Biochemical Pharmacology 78 (2009) 974–982

Contents lists available at ScienceDirect

Biochemical Pharmacology

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

Distinct modes of molecular regulation of CCL3 induced calcium flux in monocytic cells

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ARTICLE INFO

Article history:
Received 7 May 2009
Accepted 2 June 2009

Keywords:
Chemokine receptor
Caveolae
Cholesterol
Microdomain
Statin

ABSTRACT

The chemokine receptor CCR5 has been shown to be targeted to cholesterol- and sphingolipid-rich membrane microdomains. Here we elucidate the effects of membrane fluidity on CCR5 signalling and expression using the monocytic THP-1 cells. MCD treatment of THP-1 cells, which removes nearly all cholesterol from the plasma membrane, leads to an increase in the signalling properties of CCR5. In contrast, the prevention of cholesterol production with lovastatin and simvastatin decreases the release of intracellular calcium and also decreases receptor cell surface expression. The loss of response in lovastatin treated cells can be rescued by MCD addition, which shows that the cholesterol content in the membrane is only one factor in determining the amount of receptor response. We show that CCR5 signalling is dependent on thapsigargin-sensitive Ca^{2+} stores and on activation of ryanodine receptors as well as InsP3 receptors or store-operated channels. Cholesterol depletion with MCD changes the thapsigargin sensitivity in THP-1 cells and also alters receptor-G-protein coupling towards pertussis toxin (PTX) independent G-proteins. Cholesterol removal by MCD in THP-1 cells has far reaching consequences for receptor activation and signalling and emphasises the need for a clearer understanding of how membrane fluidity affects receptor signalling events.

1. Introduction

The chemokine receptor, CCR5, was originally characterised as a receptor that responds functionally to the CC-chemokines CCL3, CCL4 and CCL5 [1]. We have previously shown that CCR5 internalisation can be inhibited by treatment with sucrose, nystatin and filipin, which inhibit clathrin-coated pit internalisation and affect caveolae in the cell membrane, respectively [2]. There have been reports about a number of receptors, like CCR4 [3] or CCR2 [4] where caveolae are important for receptor internalisation. Furthermore it has been reported that chemokines bind preferentially to receptors which are associated with cholesterol and sphingolipid-rich lipid raft microdomains in the plasma membrane [5]. It was shown that CCR5 requires membrane cholesterol to retain ligand binding and signalling functions [6–8].

Additionally we could confirm previously that the amount of cholesterol in the plasma membrane is important for CCR5 induced signalling in stably transfected CHO and HEK cells. Both filipin and methyl-β-cyclodextrin (MCD) affect cholesterol in the membrane, however in different ways [9,10]. We showed that MCD treatment lead to failure of CCR5 to inhibit forskolin-stimulated accumulation of cAMP and it also changed the potency of CCL3 for intracellular calcium release, however cholesterol depletion had no effect on receptor internalisation. Furthermore, depletion of cholesterol changed the coupling of CCR5 from Gαi G-protein to a PTX independent G-protein, as shown by the release of intracellular calcium after PTX treatment [11].

Other studies also highlighted the importance of cholesterol for receptor conformation and signalling [6,12,13]. Recently it was revealed that cholesterol is important for chemotaxis of human neutrophils, but has no influence on early signalling by chemokine receptors on these cells [14], highlighting the need to investigate the function of membrane cholesterol further.

In this study we characterise the signalling properties of CCR5, especially the ability of CCR5 to release intracellular Ca^{2+} ions and also investigate how membrane fluidity influences receptor activation. Chemokine receptor induced calcium signalling is not completely understood. Several inhibitors, like omega-conotoxin GVIA (an N-type calcium channel blocker), thapsigargin (a sarcoendoplasmic reticulum Ca^{2+}-ATPase inhibitor) as well as phospholipase c and protein kinase c inhibitors seem to affect CCL2 and CXCL1 induced calcium response [15], giving some indication about how chemokine receptor activation is coupled to calcium signalling. However for CCR5, no characterisation of the mechanisms for the intracellular calcium release after receptor activation

Abbreviations: CHO cells, Chinese hamster ovary cells; FCS, foetal calf serum; FITC, fluorescein isothiocyanate; HEK, human embryonic kidney; InsP3, inositol 1,4,5-trisphosphate receptor; MCD, methyl-β-cyclodextrin; PBS, phosphate buffered saline; PTX, pertussis toxin; SOCs, store-operated channels.

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doi:10.1016/j.bcp.2009.06.003

has been shown so far. Here we analyse whether the endoplasmic reticulum Ca^{2+}-ATPases, inositol 1,4,5-trisphosphate (InsP3) receptors and ryanodine receptors are involved in CCR5 instigated signalling events. We also characterise the effect of membrane fluidity by either cholesterol depletion or the blockade of cholesterol production on receptor activation and expression in monocytic cells.

2. Materials and methods

2.1. Cells and materials

CHO cells expressing CCR5 were obtained as described in [2], HEK.CCR5 cells have been described previously [16] and the monocytic cell line THP-1 was obtained from the ATCC (Teddington, UK). The chemokine used for CCR5 activation was CCL3 (D26A), which was generously donated by Lloyd Czaplewski of British Biotech (UK). 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 [17] and it is referred to in the text as CCL3. The anti-CCR5 antibody, HEK/1/85a/7a has been described previously [2,17] and it has been shown that the antibody binds independently of chemokines. Secondary antibodies were obtained from Sigma–Aldrich (Poole, UK) and Santa Cruz (Heidelberg, Germany). Nystatin, filipin, sucrose, cholesterol, pertussis toxin (PTX), simvastatin and methyl-β-cyclodextrin (MCD) were purchased from Sigma–Aldrich. Cells were incubated for 1 h at 37 °C with filipin (5 μg/mL), sucrose (0.4 M), nystatin (50 μg/mL), MCD (10 mM) or cholesterol (2 mM), respectively, before an assay was performed. Lovastatin was obtained from Tocris (Bristol, UK) and activated before use as described by the manufacturer. 2-ABP, thapsigargin and ryanodine were also purchased from Tocris and cells were treated with 20 μM 2-ABP, 1 μM for thapsigargin or 10 μM for ryanodine

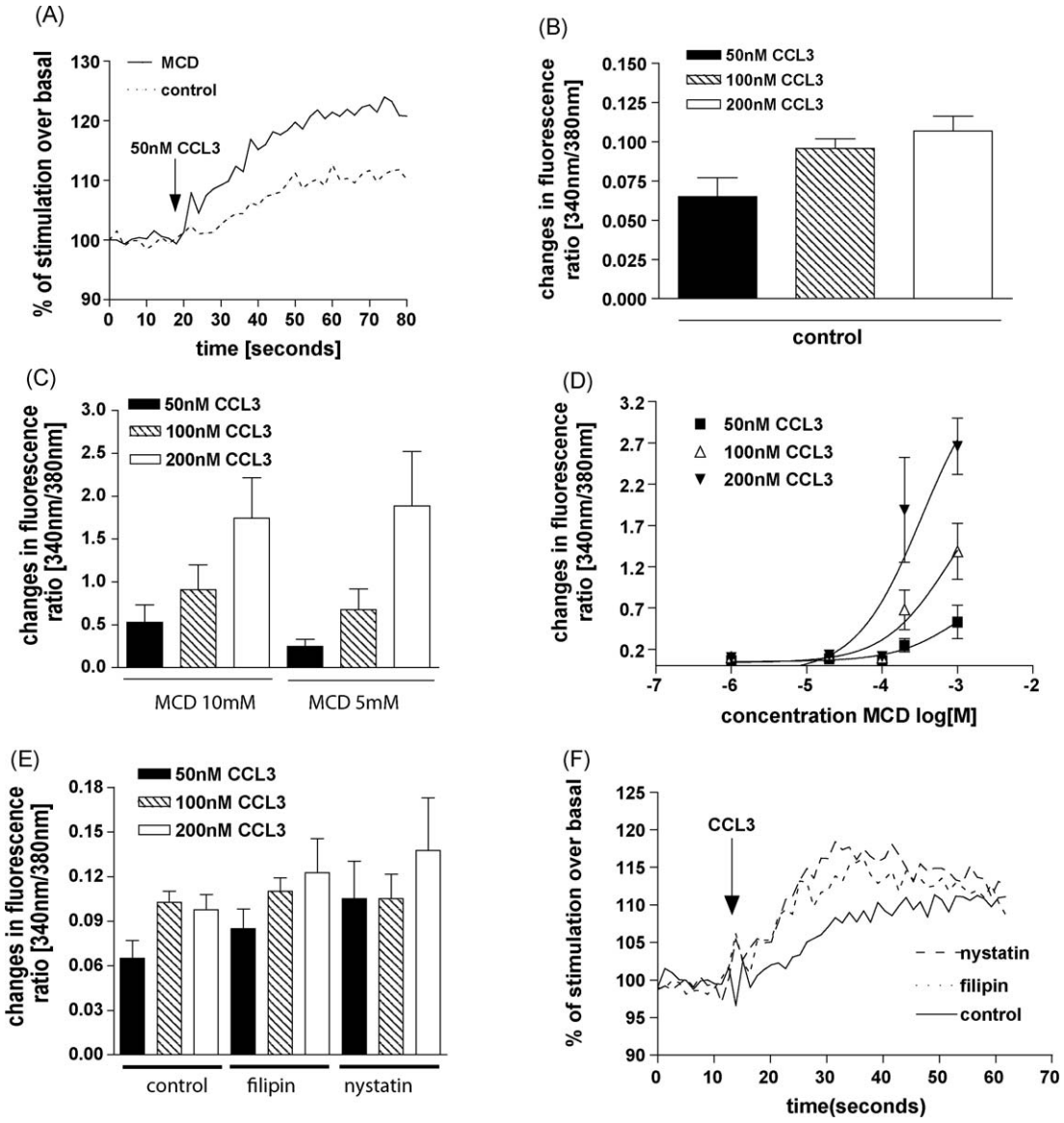

Fig. 1. Changes in intracellular Ca^{2+} in THP-1 cells in the presence of inhibitors. (A) Single traces in real time in the presence or absence of 10 mM MCD. (B) Control THP-1 cells challenged with different amounts of CCL3. (C) Cells pre-treated with different concentration of MCD and then challenged with CCL3 as indicated. (D) Concentration response curve for cells pre-treated with different concentrations of MCD. (E) Cells pre-treated with filipin, nystatin or vehicle (control) as described in materials and methods and then challenged with CCL3. (F) Single traces of THP-1 cells pre-treated with filipin, nystatin or vehicle (control) and then challenged with 100 nM CCL3. Data are expressed as either changes in fluorescence ratio [340 nm/380 nm] where the basal before addition of chemokine is subtracted from peak fluorescence after addition of chemokine or as percentage of stimulation over basal where the basal level is normalised to 100%. Data represent mean ± s.e.m. from at least three independent experiments for the bar charts and a representative tracer for the calcium flux.

unless stated otherwise for 1 h before induction of calcium flux. For PTX treatment, cells were incubated 18 h with PTX (1 μg/mL) before an assay was completed. All other chemical were purchased from Fisher Scientific (Loughborough, UK).

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 (Invitrogen, Paisley, UK) at 37 °C for 1 h. For assays in the absence of extracellular calcium, cells were washed twice in buffer containing no $CaCl_2$ and all steps of the experiment were performed in buffers without $CaCl_2$. 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 (BMGlabtech, Offenburg, Germany). In all experiments cells were challenged with different concentrations of CCL3 after 15 or 20 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 et al. [18].

2.3. Flow cytometry

Cells were treated with MCD, lovastatin or vehicle (control) cells as described, washed with PBS and stained with saturating amounts of HEK/1/85a/7a antibody or isotype control antibody for 1 h at 4 °C to prevent internalisation. To remove excess amounts of antibody, cells were washed three times with ice-cold PBS, incubated with corresponding fluorescent-labelled secondary antibody for 1 h at 4 °C, washed three times with ice-cold PBS and fixed with 4% paraformaldehyde. Receptor expression levels were determined using a Coulter Elite FACS.

2.4. Cholesterol depletion

For cholesterol depletion of cells we used two different strategies. De novo cholesterol synthesis was inhibited by lovastatin, an inhibitor of HMG-CoA reductase. Plasma membrane cholesterol was extracted by methyl-β-cyclodextrin. Methyl-β-

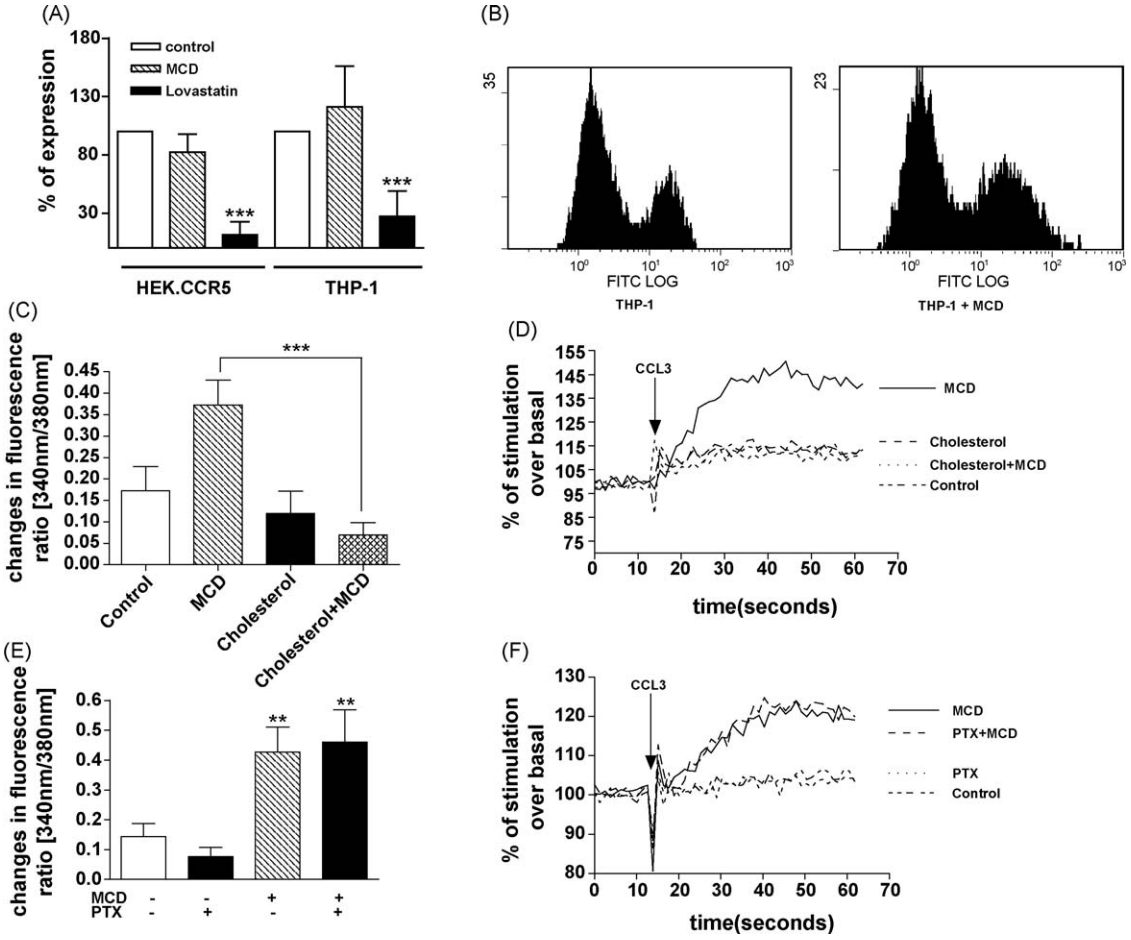

Fig. 2. Changes in receptor expression and intracellular Ca^{2+} in THP-1 cells. (A) Flow cytometry analysis of HEK.CCR5 and THP-1 cells after MCD and lovastatin treatment, cells were treated with lovastatin for 3 days and MCD for 1 h, respectively, stained with an anti-CCR5 antibody and the corresponding FITC-conjugated secondary antibody and analysed using flow cytometry, data represents percentage of Geo Mean fluorescence compared to vehicle treated (control) cells. (B) Flow cytometry analysis of CCR5 expression on control THP-1 cells and cells after MCD treatment, respectively. MCD treatment increases the expression level of CCR5. (C) THP-1 cells were treated with 10 mM MCD as described and when indicated 10 mM cholesterol was fed back to the cells before activation of receptor with 100 nM CCL3. (D) Single calcium trace of THP-1 cells either pre-treated with 10 mM MCD, 10 mM cholesterol or 10 mM MCD plus 10 mM cholesterol before receptor activation with 100 nM CCL3 when indicated. (E) Cells were treated with PTX or vehicle overnight and MCD was added 1 h before calcium flux was induced with 100 nM CCL3. (F) Single calcium trace of THP-1 cells treated with PTX overnight and/or MCD for 1 h before addition of 100 nM CCL3. Significant changes towards control cells are indicated by asterisks ($^*p < 0.05$, $^{**}p < 0.01$). Data are expressed as either changes in fluorescence ratio [340 nm/380 nm], where the basal line before addition of chemokine is subtracted from the peak fluorescence after addition of chemokine or as percentage of stimulation over basal, where the basal level is normalised to 100%. Data represent mean ± s.e.m. from at least three independent experiments for the bar charts and a representative tracer for the calcium flux.

cyclodextrin is a well established tool that selectively and quickly extracts cholesterol from plasma membranes in preference to other lipids [19]. These treatments reduce intracellular cholesterol without affecting cell viability within the time window selected for the experiments. Control cells were left untreated.

2.5. Cholesterol determination

Cholesterol levels were determined using the Amplex Red cholesterol assay (Invitrogen) in accordance to the manufacturers protocol. Cells, which were adjusted to the same cell amount, were pre-incubated with cholesterol oxidase and horseradish peroxidase and Amplex red (all obtained from Invitrogen in amounts described by the manufacturer) for 20 min in the absence of light at 37 °C. Fluorescence was measured using an excitation wavelength of 530 ± 10 nm and emission wavelength of 590 ± 10 nm.

2.6. Analysis of data

Data were analysed using GraphPad Prism (GraphPad Software, LaJolla, CA, USA). 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 one-way ANOVA with Bonferroni's multiple comparison as a post-test were appropriate. Otherwise 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

3.1. MCD depletion of cholesterol increases signalling in THP-1 cells

We previously showed that depleting the plasma membrane of cholesterol by using methyl-β-cyclodextrin (MCD) inhibits CCR5 induced calcium release in stably transfected CHO and HEK cell lines, whereas complexing cholesterol with filipin or nystatin does not affect signalling by the receptor [11]. Here we investigate in more detail how cholesterol and resulting changes in membrane fluidity affect receptor signalling in a monocytic cell line. Treatment of THP-1 cells with MCD and subsequent activation of CCR5 with CCL3 leads to a substantial increase in the release of intracellular calcium (. 1A) compared to untreated control cells (Fig. 1A and B). We therefore investigated whether this multiplication of the signal is dependent on the dosage of MCD used. Indeed there is a perfect concentration–signal relationship between the amount of MCD used to treat THP-1 cells and the signal that is induced by CCL3 (Fig. 1C and D). This result is a contradiction of our previous data, where MCD treatment significantly blocks the release of calcium [11]. To determine whether this increase can be observed with cholesterol complexing agents, we also used nystatin and filipin to complex cholesterol in the membrane and observed that these compounds lead to a slight increase in the release of calcium, however this increase is not significant (Fig. 1E and F). This is in congruence with data from CHO and HEK cells [11].

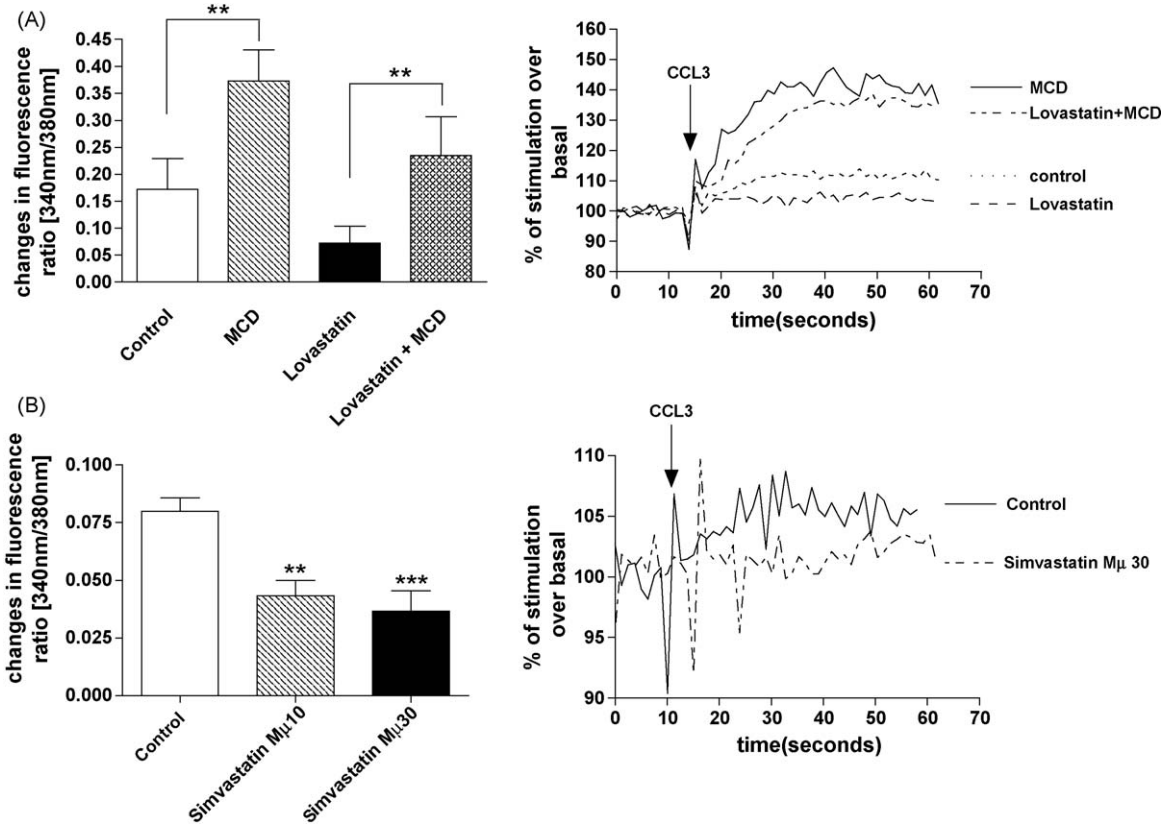

Fig. 3. Effect of statins on release of intracellular calcium in THP-1 cells. Cells were incubated with different statins for 3 days before cells were stimulated with 100 nM CCL3. (A) THP-1 cells pre-treated with lovastatin for 3 days, MCD for 1 h or vehicle (control) before receptor was activated with 100 nM CCL3, and single trace of THP-1 cells pre-treated with vehicle (control), MCD, lovastatin or MCD and lovastatin before the addition of 100 nM CCL3 as indicated. (B) The change of intracellular calcium in THP-1 cells, pre-treated with different concentrations of simvastatin for 3 days or vehicle (control) and challenged with 100 nM CCL3 and single trace of THP-1 cells pre-treated with vehicle (control) or simvastatin before the addition of 100 nM CCL3 when indicated. Significant changes to control data are shown by asterisks (**$p < 0.01$), Data are expressed as either changes in fluorescence ratio [340 nm/380 nm], where the basal line before addition of chemokine is subtracted from the peak fluorescence after addition of chemokine or as percentage of stimulation over basal, where the basal level is normalised to 100%. Data represent mean ± s.e.m. from at least three independent experiments for the bar charts and a representative tracer for the calcium flux.

To understand why MCD increases the signal and does not reduce it, we analysed the CCR5 receptor expression on different cells after treatment with MCD or lovastatin, which prevents cholesterol production in cells. MCD treatment has no significant diminishing effect on receptor expression on either CHO.CCR5 (data not shown) or HEK.CCR5 cells as analysed by immunofluorescence (data not shown) and flow cytometry (Fig. 2A and B) and a loss of receptor expression is therefore not the reason why the signal in those cells is reduced. On the other hand in THP-1 cells, the receptor expression is slightly increased after MCD treatment (Fig. 2A and B). This is in stark contrast to lovastatin, which blocks cholesterol production. Lovastatin diminishes the number of receptors on the cell surface significantly (Fig. 2A), as has been described by other groups for CCR2 receptors [20]. The THP-1 cells used in this study do not consist of a uniform population as can be seen in Fig. 2B, some cells express consistently higher levels of CCR5, this might be a reflection of a different maturation state of the cells.

The increase in receptor expression is not the sole reason why the signal in THP-1 cells increases, the loss of cholesterol and therefore changes in the fluidity of the membrane are also important. Re-introducing cholesterol into the membrane after depleting it first with MCD diminishes the signal to its basal level (Fig. 2C and D).

The loss of cholesterol not only alters the signal, it also changes the coupling of receptor to G-protein. This is similar to the results we observed earlier in CHO.CCR5 and HEK.CCR5 cells [11]. Treatment of THP-1 cells with PTX abolishes the signalling properties of CCR5 via PTX dependent G-proteins. Treatment of cells with PTX and MCD results in the release of intracellular calcium, which is due to CCR5 signalling via PTX independent G-proteins in the absence of cholesterol (Fig. 2E and F).

3.2. Statin treatment of THP-1 cells leads to different calcium response than MCD

This increase of signal in THP-1 cells is totally unexpected and therefore the question whether cholesterol levels are the determining factor for the efficacy of receptor signalling or whether MCD itself changes the signalling properties of the receptor remain. Hence statins, which block the cholesterol production in cells were used to investigate this further (Fig. 3). In THP-1 cells, lovastatin as well as simvastatin treatment over 3 days decreased the CCL3 induced release of Ca^{2+} and treatment of cells with MCD after statins treatment rescued receptor signalling (Fig. 3A and B). Similarly in HEK.CCR5 and CHO.CCR5 cells, lovastatin inhibited the CCR5 induced calcium release to similar extent as MCD and in HEK.CCR5 cells the treatment of cells with lovastatin and MCD had an accumulative effect (Fig. 4A and B). Since lovastatin decreases receptor expression to some extent in THP-1 cells (Fig. 2A), we cannot distinguish whether the loss of signalling is due to the loss of cholesterol or the decrease of receptor, although there is CCR5 receptor left on the THP-1 cells which should be sufficient to transduce a signal.

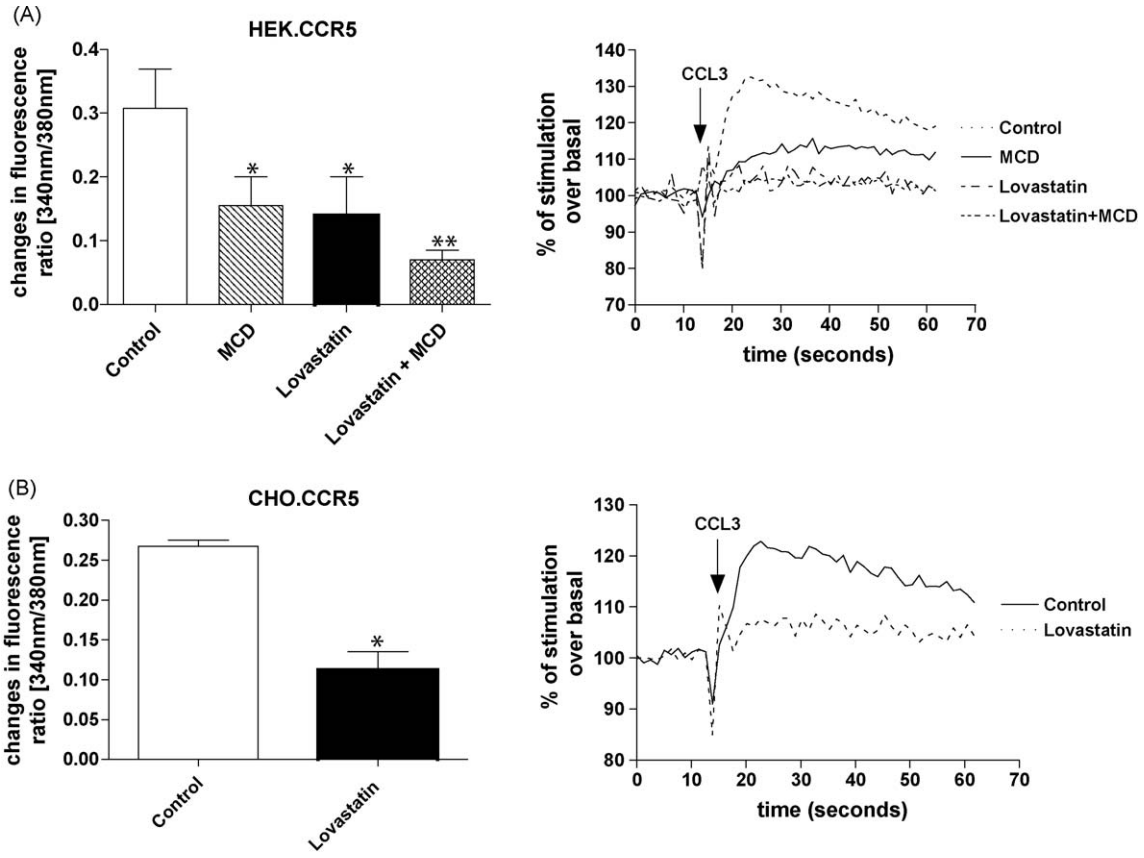

Fig. 4. Effect of statins on release of intracellular calcium in HEK.CCR5 and CHO.CCR5 cells. (A) HEK.CCR5 cells pre-treated with vehicle (control), lovastatin for 3 days, MCD for 1 h, or both before the receptor is activated with 100 nM CCL3. Significant changes to control data are shown by asterisks (*$p < 0.05$, **$p < 0.01$). (B) CHO.CCR5 cells pre-treated with vehicle (control), lovastatin for 3 days, MCD for 1 h, or both before the receptor is activated with 100 nM CCL3. Data are expressed as either changes in fluorescence ratio [340 nm/380 nm], where the basal line before addition of chemokine is subtracted from the peak fluorescence after addition of chemokine or as percentage of stimulation over basal, where the basal level is normalised to 100%. Data represent mean ± s.e.m. from at least three independent experiments for the bar charts and a representative tracer for the calcium flux.

3.3. MCD as well as statins lower the amount of cholesterol in the membrane

We already showed previously that different agents that affect cholesterol in cells have different effect on signalling by receptors [11]. We therefore were interested in understanding how these different compounds affect the overall levels of cholesterol in the cells. We determined the amount of cholesterol in untreated control cells, MCD, lovastatin, simvastatin and filipin treated cells (Fig. 5). Increasing concentrations of the statins, e.g. lovastatin and simvastatin, significantly lower the amount of cholesterol that can be detected in cells, with only 50–75% of cholesterol as compared to control cells present. MCD also decreased the amount of cholesterol significantly with less the 50% of control cell cholesterol present in MCD treated cells (Fig. 5C and D). MCD reduces the amount of cholesterol, whereas filipin complexes the cholesterol in the membrane without reducing the net amount of it.

Overall there is no marked difference between the three different cell lines tested. These results show that the amount of cholesterol levels alone cannot be responsible for the difference in signalling that is observed in different cells, on the contrary, it seems as if MCD has a second effect on THP-1 cells, apart from lowering the cholesterol level, which predicts and enhances chemokine receptor signalling. There seems to be a fundamental difference in the way CHO, HEK and THP-1 cells generate the release of intracellular calcium in response to receptor activation.

3.4. Ca^{2+} responses require a thapsigargin-sensitive Ca^{2+} store and activation of InsP3 receptors or SOCs

We wanted to clarify in more detail where the calcium released by CCR5 activation originates and therefore cells were treated with the endoplasmic reticulum Ca^{2+}-ATPase inhibitor thapsigargin before stimulation with CCL3. Thapsigargin abolished the Ca^{2+} responses in THP-1, HEK.CCR5 cells (Fig. 6) and CHO.CCR5 cells (data not shown), indicating that the released calcium originates from the endoplasmic reticulum. Interestingly, in MCD treated THP-1 cells, thapsigargin inhibition of calcium release is overridden by the MCD treatment and an increased signal can be observed (Fig. 6A). This was confirmed in experiments where THP-1 cells were incubated with calcium free medium for the duration of the experiment (Fig. 6C), therefore indicating that MCD alters the endoplasmic reticulum membrane and calcium release originates from the endoplasmic reticulum and not from the medium via transport through the plasma membrane. Incidentally, thapsigargin treatment had no effect on the viability of the cells treated (data not shown).

To test whether Ca^{2+} stores gated by activation of ryanodine receptors are involved in the release of intracellular Ca^{2+} in response to activation of CCR5 receptor, cells were incubated for 30 min in the presence of 10 or 100 μM ryanodine, respectively before addition of CCL3 (Fig. 7). Ryanodine treatment resulted in a slight increase of the CCL3 induced response. In CHO.CCR5 cells, after treatment with 10 μM ryanodine, this increase was

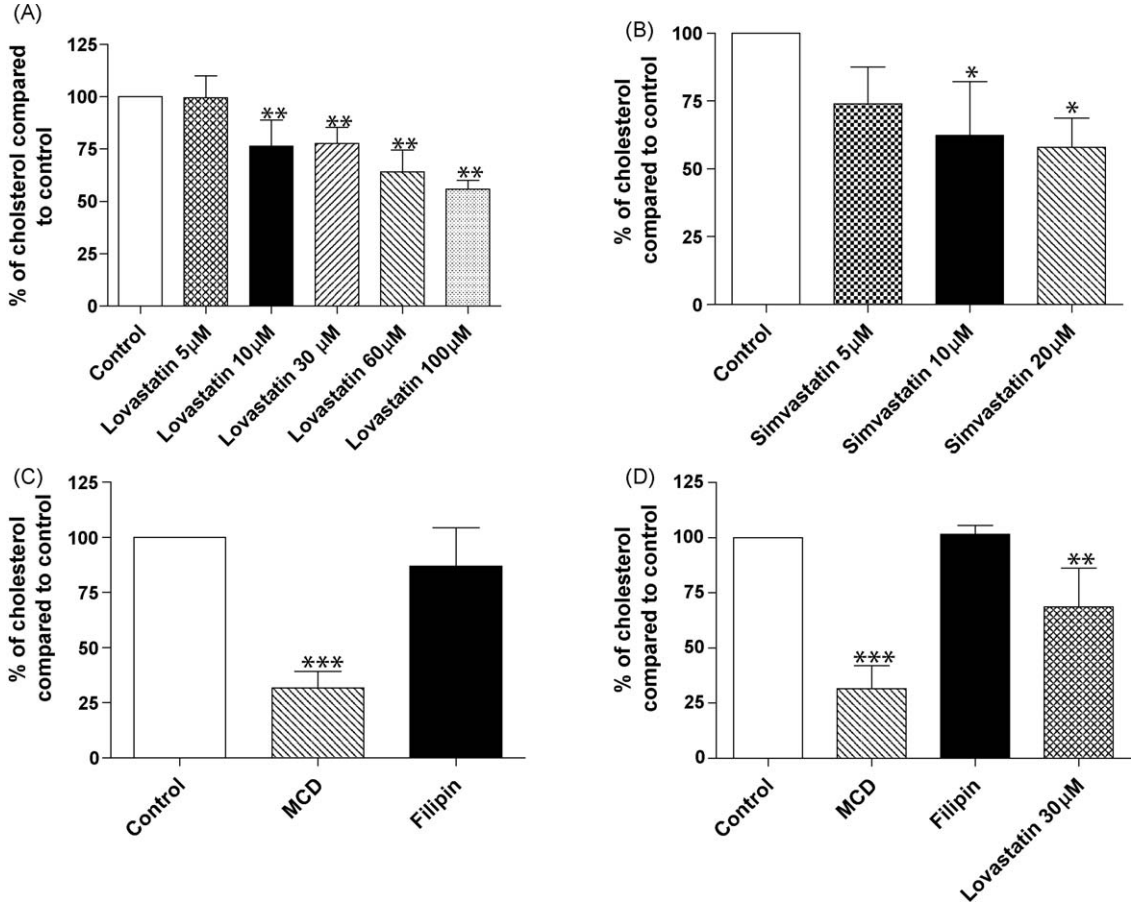

Fig. 5. Quantification of cholesterol levels in the plasma membrane of different cells. (A) THP-1 cells were incubated with different concentrations of lovastatin for 3 days and cholesterol levels in the cells were determined using the Amplex Red cholesterol assay as described. Increasing concentrations of lovastatin decrease the amount of cholesterol present in the cells. (B) THP-1 cells were treated with different concentrations of simvastatin for 3 days. (C) THP-1 cells were treated with MCD and filipin for 1 h. (D) HEK.CCR5 cells were treated with either MCD or filipin for 1 h or lovastatin for 3 days. Data are represented as percentage of cholesterol content compared to vehicle treated (control) cells. Significant changes towards control are shown by asterisks ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). Data represent mean ± s.e.m. of at least three independent experiments.

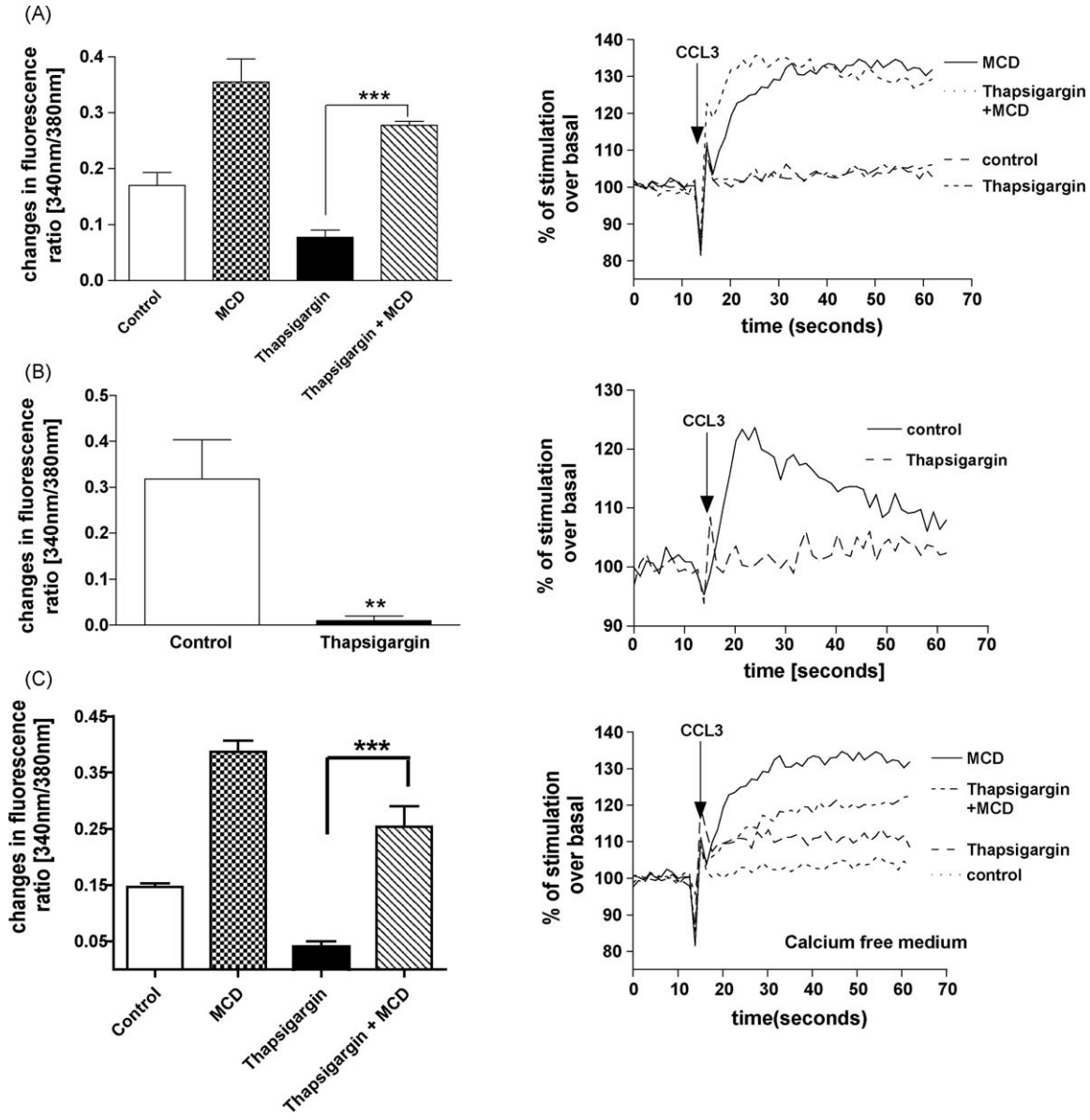

Fig. 6. Release of intracellular calcium in THP-1, HEK.CCR5 and CHO.CCR5 cells is dependent on sarcoendoplasmic reticulum Ca^{2+}-ATPase. (A) THP-1 cells and (B) HEK.CCR5 were treated with vehicle (control), MCD, thapsigargin or thapsigargin plus MCD for 1 h. Thapsigargin significantly inhibits the release of calcium in all cell lines compared to vehicle treated control cells. MCD treatment in THP-1 cells reverses thapsigargin inhibition. (C) THP-1 cells treated as in (A) in a calcium free buffer. Significant changes towards control are shown by asterisks ($^{**}p < 0.01$, $^{***}p < 0.001$). Data are expressed as either changes in fluorescence ratio [340 nm/380 nm], where the basal line before addition of chemokine is subtracted from the peak fluorescence after addition of chemokine or as percentage of stimulation over basal, where the basal level is normalised to 100%. Data represent mean ± s.e.m. from at least three independent experiments for the bar charts and a representative tracer for the calcium flux.

significant, however at 100 μM ryanodine, which should inhibit activation of ryanodine receptor, the observed changes were not considerable (Fig. 7B). These results show that 100 μM ryanodine does not block ryanodine receptors or if it does, this effect is independent of InsP3 receptors. Interestingly, like in CHO.CCR5 cells, when THP-1 cells were treated with ryanodine, a slight increase in calcium release could be observed (Fig. 7A) and ryanodine increased even further the MCD induced calcium signalling.

We used 2-APB, an inositol 1,4,5-trisphosphate (InsP3) receptor antagonist and blocker of store-operated channels (SOCs) [21] to analyse the involvement of InsP3 in release of intracellular calcium in THP-1 cells (Fig. 7C). 2-APB brings the enhanced MCD-signal back to basal levels, which suggest that MCD treatment of THP-1 cells results in an increased activation of either InsP3 or SOCs. These results indicate that MCD changes the endoplasmic reticulum membrane and increases the open probability of all ryanodine and inositol 1,4,5-trisphosphate (InsP3) receptors.

4. Discussion

The detailed mechanisms of how chemokine receptors induce the release of intracellular calcium are still not completely identified. Previous reports by us and others highlighted the importance of cholesterol in the plasma membrane for effective CCR5 signalling [6,8,11,22]. Altogether there is a certain problematic with comparing the different literature values for CCR5. Most of the experiments were performed in different cell lines and there is no conclusive study so far, whether the release of calcium ions in cell lines like CHO cells is actually similar to monocytic cells like THP-1 cells. Cell type specificity has been shown for some chemokine receptor signalling events. For example chemokine

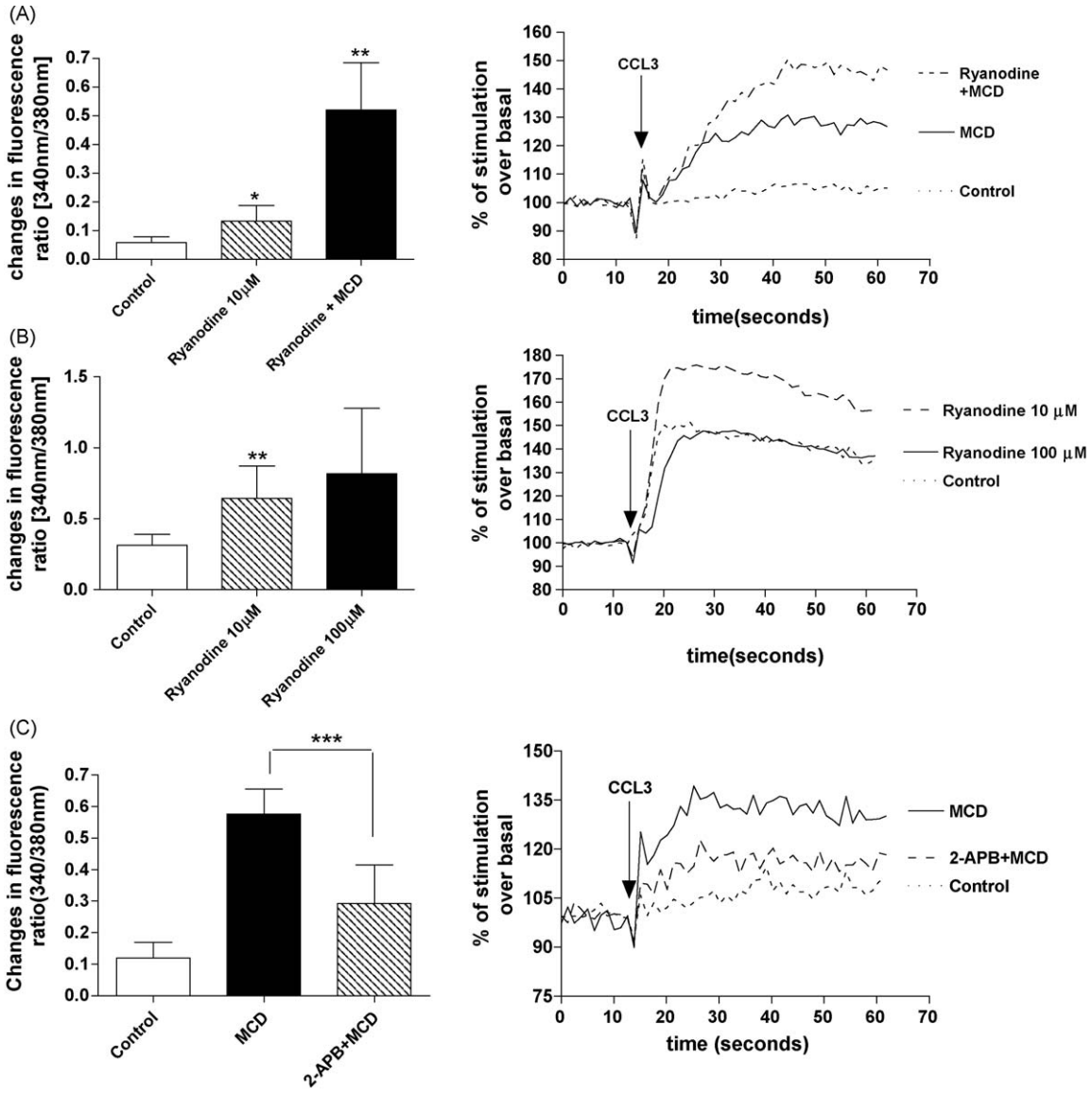

Fig. 7. Differential regulated release of intracellular calcium in THP-1, HEK.CCR5 and CHO.CCR5 cells. (A) THP-1 cells and (B) CHO.CCR5 cells were treated with vehicle (control), ryanodine or ryanodine plus MCD for 1 h before receptor activation with 100 nM CCL3. Ryanodine slightly increases the release of intracellular calcium in these cells. Significant changes towards control are indicated by asterisks ($^{*}p < 0.05$, $^{**}p < 0.01$). (C) THP-1 cells were pre-treated with vehicle (control), MCD or 2APB plus MCD for 1 h before addition of 100 nM CCL3. 2-APB reduces the release of intracellular calcium in the presence of MCD. Data are expressed as either changes in fluorescence ratio [340 nm/380 nm], where the basal line before addition of chemokine is subtracted from the peak fluorescence after addition of chemokine or as percentage of stimulation over basal, where the basal level is normalised to 100%. Data represent mean ± s.e.m. from at least three independent experiments for the bar charts and a representative tracer for the calcium flux.

receptors can be crosslinked to purinergic receptors like P2Y, when pre-treatment of cells with ATP increases the signal via the chemokine receptor [23]. This crosslinking seems to be cell type specific, since it can be observed in CHO.CCR5 cells, but not in THP-1 cells [24]. Therefore we analysed in this study the release of intracellular calcium in different cell lines with an emphasis on understanding the receptor activation pattern in the monocytic cell line THP-1.

Changing the cholesterol concentration in the plasma membrane of cells using MCD or similar agents generally decrease the signalling properties of the receptor [6,8,11], but it can also disrupt lipid rafts and modulate the activity of multiple signalling pathways, like activating the Ras-Erk pathways in T-lymphocytes [25]. Unlike other cell types, treatment of THP-1 cells with MCD leads to a vast increase of the CCL3 induced signal. This actually contradicts the results obtained by Nguyen et al. in CEM-NKR-CCR5 cells and shows that cholesterol extraction has distinct effects in chemokine receptors signalling depending on the cell type. The increase in calcium release observed here is not due to the lack of cholesterol, since lovastatin and simvastatin treatment, which both decrease the amount of cholesterol in the membrane, have the opposite effect as MCD. In fact lovastatin and simvastatin significantly decrease CCL3 induced signal in THP-1 cells, similarly to the results observed in HEK.CCR5 and CHO.CCR5 cells. However unlike MCD, lovastatin also decreases the amount of receptor on the cell surface, which could account for inhibitory effect of statins when compared to MCD.

The question remains how MCD treatment increases the signal. There is a suggestion that MCD treatment leads to a loss of Gαi expression in THP-1 cells [26], this might then lead to preferential coupling of CCR5 to Gαq and therefore to an enhanced release of calcium. We already showed for CHO.CCR5 and HEK.CCR5 cells that MCD treatment changes coupling of CCR5 from Gαi to PTX independent G-proteins [11] and there is the possibility that in

THP-1 cells this change in coupling is more distinct and effective than in other cells. PTX treatment in THP-1 cells does not completely block CCL3 induced calcium flux and in the presence of MCD there is no effect of PTX on receptor induced Ca^{2+} release.

Inhibition of endoplasmic reticulum Ca^{2+}-ATPases with thapsigargin completely blocks calcium release which is a clear indicator that in all cell lines tested the intracellular calcium stores used are situated in the endoplasmic reticulum. However MCD completely reversed thapsigargin effect in THP-1 cells, suggesting that a more fluid membrane in these cells might change the ability of this compound to block Ca^{2+}-ATPases. The involvement of ryanodine receptor on the calcium release is less clear however and it is distinct in the different cell systems. Ryanodine locks the ryanodine receptors (RyRs) at half-open state at nanomolar concentrations, yet fully closes them at micromolar concentration [27]. Importantly, ryanodine at 10 μM significantly enhances calcium release in THP-1 cells and this increase is even more pronounced in MCD-treated THP-1 cells. This implicates that MCD changes RyR function. Whereas in CHO and HEK cells ryanodine at low concentrations open up the calcium release channel and therefore increase the amount of Ca^{2+} ions that are entering the cytoplasm in response to CCR5 activation, high concentrations of ryanodine, which should close the calcium channels, do not seem to affect CCR5 induced calcium release significantly. These findings suggest that although RyR activation can potentiate calcium release, its open state does not seem essential for chemokine-induced signal transduction. This leaves a role for inositol 1,4,5-trisphosphate (InsP3) receptors in transducing CCR5 induced calcium release. InsP3 diffuses in the cytoplasm and binds to a receptor, the InsP3R, a polytopic integral membrane protein in the endoplasmic reticulum (ER), activating it as a Ca^{2+} channel to liberate stored Ca^{2+} from the ER lumen into the cytoplasm [28,29]. Some studies have shown that MCD treatment of cells resulted in phosphorylation and activation of PLC [30]. This increase in PLC activity could directly enhance the amount of calcium released due to an increase of InsP3 formation. 2-APB, an InsP3 antagonist and SOCs inhibitor reduces the calcium released in MCD treated THP-1 cells, which shows that at least in MCD treated cells part of the calcium released after activation is due to action of either the InsP3 receptor or SOCs.

Altogether this study shows the release of intracellular calcium is regulated in a distinct manner in different cells and highlights the importance of cholesterol depletion for optimal signal transduction in THP-1 cells and the involvement of ryanodine and InsP3 receptors in chemokine receptor responses. This work therefore offers more insight into key molecular mechanisms of chemokine receptor signalling

Acknowledgments

We thank Darren Sexton for help with flow cytometry analysis. We thank Maria O'Connell and David MacEwan for critically reading the manuscript and UEA for funding a studentship for CMC.

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