manner.

Even though some PKC inhibitors block calcium release, they do not affect migration towards CCL3. THP-1 cells migrate towards CCL3 in a concentration dependant manner (Fig. 4) and pre-incubation of cells with GF109203X or rottlerin does not affect migration at all. Similarly Go6976, which blocks any release of intracellular calcium, has no effect on cell migration. (Fig. 4A), which is proof that calcium release is not a pre-requisite for cell migration towards CCL3.

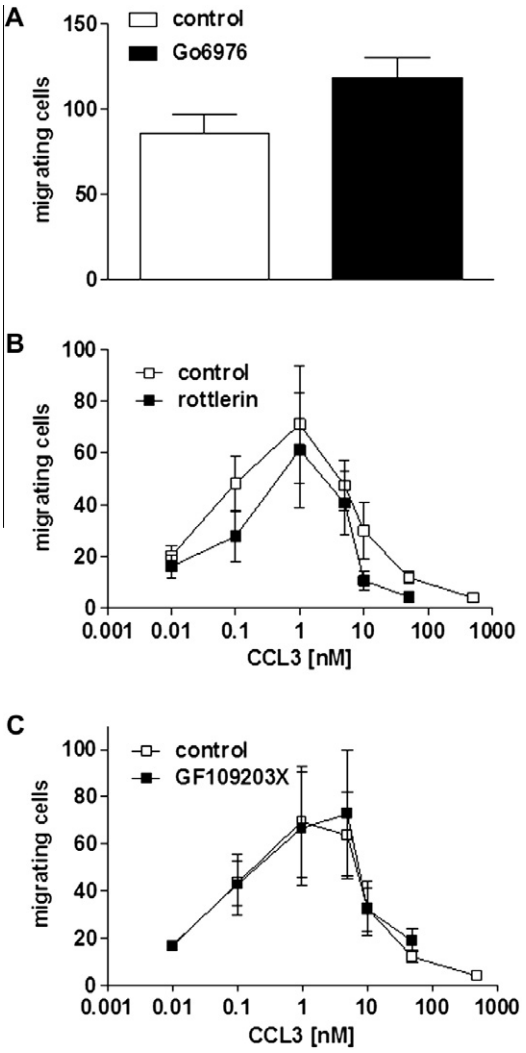

Fig. 4. The migratory responses of THP-1 cells occur independently of calcium release. (A) Shows migratory response of THP-1 cells towards 1 nM CCL3 in untreated control cells or Go6976 pre-treated cells. The migratory responses of THP-1 cells to increasing concentrations of CCL3 are shown following pre-treatment of the cells with GF109203x (B) or rottlerin (C) compared to untreated cells. Data shown are the mean ± S.E.M. of at least three experiments.

4. Discussion

In this work we have shown that the release of intracellular calcium is not essential for the migration of cells towards a CCL3 stimulus. We also highlight that the use of some PKC inhibitors is not recommended in calcium flux assays, since they deplete intracellular calcium stores independently of receptor activation and therefore might lead to false positive results. CCR5 can stimulate cell migration through a mechanism that is not fully understood. This ability is especially important in certain types of cancer where CCR5 is one of the main characters implicated in stimulating cell migration and tumour metastasis [15–18]. Numerous studies have proven that ligand binding to CCR5 results in cell movement in a mechanism dependent on actin mobilisation and PI3K recruitment to the leading edge of cells [12,19,20]. However the molecular mechanism through which CCR5 promotes cell migration is not clear. A recent report shows that oral cancer cells migrate upon CCL5 stimulation through a mechanism involving PLC, PKCγ, NF-κβ and the matrix metalloproteinase 9 (MMP-9) [21]. Also, a study performed on HIV-infected macrophages demonstrated that viral-induced cell migration through CCR5 binding could be blocked by PKC inhibition [22]. These studies suggest that in some cases, CCR5-induced cell migration is dependent on PKC activity. Additionally, several studies have reported the importance of PKCs for cell migration through other chemokine receptors. For instance, it was demonstrated that PKCα is essential for cell migration and tumour growth progression of Ishikawa endometrial adenocarcinoma cells [23,24]. Likewise, two recent papers reported that in T lymphoblastoid leukaemia cells and eosinophils, chemotaxis was blocked with rottlerin indicating that PKC δ was needed for cell migration [24,25].

However in our study we did not find any indication that PKC activation is essential for cell migration at all. Whereas there is clearly a need for PLC activation to achieve cellular migration, three different PKC inhibitors did not affect the migratory response.

To date, there is no information about the connection between calcium release and chemotaxis for CCL3 or CCR5. There are a few examples in the literature on this subject for other GPCR. For instance, chemotactic responses of T cells were impaired in PLC knock-out mice when compared with wild-type mice [26]. Also, when experiments were done with T cells and calcium was chelated using a pharmacological approach, chemotaxis was blocked resembling the results obtained in PLC knock-out mice and indicating that calcium release is needed for T cell migration. However a different study [25] showed that in the CEM leukaemic T cell line and human Th2 cells, chemotaxis was dependent on PLC but not on calcium release from ER stores and a different group showed that PLC isoforms were not needed for cell migration of neutrophils [27]. In our study we could show that for CCL3 activation of migration, no release of intracellular calcium is necessary.

We observed however that Go6976 and rottlerin deplete intracellular calcium stores and therefore give the impression as if PKC activation is needed for the release of calcium, whereas in fact the lack of calcium response is due to the unspecific phenomenon of ER store depletion. Bearing in mind the high interest that is being put on these inhibitory molecules due to PKC indisputable role in certain cancers and how often they are used in experiments, the fact that they can alter signal transduction pathways through calcium modification should be taken into consideration when using them to analyse signalling pathways.

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