ELUCIDATING THE SIGNALLING MECHANISMS OF THE CC CHEMOKINE RECEPTOR 5 UPON CHEMOKINE STIMULATION

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ABSTRACT

Aim: CCR5 is a key receptor for a wide array of human pathologies such as Rheumatoid Arthritis and HIV-1 infection. The way CCR5 membrane domains composition affects CCR5 function and the mechanisms involved in CCR5 signalling are not fully understood yet. This study intends to characterise the signal cascades initiated by CCR5 activation with a special emphasis on understanding the role of cell membranes fluidity and certain CCR5 downstream proteins.

Methodology: Experiments were performed in CCR5 stably transfected CHO and HEK cells and in THP-1 cells. Calcium mobilization, cAMP accumulation and chemotaxis assays have been used to measure receptor activation. Flow cytometry and immunocytochemistry were used to measure proteins expression levels. Changes in gene expression were measured by qRT-PCR and analysis of proteins was conducted by Western blot. Small interfering RNA sequences were employed to knock down a specific protein.

Results: CCR5 signalling behaviour upon cholesterol depletion is different depending on the cell line used. Cholesterol depletion blocks calcium release in CCR5 transfected cells whereas in THP-1 cells it massively enhances calcium mobilization but blocks chemotaxis. Interestingly, the change in membrane fluidity by Methyl-β-Cyclodextrin MCD arrests CCR5 signalling through Gαi proteins in both cell systems. Cholesterol depletion has no effect on the expression and internalisation of the receptor in stably transfected cells but MCD significantly increases CCR5 levels in THP-1 cells. In addition, CCR5 calcium and chemotaxis responses are enhanced by the blockage of PKC ε and δ.

Conclusions: This study has highlighted that CCR5 signalling function can be highly modulated by drugs intended to cure CCR5-independent pathologies. We have shown that cholesterol modulating drugs and PKC inhibitors can alter CCR5 signalling pathways. Taken together, the results here described may be relevant for future therapies targeting this chemokine receptor.

ABBREVIATIONS

AC	Adenylyl Cyclase
ANOVA	Analysis of Variance
ATP	Adenosine Triphosphate
Bp	Base pair
Ca2+	Calcium
cAMP	Cyclic Adenosine Mono Phosphate
cE	Calculated Efficacy
CCR5	Chemokine Receptor 5
cDNA	Complementary Deoxyribonucleic Acid
cADPR	Cyclic Adenosine Diphosphate Ribose
CHO	Chinese Hamster Ovary
DAG	Diacylglycerol
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
EDTA	Ethylenediaminetetraacetic Acid.
ER	Endoplasmic Reticulum
ERK1, ERK2	Extracellular signal-regulated kinases
FAK	Focal Adhesion Proteins
FITC	Fluorescein Isothiocyanate
Fura2AM	Acetoxymethyl 2-[5-[bis[(acetoxymethoxy-oxo- methyl)methyl]amino]-4- [2-[2-[bis[(acetoxymethoxy-oxo-methyl)methyl]amino]-5-methyl-phenoxy]ethoxy]benzofuran-2-yl]oxazole-5-carboxylate.
G418	Geneticin
GDP	Guanine Diphosphate
GRKs	G Receptor Kinases
GTP	Guanine Triphosphate
GPCR	G-protein Coupled Receptor
HEK	Human Embryonic Kidney
HeLa	Henrietta Lacks Derived Cell Line
JAK	Janus Kinase
IP3	Inositol 1,4,5-Triphosphate

IP3R	Phosphotidylinsitol 1,4,5 Triphosphate Receptor
MAPK	Mitogen-activated Protein Kinases
MCD	Methyl-β-Cyclodextrin
NADPH	Nicotinamide Adenosine Dinucleotide Phosphate
NAD+	Nicotinamide Adenine Dinucleotide
PBS	Phosphate Buffered Saline
PDE	Phosphodiesterase
PH	Pleckstrin Homology
PI3K	Phosphoinositide 3-Kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-triphosphate
PKA	Protein Kinase A
PKC	Protein Kinase C
PLC	Phospholipase C
PTEN	Phosphatase and Tensin Homolog
PTX	Pertussis Toxin
qRT-PCR	Quantitative Real-time Polymerase Chain Reaction
RAFTK	Related Adhesion Focal Tyrosine Kinase
RyR	Ryanodine Receptor
RPM	Revolutions per Minute
S1P	Sphingosine 1 Phosphate
SDS	Sodium Dodecyl Sulphate
siRNA	Small Interfering Ribonucleic Acid
STAT	Signal Transducers and Activators of Transcription
TG	Thapsigargin
THP-1	Human Acute Monocytic Leukaemia Cell Line
TM	Transmembrane
TRITC	Tetramethyl Rhodamine Iso-thiocyanate

CONTENTS

TABLE OF TABLES

TABLE OF FIGURES

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 6

Chapter 7

Chapter 8

PUBLICATIONS

- **Cardaba CM**, Kerr JS, Mueller A (2008). *"CCR5 internalisation and signalling have different dependence on membrane lipid raft integrity"*, Cell Signal. 2008 Sep;20(9):1687-94. Epub 2008 Jun 3. PMID: 18573334.

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- Mueller A, Lalor R, **Cardaba CM** and Matthews SE (2010). *"Stable and sensitive probes for lysosomes: cell-penetrating fluorescent calix]arenes accumulate in acidic vesicles"*, Cytometry Part A. 79(A):126-136.

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CHAPTER 1- INTRODUCTION

1.1 G Protein Coupled Receptors

G protein coupled receptors (GPCR) are a large family of proteins expressed in numerous cells in the human body which share the same basic structural design of seven transmembrane domains, an extracellular amino-terminal segment and an intracellular carboxy-terminal tail. As its name indicates, GPCR are receptors coupled to G proteins, which are guanine nucleotide regulated proteins that transmit the external stimuli to the cytosol, connecting important signalling pathways (Rosenbaum et al., 2009).

The most commonly used system of classification divides the GPCRs into six classes: Class A: rhodopsin-like receptors, which includes over 80% of all GPCRs in humans and has been classified into 19 subgroups (A1-A19) based on a phylogenetic analysis. It comprises the Rhodopsine, cannabinoid, olfactory, amine, peptide, hormone, protein and platelet activating receptor-like GPCR (Kumari et al., 2009). Class B: secretin-like receptors; Class C: metabotropic glutamate receptors; Class D: pheromone receptors; Class E: cAMP receptors; and Class F: Frizzled/smoothened family, which represents the smallest class of GPCRs (Horn et al., 2003).

GPCR are a very diverse group of receptors that react to different stimuli, creating a wide range of cellular responses. Hormones, peptides, growth factors and cytokines are just some examples of the large number of molecules able to trigger GPCR activation. Stimulation of GPCR can alter physiological functions as vital as heart rate, white blood cell activation, and nervous connections (Millar and Newton, 2009).

Although GPCRs only account for 3% of the genes in the human genome (Fredriksson and Schioth, 2005), around 50% of the drugs that are currently on the market, target, one way or another, this huge family of transmembrane proteins (Flower, 1999). This is partly because of the wide range of ligands that bind to these receptors originating a huge variety of responses. There are about 800 genes in the human genome coding for GPCRs, half of which code for olfactory receptors that are responsible for our ability to smell and are not essential for human pathologies

(Klabunde and Hessler, 2002). Remarkably, only 15% of the considered "essential GPCR" have been successfully targeted with drugs (Flower, 1999), indicating that a great deal of research in this area still remains to be completed.

Two examples of GPCRs with a key role in human pathologies are adrenergic receptors, which control muscle contraction in several organs, and serotonin receptors, responsible for the mediation of inhibitory and excitatory effects in neurotransmission. Specifically targeting some types of adrenergic receptor represents one of the major approaches to treat diseases such as hypertension (Wiysonge et al., 2007), and asthma (Ortega et al., 2007) whereas serotonin receptors are the target of drugs such as antidepressants, antipsychotic or antimigraine agents (Carter et al., 2009). These examples illustrate the large variety of responses that can be initiated by one type of GPCR.

Table 1.1 Example of GPCR used as target of commonly prescribed drugs

Drug name	Target receptor	Pathology
Salbutamol	β2 Adrenergic receptor (agonist)	Asthma
Atenolol	β1-Adrenergic receptor (Antagonist)	Hypertension
Losartan	Angiotesin AT1 receptor (Antagonist)	Hypertension
Ranitidine	Histamine H2 (Antagonist)	Stomach Ulcer
Morphine	μ-Opioid receptor (Agonist)	SNC (Pain)
Ipatropium	Muscarinic receptors (Antagonist)	Asthma
Montelukast	Lekotriene receptor (Antagonist)	Asthma
Sumatriptan	Serotonin (5HT) receptor (Agonist)	Migraines

1.2 Chemokine receptors

Chemokine receptors are a special type of GPCR, characterised by their response to a series of small peptides called chemokines. Chemokines are small chemotactic cytokines (8-15 KDa) secreted by a wide number of cells upon certain stimuli

(Baggiolini et al., 1997). Among the main functions of this family of cytokines are recruitment of white blood cells to sites of inflammation through rearrangement of the cell cytoskeleton and stimulation of vital signalling pathways in white blood cells through chemokine receptors activation (Thelen, 2001). Chemokine receptor coordinate leukocytes biological activity by directing their movement towards a chemokine gradient and promoting numerous signalling events required for the important role of leukocytes in the immune response. Most chemokine receptors are primarily expressed in leukocytes, the two exceptions being CXCR4, that is also expressed in platelets, and Duffy (DARC), which is mainly expressed in erythrocytes.

Chemokine receptors belong to class A1 and class A2 of GPCR (Murphy et al., 2000). They are classified by their ability to signal on binding one or more members of the chemokine superfamily into 18 different types. In order to comprehend chemokine receptors nomenclature, chemokines classification should be described first.

There are four main subfamilies of chemokines which are classified according to the number and position of cysteine molecules. The CC chemokines (or β chemokines) have four cysteines, two of them adjacent; the CXC chemokines (or alpha chemokines) have four cysteine residues, two of them separated by one amino acid. The third subfamily comprises the C chemokines (or γ chemokines) and they only have two cysteines which are situated in the N-terminus and the C-terminus. The last group, that of the CX3C chemokines (or d-chemokines), have four cysteines, two of them separated by three amino acids (Thelen, 2001).

The majority of chemokine receptors are classified into those binding CXC chemokines, CXCR1 to CXCR5 or those binding CC chemokines which consists of 9 receptors, CCR1 to CCR9. A further receptor, initially designated D6 binds to CC chemokines and has thereby been suggested to be termed CCR10, whereas the receptors CX3CR1 and XCR1 bind to CX3CL1 (fractalkine) and XCL2 (lymphotactin) chemokines respectively. An additional chemokine receptor, known as the Duffy antigen receptor for chemokines (DARC) has been shown to bind both CC and CXC chemokines (Murphy et al., 2000).

Chemokine receptors are formed of 340-370 amino acids that create seven transmembrane hydrophobic loops, with an external N-terminal domain and an internal C-tail domain. All chemokine receptors share the following structural characteristics: several cysteines residues in all the extracellular domains; a conserved DRYLAVVHA sequence in the second intracellular loop which seems to be essential for G protein interaction with the receptor; and a short and positively charged third intracellular loop which has been found not to be essential for the receptor function (Oppermann, 2004). The cysteine residues located in the second and third extracellular loops form a disulfide bridge found in all GPCR. Chemokine receptors form an extra disulfide bond which is believed to be important for receptor conformation in the membrane and ligand binding (Baggiolini et al., 1997). Ligand binding occurs through a first interaction with the N-terminus of the receptor followed by contact with the second extracellular loop, generally responsible for ligand specificity (Samson et al., 1997).

Chemokine receptors are known to participate in several human pathologies. To enumerate a few of them: CCR1 and CCR2 receptors play an important part in inflammatory diseases such as RA or atherosclerosis (Bhat et al.; Conductier et al.; Pease and Horuk, 2005) whereas CXCR1 and CXCR2 have been involved in psoriasis, asthma and other skin conditions (Murdoch and Finn, 2000). CCR3, expressed in the cell surface of eosinophils, Th2 lymphocytes, basophils and mast cells and binding the chemokines CCL11, CCL24 and CCL26, has been widely linked to the recruitment of inflammatory cells in allergic conditions (Pease, 2006). Additionally, the chemokine receptors CCR5 (Azenshtein et al., 2002; Vaday et al., 2006) and CXCR4 (Salcedo and Oppenheim, 2003) are clearly involved in certain cancers and inflammatory conditions as well as in facilitating HIV-1 virus entry into macrophages and T cells respectively (Mosier, 2009). In line with this characteristic of CCR5 and CXCR4, DARC expression in erythrocytes is required for the entry of *plasmodium vivax (p.vivax)* and *plasmodium knowlesi (p.knowlesi)* into these cells. Individuals that due to a genetic condition that causes a single G-to-A nucleotide substitution, producing a Gly44Asp substitution in the polypeptide chain, do not express DARC in erythrocytes, are resistant to these two parasites (Murdoch and Finn, 2000). Next, the characteristics and functions of CCR5 will be analysed in more detail.

1.3 CC Chemokine receptor 5

The chemokine receptor 5 (CCR5) is a member of the seven transmembrane G protein coupled receptor (GPCRs) family. It belongs to class A, the largest of the GPCR superfamilly classes which shares homology with rhodopsin, to the A1 subclass of GPCR (Horn et al., 2003). CCR5, along with other chemokine receptors, is activated upon binding to chemokines.

CCR5 is expressed in T cells, natural killer cells, monocytes, macrophages and langerhans cells (Murphy et al., 2000). Although CCR5 was initially believed to be only expressed in these cells, its presence has also been shown in the human brain in a variety of cell types including microglia, astrocytes, neurons, and vascular endothelial cells (Lavi et al., 1998). Chemokines binding to CCR5 promote important cellular changes such as calcium release, cell movement or secretion of different types of cytokines (Oppermann, 2004). These processes are essential for the body's immune response and drive the distribution of effector cells expressing CCR5 to sites of inflammation. There, upon interaction with antigens, these cells will secrete further CCR5 ligands, attracting more CCR5 expressing cells. This inflammation process will, under normal conditions, lead to infection control. Yet, on other occasions, if the recruitment of cells becomes excessive, it may lead to numerous pathologies such as Cancer, Rheumatoid Arthritis (RA), Multiple Sclerosis (MS), Alzheimer's disease (AD) or transplant rejection as will be analysed in more detail in this thesis (Ajuebor et al., 2006; Coussens and Werb, 2002).

CCR5 became a key receptor in cell signalling research when found to act as a co-receptor for the entry of the HIV virus into the cell. This finding was backed up by the discovery that people expressing a mutant form of the receptor with a 32-base-pair deletion (CCR5- d32), that is not expressed in the membrane, are highly resistant against initial infection by HIV-1 (Samson et al., 1996).

1.3.1 Structure

The complete crystal structure of rhodopsin, obtained in 2000 (Palczewski et al., 2000), was the first three dimensional GPCR crystal structure to be solved and was subsequently used as a template to elucidate the structural features of CCR5. Recently, the crystal structures of the ligand-activated human A_{2A} adenosine receptor, the human β_2 adrenergic receptor (β_2AR) and the avian β_1AR as well as the structures of an active

form of rhodopsin have been obtained (Rosenbaum et al., 2009). More importantly, the crystal structure of the first chemokine receptor has also been resolved recently. Wu et al. obtained the X-Ray structure of the chemokine receptor CXCR4 in its active conformation (see Figure 1.1). These discoveries open new fields of research as they will allow clarification of the conformational changes occurring upon GPCR stimulation that, in turn, will help to elucidate signal transmission through G proteins.

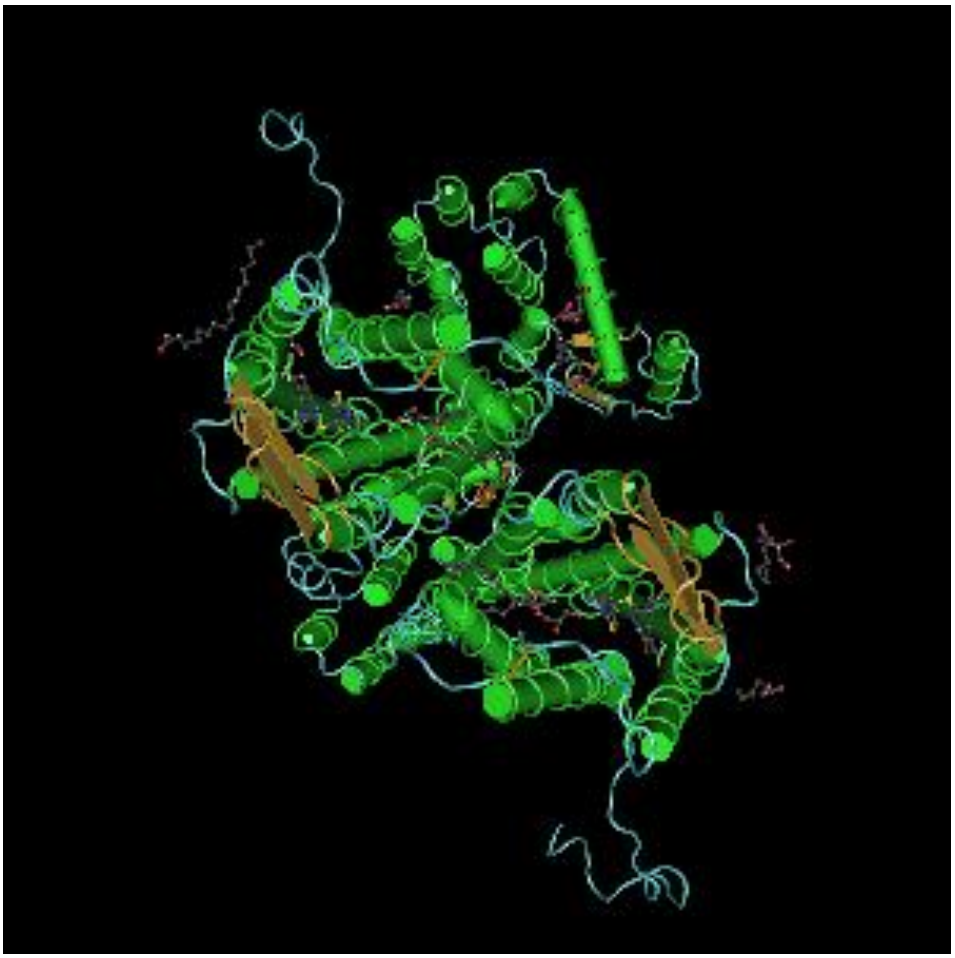

Figure 1.1 X-Ray structure of the CXCR4 chemokine receptor in complex with small molecule antagonist IT1t. Adapted from Wu et al. by NCB protein database program.PDB ID:3ODU. (Wu et al., 2010).

Figure 1.2 Two-dimensional structure of the human CCR5 sequence. Membrane topology of CCR5 with the extracellular space at the top and the intracellular space at the bottom. Amino acids shown to be critical for CCR5 function, such as the extracellular cysteines, the internal serines or the DRYLAVVHA sequence expressed in the second intracellular loop, are highlighted by filled circles. (Oppermann, 2004).

CCR5 has 352 amino acids, a molecular mass of 40.6 KDa, it is located in the chromosome 3p21 and possesses all the characteristics of chemokine receptors described above CCR5 undergoes several posttranslational modifications both in its amino-terminus and in its carboxyl-terminus: the three tyrosine residues in its N- terminus are modified by a sulphate group and the serine residues situated close to the amino-terminus have been shown to be O- glycosylated. These modifications in the extracellular domain of CCR5 strongly contribute to ligand binding and efficient signalling (Bannert et al., 2001). Similarly, CCR5's C-tail undergoes a small number of key post-translational modifications which are essential for its function. For instance, the three cysteines in the C-tail are palmitoylated, anchoring the receptor to the plasma

membrane and thus forming a fourth intracellular loop (see Figure 1.2). A body of evidence indicates that substitution of these cysteines by alanines abrogates receptor palmitoylation, phosphorylation, and internalisation (Blanpain et al., 2001; Kraft et al., 2001). Furthermore, Kraft's group demonstrated that the two contiguous leucine residues near the C-tail were largely accountable for receptor endocytosis whilst amino acids from 308 to 320 in the C terminus were essential for CCR5 coupling to G proteins (Kraft et al., 2001).

1.3.2 Signalling

CCR5 signalling pathways encompass a series of processes leading to activation of numerous molecules which trigger or block important cellular mechanisms. The first step in CCR5 signalling involves the binding of a chemokine to the N-terminus of the receptor, producing a change in the conformation of the receptor and the activation of heterotrimeric G proteins. As a result of CCR5 activation, cells start migrating, proliferating or transcribing new genes.

1.3.2.1 CCR5 ligands

In 1995, CCL3 (also named MIP-1α), CCL4 (MIP-1β) and CCL5 (RANTES) chemokines were identified as HIV suppressive factors secreted by CD8+ T lymphocytes (Cocchi et al., 1995). Following this extraordinary discovery, CCR5 was identified as the main co-receptor for HIV infection. CCR5 was then classified as the receptor for the CC chemokines CCL3, CCL4, CCL5, which are known to act as full agonists. Yet, CCR5 has also been shown to bind other chemokines such as CCL2, CCL8, and CCL13 with less affinity and efficacy (Blanpain et al., 1999; Mueller et al., 2001). Certain chemokines were later shown to block some of the responses initiated by stimulation of CCR5, providing evidence that the body has a regulatory system to decrease CCR5 activity.

For instance, CCL7 (Blanpain et al., 1999) and CXCL11 (Petkovic et al., 2004) have been shown to act as CCR5 natural antagonists.

One of the main characteristics of chemokine receptors is their versatility. Normally, one chemokine receptor can be activated by several different chemokines, each of which is also able to stimulate more than one chemokine receptor. The ability of a ligand to cause similar responses through the activation of different chemokine

receptors explains why, with the exception of CXCR4, chemokine receptors are not essential for life (Murphy et al., 2000). In this respect, an excellent example of the low specificity of chemokine receptors can be found in the fact that individuals who are homozygous for CCR5 delta 32 do not have any cellular dysfunction. In a system where multiple chemokine receptors are expressed, it is of great utility for the research field that CCL4 is specific for CCR5 (Murphy et al., 2000) since it allows the characterization of CCR5-specific pharmacological responses.

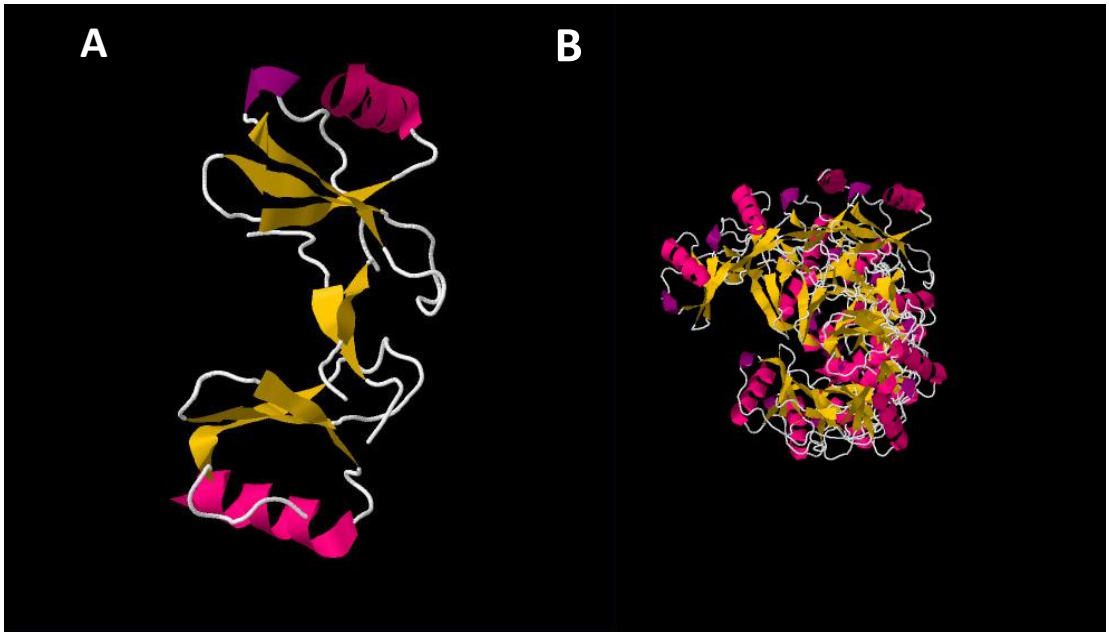

Figure 1.3 X-Ray structure of CCL3 (D27A) (A) and CCL4 (B) showing its biological assembly conformation. Adapted from Ren et al. by the program Protein Data Bank http://www.pdb.org/pdb/home/home.do. (Conductier et al., 2010).

1.3.2.2 Heterotrimeric G proteins

As their name indicates, GPCR transmit their signal by coupling to heterotrimeric G proteins. These membrane bound proteins are guanosinetriphosphatases (GTP-ases) that exist in an inactive GDP-bound form. They comprise a α and a βγ subunits which dissociate upon chemokine binding to the receptor. Although 27 Gα subunits, 5 Gβ and 14 Gγ have been identified to date, G proteins are mainly classified into 4 different groups ($G\alpha_s$, $G\alpha_i$, $G\alpha_q$ and $G\alpha_{12/13}$) depending on the G alpha-subunit involved in the signalling (Simon et al., 1991).

$G\alpha_s$ stimulates the enzyme adenylylcyclase (AC), increasing the concentration of cAMP in the cytosol. On the other hand, receptor coupling to $G\alpha_i$ produces inhibition of

cAMP production due to AC inhibition (McCudden et al., 2005). As for $G\alpha_q$, it has been shown to activate PLC (which is described in detail below) whereas $G\alpha_{12/13}$ is involved in cell migration through cytoskeleton rearrangement and actin reorganization (Oppermann, 2004; Tanabe et al., 2004). The Gα subunit is composed of two domains: a nucleotide binding domain with GTPase activity and a α-helical domain. The combination of both domains forms a pocket for the binding of the guanine nucleotide. The GTP-ase domain contains three flexible regions which change conformation upon both GTP binding and hydrolysis. Upon ligand activation of the receptor, GDP is exchanged by GTP and the latter changes the conformation of these regions, decreasing the affinity for the Gβγ subunit and allowing the binding of other intracellular molecules to the Gα subunit (Lambright et al., 1996). Subsequently, GTP is hydrolysed by the catalytic subunit of the nucleotide´s binding domain, that promotes the re-association of Gα with GDP and the Gβγ subunit. The re-coupling of both subunits terminates the signalling process due to a loss in affinity for effector molecules (McCudden et al., 2005).

Gα subunit behaviour upon treatment with pertussis toxin (PTX) allows classification of G proteins as PTX-resistant or PTX-sensitive proteins. PTX catalyses the ADP ribosylation of a cysteine residue situated at position 4 from the C-terminus of some Gα subunits (Krueger and Barbieri, 1995). This chemical modification locks the proteins in their inactive-GDP bound state and thus impairs the activation of downstream proteins. Almost all members of the $G\alpha_i$ family are PTX-sensitive. The rest of the G proteins, $G\alpha_s$, $G\alpha_{q/11}$ and $G\alpha_{12/13}$, are not affected by treatment with this toxin (Fields and Casey, 1997).

Table 1.2 Characterization of G proteins downstream effectors activation and sensitivity to PTX.

Subfamily	**Gα**	**Signalling**	**PTX –sensitive?**
Gi	Gz	AC	No
	Gi1 Gi2 Gi3 G0 Gt		Yes
Gs	Gs Golf	AC	No
G12/1	G12 G13	Stimulation cell growth/ migration	No
Gq	Gq G11 G14 G15 G16	PLCβ	No

The Gβγ subunit contains seven tryptophan-aspartatic acid sequences that repeat every 40 amino acids forming antiparallel β strands which gives a characteristic "torus-like structure" (McCudden et al., 2005). This subunit has traditionally been thought to be only involved in keeping Gα subunit inactive, but recent studies have shown its capacity to stimulate several signalling pathways. For instance, Gβγ subunit directly interacts with and activates PLC (Blank et al 2005, Boyer et al., Chen et al 2005), causing IP3 production and subsequent calcium mobilization. Other studies have shown the ability of Gβγ to activate ERK1/2, JNK, and p38 mitogen-activated protein kinases (MAPKS) (Aramori et al., 1997b; Obara et al., 2008).

Most notable among proteins able to be activated by Gβγ is PI3K-γ, which is responsible for the induction of important pathways such as chemotaxis (McCudden et al., 2005).

CCR5 is mainly known to associate with $G\alpha_i$ and, therefore, all cellular signalling pathways not related to cAMP inhibition are believed to be activated through the Gβγ subunit. However, some recent evidence (Cardaba et al., 2008; Cardaba and Mueller,

2009; Harmon and Ratner, 2008; Mellado et al., 2001) suggest that CCR5 also induces its response through $G\alpha_{q/11}$ or $G\alpha_{12/13}$ proteins which might share some of the pathways activated by $G\beta\gamma$.

1.3.2.3 Pathways activated by G proteins

CCR5 challenge with a chemokine triggers the activation of numerous intracellular cascades, some of which are independent of G protein activation (Mueller and Strange, 2004b). In this section we have focussed on the processes initiated by the dissociation of heterotrimeric G proteins.

1.3.2.3.1 Calcium mobilization

Heterotrimeric G proteins can stimulate several effector proteins when in an active state. Table 1.2 represents the main proteins activated by the different $G\alpha$ subunits. Calcium mobilization from intracellular stores upon chemokine receptor stimulation has been widely used to test the receptiveness of the receptors to different ligands. Three different pathways that lead to an increase in intracellular calcium mobilization have been described (Maghazachi, 2000). The first one involves the activation of PLC and consequent generation of inositol triphosphate (IP3) which releases calcium through the inositol triphosphate receptor (IP3R). The second pathway releases calcium through ryanodine receptors (RyR) in response to the formation of the second messenger cyclic adenosine diphosphate ribose (cADPR) which is produced by $G\alpha_s$ proteins activation from the product nicotinamide adenine dinucleotide (NAD^+).

The third pathway requires the formation of sphingosine 1 phosphate (S1P) by $G\alpha_i$ proteins, but the precise mechanisms through which this second messenger induces calcium release remain uncertain.

It is generally accepted that GPCR which couple to $G\alpha_i$ proteins, like CCR5, activate PLC through the $G\beta\gamma$ subunit (Oppermann, 2004). PLC catalyses the hydrolysis of phosphatidylinositol 4,5 diphosphate (PIP2) into inositol triphosphate (IP3) and diacylglicerol (DAG), the former being responsible for calcium release through ER stores. PIP2 is a membrane phospholipid that plays a central part in cell signalling by serving as substrate for enzymes like PLC or PI3K; acting as membrane anchor for many proteins; and helping in cell transport of vesicle exo- and endocytosis and

accompanying actin cytoskeletal rearrangements (Blazer-Yost and Nofziger, 2005).

There are five classes of PLC isozymes: PLCβ, PLCδ, PLCγ, PLCε, and PLCζ. CCR5 has been mostly shown to signal trough PLCβ (Oppermann, 2004). Hallmarks of PLC family members include a N-terminal pleckstrin homology (PH) domain which binds the Gβγ subunit, and EF, X, Y and C2 domains that form the catalytic core for phosphoinositide hydrolysis (McCudden et al., 2005). Bony et al. found that in cardiac cells, phosphatidylinositol 3-kinase (PI3-K) was needed for PLC-γ activation, translocation to the membrane and phosphorylation (Bony et al., 2001), linking the IP3 pathway with this important enzyme. The two products of the PLC reaction follow different pathways: IP3 binds the IP3R releasing calcium from the endoplasmic reticulum (ER) into the cytosol whereas DAG remains in the membrane and activate certain isoforms of PKC, which starts other important signalling pathways (Spitaler and Cantrell, 2004).

In the past few years, the way receptors coupled to $G\alpha_i$ proteins might promote intracellular calcium responses has become a matter of debate. As explained, $G\alpha_i$ proteins have been described as proteins capable of inducing inhibition of the enzyme AC which does not involve activation of calcium related pathways. Consequently, it has been hypothesised that GPCR coupled to $G\alpha_i$ proteins are able to promote calcium mobilization by stimulation of the βγ subunit. As outlined above, this subunit can engage PLC pathways and promote intracellular calcium mobilization (Blank et al., 1992; Boyer et al., 1992). Further experiments carried out by Cartier et al. (Cartier et al., 2005) complemented this work by showing that the βγ subunit had a special PLC-β-binding region. Furthermore, it is thought that the βγ subunit binds all its effectors (PLC-β, Gα-GDP and AC) through the same amino acids, which explains why dissociation of heterotrimeric G proteins is necessary for all the signalling pathways initiated by G proteins activation (Ford et al., 1998).

In basal conditions, the cytosolic free calcium concentrations are kept at 100 nM. However, calcium concentration increases more than tenfold after calcium release from ER stores (Berridge et al., 2000). This change in calcium concentrations causes activation of membrane channels called store operated calcium channels (SOCs) through a mechanism that has been termed capacitative calcium entry (CCE). This high increase in intracellular calcium concentration is necessary for the ER stores to be filled up again,

so that the next stimulus can trigger a new calcium flux (Marks, 1997). Changes in cytosolic calcium levels cause activation of calcium dependent pathways which are basic for many processes such as cell proliferation, apoptosis, metabolism and gene expression (Berridge et al., 2000). Rises in intracellular calcium, cause important cellular effects, including activation of proteins like protein kinase c (PKC), calmodulin, IP3R, and Ca2+ ATP-ases. Similarly, calcium binding to calcium sensor proteins promotes changes in the activity of several intracellular proteins (Roderick and Cook, 2008). In line with these data showing the important role of calcium for cellular signalling, it has recently been learned that in certain cancerous cells, calcium pathways leading to cell proliferation are over-stimulated. Alternatively, calcium pathways related to apoptosis are decreased. In this respect, some groups suggest that an increase in cytosolic calcium due to ER channels activation has apoptotic effects whereas an increase in intracellular calcium caused by calcium entry through membrane channels would have pro-proliferative effects (Roderick and Cook, 2008). These data provide perspective on the significance and versatility of calcium responses, pointing at the importance of understanding the way CCR5 modulates this signalling pathway.

1.3.2.3.2 Inhibition of cAMP accumulation

cAMP is an important second messenger produced by stimulation of the enzyme adenylyl Cyclase (AC). Mammalian ACs comprise a family of 10 members all of which share a similar structure which encompasses a short variable intracellular N-terminus; six transmembrane domains; a cytoplasmic domain of 360-390 amino acids; and other six transmembrane domains followed by another large cytoplasmatic domain of 255-330 amino acids (Sunahara et al., 1996).

cAMP levels are mainly regulated by $G\alpha_s$ and $G\alpha_i$ proteins. Yet, other intracellular elements such as the $\beta\gamma$ subunit, PKA or certain types of PKC have also been reported to modulate AC (Sunahara et al., 1996). $G\alpha_s$ stimulates AC increasing cAMP levels. On the other hand, $G\alpha_i$ activation is responsible for inhibition of AC and thus for inhibition of cAMP accumulation in the cell. In accordance with this evidence, CCR5 activation is known to be involved in reducing the concentration levels of cAMP (Aramori et al., 1997a; Cardaba et al., 2008; Oppermann, 2004; Rodriguez-Frade et al., 1999).

cAMP is derived from adenosine triphosphate (ATP) and used for intracellular signal transduction in many different organisms. Among the main functions of cAMP,

activation of PKA is one of the most relevant ones. PKA is a serine/threonine kinase consisting of two catalytic and two regulatory subunits. This enzyme is in a steady state until cAMP levels rise and this second messenger binds the cAMP sites in the regulatory subunits, automatically releasing the catalytic subunits. These subunits subsequently bring about the phosphorylation of serine/threonine residues of PKA substrates (Sveshnikov et al., 2002). Among these substrates, PKA phosphorylates a phosphodiesterase (PDE), an enzyme responsible for the down-regulation of cAMP levels and consequent PKA inactivation (Baillie and Houslay, 2005; Keravis and Lugnier, 2010).

PKA is involved in multiple intracellular functions, of which the following are worth highlighting: modulation of transcription factors (Goldman et al., 1997), regulation of some members of the MAP kinase family (Torgersen et al., 2002); and numerous substrates like RhoA (Meiri et al., 2009), IP3R (DeSouza et al., 2002) or GPCR (Gehret and Hinkle). Interestingly, there is evidence of PKA interaction with members of the PLC family, linking cAMP and calcium pathways (Benaud et al., 1998; Yoshida et al., 1996). On account of its versatility, PKA acts as a key modulator of immune responses, cell growth and cell migration (Torgersen et al., 2002). Considering the high number and diversity of its substrates, this kinase would be expected to affect several other cellular processes that are yet to be investigated. On top of this, PKA has been suggested to play a role in HIV-1 infection given that a reduction in its activation through cAMP significantly inhibits synthesis of HIV-1-specific DNA without affecting virus entry (Amella et al., 2005).

1.3.2.3.3 Cytoskeleton rearrangement and cell migration.

One of the main functions of chemokine receptors is to promote cell migration towards a chemokine gradient. The organised migration of cells following extracellular signals requires a series of changes in cell morphology which are initiated by asymmetrical distribution of key intracellular proteins. This initial process is called cell polarization. It involves transformation of cells from spherical to elongated structures with a leading edge formed by membrane protrusions and a rear edge (Hirata Terra et al., 2004). Chemotaxis is initiated by chemokine receptor stimulation but, since these receptors are homogenously distributed in the membrane, it seems clear that other mechanisms need to be present to control the direction of the movement (Barber and

Welch, 2006). There are other key molecules involved in cellular polarization that can explain why only some parts of the membrane suffer modifications that allow them to generate movement towards a defined direction.

Chemotactic processes are believed to be initiated by the activation of Ras through the free Gβγ subunit of G proteins which binds to PI3Ks promoting its activity (Freedman et al., 2008). PI3Ks, constitute a lipid kinase family that can be divided into three different classes, PI3K class I, II and III. Class I PI3Ks are involved in the formation of PIP3. There are two different subclasses of PI3Ks, the PI3K class I A, which includes PI3K α β and γ and PI3K class I B.

Class-I PI3Ks are heterodimers composed of a catalytic subunit (p110) and a regulatory subunit (p85) (Barber and Welch, 2006).

PI3Ks are characterized by their ability to phosphorylate the inositol ring 3'-OH group in inositol phospholipids. PI3K synthesises PIP3 in the inner membrane from the membrane lipid PIP2. It is known that PI3K is asymmetrically distributed in the cell (representing, therefore, the first asymmetrical event occurring during chemotaxis) allowing its product, PIP3, to accumulate at the leading edge of cells (Ward, 2004). PIP3 accumulation recruits Rho GTP-ases which are the main characters in regulating cytoskeleton reorganisation; a process needed for cellular chemotaxis (Barber and Welch, 2006). Small GTPases are monomeric proteins that belong to the family of GTPases and are similar to the α subunit of heterotrimeric G proteins. Like heterotrimeric G proteins, small GTPases have an inactive GDP-bound and an active GTP-bound state which is regulated by upstream proteins (Pertz, 2010). They are divided into 5 subfamilies: Ras, Rho, Rab, Ran and Arf, the Rho subfamily being the most studied in cell migration (Kandpal, 2006). Three members of the Rho subfamily of GTPases, RhoA, Cdc42 and Rac are the best characterised and are especially important in cell polarity regulation, lamellopodia formation and reorganization of the actin cytoskeleton (Evers et al., 2000).

Contrary to the recruitment of PI3K to the leading edge of cells, phosphoinositide phosphatase PTEN (phosphatase and tensin homolog deleted on chromosome 10 protein) and SHIP (SH2-containing inositol 5-phosphatase) are important enzymes responsible for the catalysis of PIP3 into PIP2, which accumulate at the rear of the cell.

PTEN activation is potentiated by the action of Rho A, leading to PIP2 accumulation and the initiation of actin-myosin reorganization events, that eventually will promote cell contraction at the rear of the cell (Barber and Welch, 2006).

CCR5 involvement in chemotaxis has been demonstrated on numerous occasions. For instance, there is evidence showing the co-partition of PI3K and CCR5 to the leading edge of migrating cells upon stimulation with RANTES (Gomez-Mouton et al., 2004). Additionally, numerous reports have shown that blockage of PI3K completely abrogates CCR5-dependent cell migration (Cheung et al., 2009; Huang et al., 2009a; Shideman et al., 2006). Some examples which support that CCR5 actively participates in cytoskeleton rearrangement are illustrated by the fact that Rac activation upon CCR5 stimulation is required for actin polymerization and cell migration (Di Marzio et al., 2005) and by the fact that CCR5 pathways engage Rho A intracellular cascades, clearly linking CCR5 with actin polymerization and cell movement (Man et al., 2007; Oppermann, 2004).

In contrast to the well characterised role of PI3K, the signalling cascades linking PLC with chemotaxis are less understood. A recent study (Chuang et al., 2009) proposes CCR5 chemotactic responses to be dependent on PLC activation, yet the mechanism behind it is completely unknown. Moreover, PLC is apparently critical for the migration of T cells (Bach et al., 2007) and for the activation of numerous second messengers that lead to activation of important proteins such as PKC, which is involved in cell migration in general (Abeyweera et al., 2009; Langlois et al., 2009) and in chemotaxis processes triggered by CCR5 stimulation in particular (Chuang et al., 2009; Langlois et al., 2009; Liu et al., 2009). In contrast to the important role of PLC in these cells, PLC is not required for the migration of neutrophils (Murphy et al., 2000). Putting these results together, it appears that so far there is not a clear mechanism that links PLC-dependent pathways with chemotactic processes. This effect might be explained by differences in the mechanisms leading to cell migration in T cells and neutrophils.

In general terms, whether calcium responses initiated by PLC are required for cell migration is still a matter of debate.

Other proteins known to play a role in CCR5 induced polymerization are the Focal Adhesion Kinases (FAKs). This group of proteins are non-receptor tyrosine kinases that

act as primary regulators during chemotactic processes. FAKs link integrins and proteoglycans to the actin cytoskeleton, regulating cell migration, proliferation and differentiation (Schlaepfer and Hunter, 1998). CCR5 activation through HIV or chemokines has been shown to activate FAKs pathways (Cicala et al., 1999; Mueller et al., 2002), which raises the possibility of CCR5-dependent cell migration involving FAKs proteins.

Moreover, CCL3 stimulation of CCR5 leads to phosphorylation and activation of RAFTK, one of the newest members of the FAK family, with subsequent activation of the cytoskeletal protein paxillin (Ganju et al., 1998), which is believed to help in cell migration processes.

Overall, there is abundant evidence to demonstrate that CCR5 initiates cell migration in a process that involves activation of PI3K, Rho GTP-ases and FAK proteins.

1.3.2.3.4 Gene regulation

CCR5 signalling activates different protein kinases that lead to regulation of transcription factors involved in gene regulation and cell proliferation. These pathways are of great importance in cancerous processes where an excess in cell proliferation is a fundamental element.

CCR5 can stimulate a number of mitogen activated protein kinases (MAPK) pathways. These proteins get activated both through stress stimuli as well as pro-inflammatory cytokines. They are involved in promoting gene regulation, cytokine secretion, and in inducing cell proliferation or cell survival/apoptotic responses (Huang et al., 2009b). Among the different types of MAPK, CCR5 has been shown to activate the ERK1/2, also known as extracellular regulated kinases, the JNK/SAPK and the p38 mitogen-activated protein (MAP) kinase (Mettling et al., 2008; Paruch et al., 2007; Popik and Pitha, 1998; Rahbar et al., 2006; Wong et al., 2001)

CCR5 up-regulation of the JAK-STAT pathway has also been widely documented (Gomez-Mouton et al., 2004; Mueller and Strange, 2004a; Wong et al., 2001). STAT proteins or signal transducers and activators of transcription, as its name indicates, are

proteins that transmit extracellular stimuli to the nucleus. Their activation leads to the up-regulation of some genes such as the proto-oncogene c-fos (Wong et al., 2001) or the modulation of the p53 transcriptional activity (Manes et al., 2003).

It is also known that stimulation of RAFKT proteins is implicated in MAPK and JAK pathways activation in a process initiated by CCR5 (Ganju et al., 1998).

To conclude this section; there is sound evidence of a significant link between CCR5 activation and the stimulation of important pathways that predominantly lead to inflammatory cytokines secretion and cell migration, survival and proliferation (see Figure 1.4).

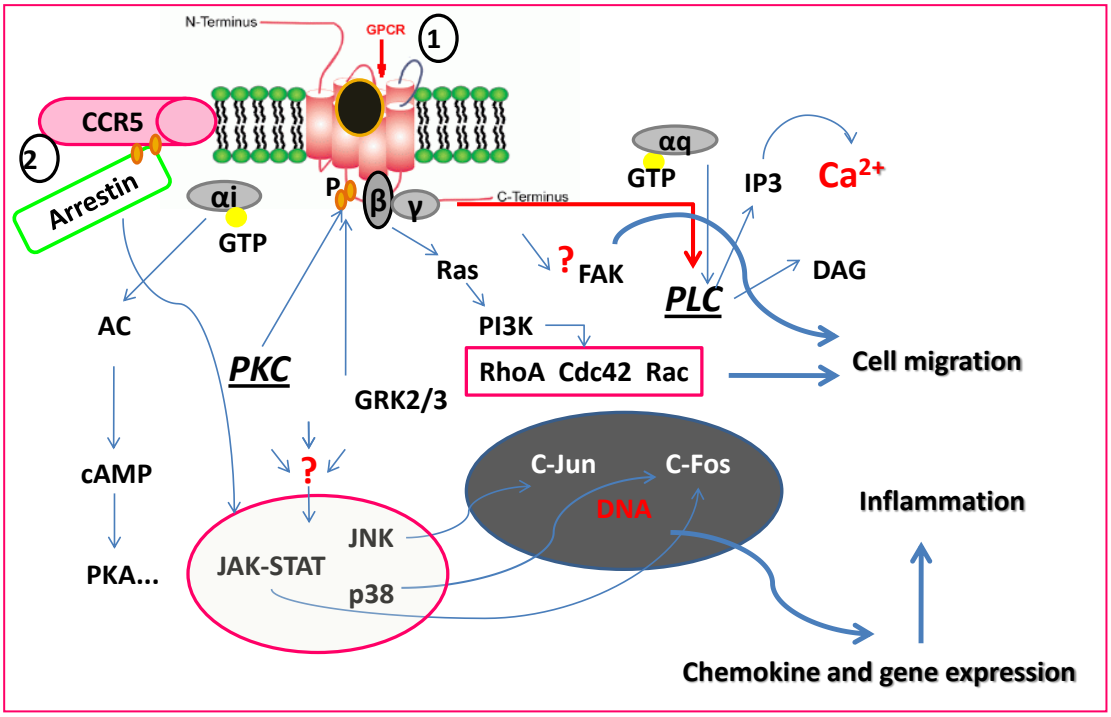

Figure 1.4 Schematic representation of some of the signalling pathways activated by CCR5. 1) upon chemokine binding the Gα and βγ subunits dissociate and activate different downstream effectors as indicated by thin arrows. CCR5 can activate $G\alpha_i$ or PTX-resistant Gα proteins here illustrated by $G\alpha_q$. Only some of the signalling connections have been indicated. For example DAG and calcium can activate PKC which is involved in more signalling pathways than just receptor phosphorylation. The question marks indicate that the exact mechanism leading to activation of JAK-STAT, MAPK (p38 and JNK) and FAKs upon CCR5 stimulation is not fully understood. 2) CCR5 phosphorylation by PKC and GRK2/3 induces the recruitment of β-Arrestin which sequesters the receptor to the cytosol. β-Arrestin activates numerous intracellular signalling cascades. CCR5 signalling leads to calcium release, cell migration, cytokines secretion, induction of gene expression and inflammation.

1.3.3 CCR5 desensitisation, internalisation and recycling

GPCR activation by a ligand generates a series of intracellular responses that prompt important changes in cell functions. In order to prevent these mechanisms from perpetuating indefinitely and, therefore, enabling the receptor to be reused, GPCR are desensitised, sequestered to endosomes and taken back to the cell membrane, where they become functional again.

CCR5, similarly to other chemokine receptors, is regulated by a desensitisation mechanism that starts with receptor phosphorylation in its C-terminus which leads to the binding of a molecule called β-arrestin that stops the signal transduction and promotes CCR5 internalisation into early endosomes. There, CCR5 is dephosphorylated to be recycled back to the plasma membrane (Oppermann, 2004).

CCR5 down modulation has been broadly studied due to its importance in HIV infection. CCR5 is phosphorylated by protein kinase c (PKC) and G protein coupled receptor kinases (GRKs) in serine residues of the C-terminus (Huttenrauch et al., 2005). PKCs are serine/threonine kinases divided into 10 different isozymes represented by 3 families (Newton, 2009) which are stimulated by phorbol esters and intracellular second messengers. The PKC isoforms involved in CCR5 phosphorylation have not yet been identified despite their importance in such a key physiologic process such as CCR5 desensitisation. Chapter number 7 will look at the mechanisms initiated by these enzymes in more detail.

GRKs are also a family of serine/threonine kinases that regulate the phosphorylation state of GPCRs. There are seven types of GRKs (GRK1-GRK7) from which only GRK1, GRK2, GRK5 and GRK7 are homogenously distributed in mammalian tissues (Barki-Harrington and Rockman, 2008). CCR5 is believed to be desensitized by GRK2 and GRK3 since blockage of these two isoforms with monoclonal antibodies impairs receptor phosphorylation (Oppermann et al., 1999). In addition to this, Aramori and co-workers succeeded in demonstrating that GRK2, GRK3, GRK5 and GRK6 over-expression in HEK.CCR5 cells highly increased CCR5 phosphorylation upon MIP-1β treatment, this effect being especially evident in GRK2 and GRK3 over-expressed cells (Aramori et al., 1997a). This finding points again at the special role these

two isoforms play in CCR5 desensitisation.

GRKs consist of three different domains, a N-terminal RH (regulators of G-protein signalling homology) domain, a central kinase domain and a C-terminal domain. Some reports which focussed on GRK2 provided deep information about its binding characteristics. It has been demonstrated that the RH domain of GRK2 binds to some Gα subunits, whereas the catalytic domain binds to the GPCR and a PH (pleckstrin homology) domain, situated in the C-terminal domain, binds to the Gβγ subunit (Barki-Harrington and Rockman, 2008). Interestingly, while studying the crystal structure of GRK2 it was found that all three domains are distributed in a way that allows the simultaneous interaction of each of them with its binding partner. Therefore, it appears that GRK2 phosphorylates GPCRs and, at the same time, binds and inactivates the α and βγ subunits of G proteins (Lodowski et al., 2003).

It is remarkable to note that, whereas PKC phosphorylation requires G protein activation, GRKs can phosphorylate GPCRs in the presence of PTX, what indicates that these kinases are able to directly recognise the change in conformation on the receptor induced by the ligand and proceed to receptor phosphorylation in the absence of G protein activation (Thelen, 2001).

Of interest, over the past few years, several studies have found novel GRKs-dependent signalling pathways initiated by the allosteric activation of these kinases by GPCRs activation (Cant and Pitcher, 2005; Penela et al., 2009; Pitcher et al., 1998). These discoveries indicate that GRKs have a larger number of functions in the cell than just GPCRs phosphorylation, some of these functions being responsible for facilitating cell migration or inflammation processes (Penela et al., 2010).

Serine phosphorylation of the C-terminus of CCR5 recruits β-arrestin whose binding to the receptor causes its dissociation from G proteins and consequently stops signal transduction. There are four different types of β-arrestin proteins in mammals, divided into visual and non-visual arrestins. The former are arrestins 1 and 4 which are exclusively expressed in retinal rods and cones. Arrestins 2 and 3 (also termed as β-arrestins 1 and 2) represent the non-visual ones since they are ubiquitously expressed in mammalian tissues (Barki-Harrington and Rockman, 2008). Arrestins promote receptor internalisation through the recruitment of the β2 adaptin subunit of the heterotetrameric

AP-2 adaptor complex through a sequence located between residues 394 and 396 of β-arrestin (Laporte et al., 1999). AP-2 binds to the heavy chain of clathrin proteins and promotes the formation of clathrin coated vesicles, which are formed by invagination of the inner part of the plasma membrane with the help of the molecule dynamin (Vila-Coro et al., 1999). As a result of CCR5 association with these molecules, the receptor is endocytosed into early endosomes where it gets dephosphorylated.

Then, CCR5 is returned to the cell surface in a process that is independent of protein synthesis and late endosomes (Mueller et al., 2002).

β-arrestin was initially characterised as the molecule responsible for the arrest of GPCR signal transduction. However, in the past few years this molecule has been re-characterised as being able to bind and activate other proteins (Gurevich and Gurevich, 2006). The complex formed by the receptor, β-arrestin, clathrin proteins and the 2-AP is called signalosome and has been found to recruit signalling proteins such as ERK1/2, p38 MAPK, and JNK, thus initiating new signalling pathways (Figure 1.4) (Cottrell et al., 2009; Cheung et al., 2009; McLaughlin et al., 2006). Arrestins have also been demonstrated to activate the non-receptor tyrosine kinases c-Src and PI-3K-AKT and NF-κB pathways (Yang et al., 2009).

Many studies have shown that CCR5 internalises through the mechanism described above, commonly known as clathrin coated pits (Mueller et al., 2002; Signoret et al., 2005). In addition to internalising through clathrin coated pits, CCR5 has been suggested to internalise through a different pathway that requires the presence of a special membrane structure called caveolae. The main arguments to claim the existence of an alternative internalisation pathway for CCR5 sustain that the receptor co-localises with Caveolin-1 (Venkatesan et al., 2003), the main protein of caveolae, and that specific inhibitors of this internalisation pathway can block CCR5 internalisation (Mueller et al., 2002). Furthermore, several studies have provided evidence that a CCR5 mutant unable to interact with arrestins, had no internalisation defects. This strongly supports the ability of CCR5 to follow alternative endocytosis pathways (Kraft et al., 2001; Venkatesan et al., 2003).

However, there does not seem to be an agreement on the internalisation pathways followed upon CCR5 activation. Signoret et al. (Signoret et al., 2005) have shown that

CCR5 exclusively internalises through a clathrin-dependent pathway. One of their main arguments to state that CCR5 is recruited to clathrin coated pits is its co-localisation in endocytic vesicles with the Transfering receptor (a marker of this endocytic pathway) and with clathrin proteins. This group not only demonstrated that CCR5 internalisation is via clathrin vesicles but also showed, by using diverse approaches, that CCR5 does not internalise via caveolae (Signoret et al., 2005). Similarly, optimal CCR5 internalisation processes in HEK.CCR5 cells were shown to require simultaneous transfection of GRK2 or GRK3 and β-arrestin, excluding the possibility of a high number of CCR5 molecules requiring Caveolin dependent internalisation pathways (Aramori et al., 1997a).

1.4 CCR5 implications in human pathologies

As previously outlined, CCR5 has an important role in different diseases, including acquired immunodeficiency syndrome (AIDS), cancer and some inflammatory diseases such as RA, MS or Alzheimer's disease AD.

In this section, CCR5 participation in these pathologies will be analysed and special emphasis will be put on how CCR5 signalling and expression may affect these pathologies.

1.4.1 HIV infection

In 1996, CCR5 (Deng et al., 1996) and CXCR4 (Dimitrov, 1996) were discovered as the co-receptors for the Human Immunodeficiency Virus type 1 (HIV-1). A decisive factor that led to this extraordinary discovery was the identification, one year earlier, of the so called HIV-suppressive factors (HIV-SF): CCL5, CCL3 and CCL4 secreted by CD8+ cells (Cocchi et al., 1995). HIV-1 needs the receptor CD4 and either CXCR4 or CCR5, depending on the phase of the infection, to enter into cells. HIV starts infecting macrophages through interaction with CD4 and CCR5 (macrophage tropic), being only in late states of the disease when the virus attacks CD4+ T cells by attaching itself to CD4 and CXCR4 (T cell tropic) receptors. Entry of the virus into host cells requires the anchoring of the viral envelop glycoprotein gp120 to CD4 and the N-terminus of CCR5 or CXCR4. This induces changes in the virus conformation that lead to the exposure of

another envelop glycoprotein called gp41. Gp41 assists in the fusion between the host cell and the virus. For instance, the fusion inhibitor drug enfuvirtide works by binding to gp41 and preventing HIV entry into the cell (Poveda et al., 2005).

CCR5 binding ligands act as antiviral molecules mainly due to their ability to down regulate the receptor and, to a lesser degree, due to the steric effect they cause by binding to the receptor which impairs virus binding (Cocchi et al., 1995). Small agonist molecules like TAK-777 or PSC-RANTES and derivatives were developed in order to mimic the antiviral effect of chemokines. These inhibitors, which are still under investigation, interfere with receptor trafficking, thereby inducing long-lasting intracellular sequestration and blocking its return to the cell surface (Escola et al., 2010; Este, 2003). More recently, a small molecule which antagonises CCR5, maraviroc, has been commercialised for the treatment of HIV infection. Maraviroc works by binding to CCR5 and preventing the binding of gp120 to the receptor. This entry inhibitor is the first US Food and Drug Administration-approved drug from a new class of antiretroviral agents which, instead of targeting HIV-1, are aimed at host proteins (MacArthur and Novak, 2008). Maraviroc, which is only active against macrophage tropic viruses, has been shown to effectively reduce HIV-1 infection.

Because little resistance to this compound has developed since its commercialisation, it is considered a key complement to highly active antiretroviral therapy (HAART) (MacArthur and Novak, 2008; Reuter et al.).

1.4.2 Cancer

CCR5 signalling is a key aspect in many cancers. CCR5 stimulation can lead to activation of many pathways involved in cell migration, apoptosis regulation and cell proliferation. Also, CCR5 signalling helps recruiting white blood cells to increase inflammation, which in certain conditions has been suggested to play a more important role in favouring tumour spread than in the fight against the disease (Coussens and Werb, 2002). The activities of the CCR5 ligand CCL5 are well known to be associated with several types of cancers which, in turn, introduce the possibility that CCR5 has a key role on them. For example, CCL5 is expressed in 74% of biopsy sections of breast carcinoma patients and, more importantly, this percentage increases along with disease progression (Luboshits et al., 1999). The CCR5-CCL5s axis is also very important for

prostate cancer progression as CCL5 was shown to induce migration of prostate cancer cells; the CCR5 antagonist TAK-779 was able to inhibit this process (Vaday et al., 2005).

Further studies showing CCR5 contribution to pathological cell migration were performed in oral cancer cells where Chuang et al. demonstrated that blockage of CCR5 downstream effectors like metalloprotease 9 (MMP-9) or PLC could inhibit cell migration.

Additionally, they also demonstrated that cells expressing higher levels of CCR5 migrated more aggressively (Chuang et al., 2009).

This is not the first study highlighting the importance of CCR5 in the function of MMPs. These proteins form a group of zinc-binding proteases that play key roles in cancer spread by modulating processes like cell differentiation, remodelling of the extracellular matrix (ECM), vascularisation and cell migration (Chang and Werb, 2001). Several studies have shown that CCR5 stimulation can induce cancer processes by up-regulating the expression of MMPs. For instance, the human chondrosarcoma cancer cells, which present abnormally elevated expression levels of CCR5 and migrate upon CCL5 stimulation, were shown to present increased levels of MMP-3 upon CCL5 stimulation. Moreover, MMP-3 blockage was shown to inhibit CCL5-induced migration of these cancer cells (Tang et al., 2009). In addition to this, CCL5 is known to induce MMP-9 (recognized to promote metastasis processes in breast tumours (Benaud et al., 1998)) expression levels in breast cancer cells and to elevate vascularity in vitro assays (Azenshtein et al., 2002).

The invasion of tumour cells is a complex and not completely understood process. CCR5 has been clearly demonstrated to play an important part in cancer spread but more research is needed to fully understand the extent of its effect. The studies outlined above have discovered a new possible pathway through which CCR5 promotes cancer metastasis through the engagement of MMP dependent intracellular cascades.

1.4.3 Other inflammatory diseases

CCR5 signalling has been implicated in the progression of pathologies like, RA, MS and AD, all of which are characterised by an excess of inflammation. These pathologies have in common that an excess of white blood cell recruitment is highly related to the worsening of the condition.

RA is a chronic inflammatory disease that affects multiple synovial joints and which is believed to be caused by an immense infiltration of white blood cells into the synovial tissues. The concentration of these cells into the joints appears to be essential for cartilage destruction and induction of all the machinery responsible for tissue damage characteristic of RA. The chemokine receptors CCR2, CXCR3 and CCR5 are over expressed in T-cell infiltrates in these inflamed areas, and their blockage has been associated with an improvement of disease progression (Norii et al., 2006). CCR5 ligands, in particular, have been found to be over expressed in synovial tissue of RA patients, which points to the responsibility this chemokine receptor signalling has on RA (Desmetz et al., 2007; Norii et al., 2006). Moreover, two recent studies analysed the effect on RA progression of the 32 bp deletion in the gene of the chemokine receptor, CCR5, and found negative association between the expression of this mutant receptor and the inflammatory response generated during the course of RA (Pokorny et al., 2005; Rossol et al., 2009). It must be mentioned, however, that contrary to the data just outlined, other studies found no role for CCR5 in the progression of the disease (Martens et al.; van Kuijk et al., 2010). Moreover, there is one group actually reporting anti-inflammatory effects of CCR5 signalling in RA (Doodes et al., 2009). As a result of these discrepancies more research should be done to find out the real involvement of CCR5 signalling in the inflammation processes associated with RA.

A role for CCR5 in MS has also been broadly studied. CCR5 is expressed at low levels in the brain but it has been shown to be induced in certain neurological disorders like MS, AD or certain viral infections (Cartier et al., 2005). MS is a chronic demylinising disease of the human central nervous system with a clear inflammatory component (Szczucinski and Losy, 2007). An excessive number of CCR5 expressing macrophages and microglia cells are characteristic of MS lesions (Balashov et al., 1999; Sorensen et al., 1999). More importantly, MS suffering patients have a much higher percentage of CCR5 expressing T cells in blood than control patients (Szczucinski and Losy, 2007). It has been shown that these cells migrate toward RANTES and MIP-1

alpha, chemokines that are highly expressed in MS lesions (Balashov et al., 1999), in bigger numbers than T cells from healthy patients due to over expression of CCR5 in T cells from MS patients (Zang et al., 2000).

Bearing in mind these studies it is easy to hypothesise that an enhancement or decrease in CCR5 signalling may have great influence on the progression of this pathology through modulation of cell migration towards MS lesions.

Similarly to MS, AD patients also show increased levels of cytokines by activated microglia (Steinman, 2008). AD is characterised by an increased deposition of amyloid beta peptide which is generally accompanied by an increased presence of monocytes, macrophages and T cells in the activated microglial cells in the brain (Man et al., 2007). The role of CCR5 in this disease seems to resemble the one in MS; the promotion of an excessive recruitment of T cells to the target site, in this case, across the blood brain barrier (BBB) in a CCR5 and CCR5 ligand dependent manner (Giri et al., 2003; Man et al., 2007). Further evidence of the importance of CCR5 signalling in AD has been highlighted in an interesting study showing that CCR5 and MCP-1 alpha expression is required for amyloid beta induced inflammation, as well as for amyloid beta induction of transcription factors that lead to an increase in COX-2 expression and, thereby, to inflammation through the synthesis of prostaglandins (Passos et al., 2009).

To date, there are two drugs in the market that reduce CCR5 signalling. The first one is the antiviral Maraviroc, which not only prevents HIV from binding the receptor but also prevents chemokines from doing so, thus blocking CCR5 signalling pathways (MacArthur and Novak, 2008). The second group of drugs that have a negative effect on CCR5-mediated responses are statins. As it will be explained in Chapter 6, statins are capable of reducing CCR5 expression and signalling in different cell systems.

This effect is believed to contribute to the pleiotropic effects of statins and to help in the progression of diseases like atherosclerosis (Kleemann and Kooistra, 2005) or transplant rejection (Yin et al., 2007) in a cholesterol independent way.

Taking into consideration all these reports, it is not surprising that the use of statins or other anti-inflammatory drugs is being considered for the treatment of AD as an approach to reduce the recruitment of white blood cells to active sites. Nevertheless,

no conclusive results regarding its efficacy have been obtained to date (Sabbagh, 2009; Tong et al., 2009).

In general terms, it might be stated that an excess of CCR5 stimulation can have negative effects on the progression of the above described pathologies and, therefore, medication leading to a reduction of CCR5 signalling could be beneficial. To my knowledge there is only one condition where the absence of CCR5 in patients heterozygous for the CCR5 delta32 mutation has been shown negative for the progression of the disease. This is the case of the infection caused by West Nile virus, a pathogen that can cause fatal encephalitis in humans and which, apparently, is more effective in doing so in the absence of CCR5 expression (Glass et al., 2006).

Overall, it is important to bear in mind that modulating CCR5 can affect other pathologies and treatments which may have important implications for the safety of CCR5-blocking agents or other possible drugs modulating CCR5 expression or signalling.

1.5 Aims and outline of the project

After almost 20 years of intensive research, our knowledge of the mechanisms involved in CCR5 signalling pathways has largely improved. However, considering its importance in human physiology, there is still a lot of useful information missing from these chemokine receptor intracellular pathways. For example, the connections between CCR5 stimulation and gene regulation, so important for many cellular processes, are not crystal clear yet. Besides, the exact mechanisms leading to CCR5-dependent calcium release and the machinery activated in cell migration upon CCR5 activation are not fully understood and it is still an open question whether calcium release to the cytosol is required for other intracellular processes such as chemotaxis. Also, there appears to be, to a certain degree, a lack of knowledge about the influence of some key intracellular proteins on CCR5 signal transduction.

The work presented in this thesis aims at conducting a broad analysis of the signalling characteristics of the chemokine receptor, CCR5, as well as at clarifying the involvement of several enzymes on CCR5-related intracellular cascades. This study focuses on analysing CCR5 activation patterns in different cell lines whose normal

environment has been modified by different methods, from the inhibition of enzymes working downstream of the receptor to physical and chemical alteration of CCR5 surroundings. Calcium flux assays, which provide quick information about the effectiveness of a ligand in activating CCR5´s signalling machinery, have been chosen as the principal method to measure CCR5 stimulation. Other experiments based on cAMP accumulation or cell migration have also been required to determine CCR5 activation. In addition to the above assays, techniques such as Western blot, flow cytometry, fluorescence microscopy, small interfering RNA (siRNA) transfection or RT-PCR have been essential to complete the study.

Chapter 3 analyses the importance of cellular cholesterol and lipid rafts domains in the plasma membrane on CCR5 signalling, expression and internalisation. In this section cells have been treated with different cholesterol modulating agents like the cholesterol depleting drug MCD, or deprived of some membrane proteins to understand its effect on CCR5 functions in CCR5 stably transfected CHO and HEK cells.

Chapter 4 aims at comparing the behaviour of CCR5 in stably transfected cells with its signalling pattern in the monocytic cell line THP-1, naturally expressing CCR5. Similarly to the previous chapter, great importance is given here to CCR5 activation as measured by calcium release and cell migration upon changes in cholesterol levels through the use of MCD.

Chapter 5 looks at the mechanisms involved in calcium release from the ER. It analyses the role of different ER transmembrane proteins on CCR5-dependent calcium release through their modulation with agonists/antagonists. The research presented here also looks at the involvement of an IP3 independent pathway in the calcium signals initiated by CCR5 stimulation and also studies the importance of acidic vesicles on ER stores-dependent calcium release.

Chapter 6 investigates the effect of the commonly prescribed drugs statins on CCR5 signalling. Statins have been shown to affect cellular signalling in a cholesterol independent manner and in this chapter we examine this possibility by comparing their effects to those of MCD.
Arguments about the possible consequences of administering statins in certain CCR5-related pathologies are presented.

Finally, **Chapter 7** looks at the potential involvement on CCR5 activity of PKC inhibitors, drugs that are being currently studied as new therapeutic targets especially due to their potential as anticancer agents. Whether PKC desensitisation of CCR5 decreases its signalling response and the PKC isoforms possibly involved in doing so has been carefully looked into. In line with the research carried out on statins, this study focuses on understanding CCR5 signalling dependence on PKC enzymes as an approach to obtain a better understanding of CCR5-induced intracellular cascades but also as a useful tool to analyse the side effects that potential PKC-derived anticancer drugs could have in certain conditions where CCR5 is a key element.

CHAPTER 2- MATERIALS AND METHODS

2.1 Cell culturing

2.1.1 Description of cell lines

2.1.1.1 Chinese Hamster Ovary Cells (CHO)

Chinese hamster ovary (CHO) cells had been transfected with pcDNA3 encoding CCR5 and selected for stable expression in 10% foetal calf serum (FCS)–Dulbecco modified Eagle medium (DMEM)–glutamine (2 mM) in the presence of 400 μg/ml G418. CHO.CCR5 cells were routinely cultured in complete DMEM (Invitrogen) (DMEM, 2 mM L-glutamate, 10% foetal calf serum (Invitrogen), 100 U/mL penicillin 100 μg/mL streptomycin (Invitrogen), 1 mM sodium pyruvate (Invitrogen), 100 μm nonessential amino acids (Invitrogen)) supplemented with 400 μg/ml G418.

CHO.CCR5 cells were chosen for these experiments because they stably express CCR5 and have been widely used due to its easy culturing.

2.1.1.2 Human Embryonic Kidney Cells (HEK)

Human Embryonic Kidney cells stably transfected with CCR5 (HEK.CCR5) were a kind gift from British Biotech (Oxford, UK) and were cultured in complete DMEM (Invitrogen) supplemented with 100 μg/mL hygromycin B.

2.1.1.3 HeLa.RC49 Cells

HeLa.RC49 cells were obtained from D. Kabat (Platt et al., 1998). HI-Rclone of HeLa-CD4 cells that contains a low amount of CD4 (approximately10^4 molecules/cell) had been transfected with the retroviral vector SFF-CCR5 and susceptibility to infection by the Ba-L M-tropic HIV-1 isolate was analysed. The clone RC49 was shown to be the one expressing a higher level of CCR5 membrane receptors. HeLa.RC49 cells were cultured in complete DMEM (Invitrogen).

2.1.1.4 Acute Monocytic Leukaemia Cell line (THP-1)

THP-1 cell were bought from American Type Culture Collection (ATCC)

(Teddington, UK**)** and kept in complete RPMI medium (Invitrogen) [10% foetal calf serum (Invitrogen), 100 U/mL penicillin 100 μg/mL streptomycin (Invitrogen), 1 mM sodium pyruvate (Invitrogen), 100 μM nonessential amino acids (Invitrogen)].

THP-1 cells are monocytic cells that naturally express CCR5 as well as other chemokine receptors and represent a good model for studying CCR5 signalling in a non-transfected system.

2.1.2 Routine conditions for cell culture

Cells were grown in 75cm^2 flasks (Corning) at 37C° in a humidified atmosphere of 5% CO2. When cells had reached 70-90 % confluency, cells were removed from the flask by adding PBS (1.5 mM potassium phosphate monobasic, 3 mM potassium phosphate dibasic, 150 mM NaCl; pH 7.2) supplemented with 2 mM EDTA and incubating them for 10 minutes at 37C° and 5% CO2. After this period the cells that were still attached to the flask surface were obtained by softly shaking the flask. Cells were centrifuged at 182 g for 5 minutes and the supernatant was removed. The cellular pellet was resuspended in growth medium and the cell number was determined microscopically, using a haemocytometer, to be ready for experimenting with them or re-suspended in medium and seeded into a new flask to be left growing. Cells were cryopreserved following the next steps: one million cells were resuspended into 1 ml of 10% (v/v) dimethyl sulfoxide (DMSO) in FCS and transferred into cryotubes which were first wrapped in tissue and placed at -80 ºC overnight before put in liquid nitrogen (-196 ºC) where they could be kept stored for an indefinite period.

2.1.3 Materials and reagents

Chemokines were purchased from PeproTech (Rocky Hill, NJ) with the exception of CCL3 (D26A), which was generously donated by Lloyd Czaplewski of British Biotech (Oxford, UK). The form of CCL3 used in the present study, CCL3 (2-70) (D26A), has a reduced tendency to aggregate and has been reported to retain an identical affinity to CCL3 (2-70) for CCR5, while its ability to bind the receptor, signal in Ca^{2+} mobilization assays and to induce chemotaxis or thymidine suicide assays was also unaffected (Hunter et al., 1995), suggesting that it acts similarly to CCL3 (2-70). This

isoform has been referred to in a previous publications as CCL3 (2-70) (D27A) in comparison with the full gene sequence for CCL3 (Mueller et al., 2006). In the same report, it was shown that CCL3 (2-70) and the D/A mutant at position 26/27, however, signalled with higher potency and efficacy than other CCL3 isoforms. To simplify, in this study CCL3 (2-70) (D26A) is referred to as CCL3.

The Anti-CCR5 antibody, HEK/1/85a/7a, was produced by a hybridoma cell line donated by Dr Jane McKeating and was raised against intact CCR5 expressed in CHO cells. The anti $G\alpha_{q/11}$ antibody (C-19) and the anti-Caveolin-1 antibody and all the PKC isoforms antibodies were bought from Santa Cruz Biotechnology, (Heidelberg, Germany).

Secondary antibodies were obtained from Sigma-Aldrich (Poole, United Kingdom), or Invitrogen. nystatin, filipin, methyl-β-cyclodextrin (MCD), sucrose, pertussis toxin (PTX) and cholesterol were purchased from Sigma. Thapsigargin, lovastatin, simvastatin, caffeine, Ly294002, U73122, mastoparan, rottlerin, Go6976, CID 755673, GF10923X and 2-APB were purchased from Tocris (Bristol, UK). Ryanodine and bafilomycin-A1 were purchased from Calbiochem (La Jolla, California). The CCR5 antagonist maraviroc was kindly donated by Pfizer (Sandwich, UK).

Cells were incubated for 30 minutes to 1 hour at 37°C with filipin (5 µg/mL), sucrose (0.4 M), nystatin (50 µg/mL), MCD (10 mM), cholesterol (2 mM), thapsigargin

(1 µM), caffeine (10 mM), ryanodine (10 µM), bafilomycin-A1 (100 µM), Y27632 (10 µM), Ly294002 (20 µM), U73122 (10 µM), mastoparan (10 µM), rottlerin (4 µM), Go6976 (100 nM), CID755673 (400 nM), maraviroc (100 nM), GF109203X (50 nM or 5 µM), PMA (100 nM) and 2-APB (20 µM), Lovastatin was activated prior to its use as indicated by manufacturer's instructions. Cells were incubated for 3 days at 37 °C with lovastatin and simvastatin 10 µM unless stated otherwise and for 2 hours with PTX (1 µg/ml) before the assay was performed. All other materials for cell culture and buffer composition were bought from Fisher Scientific (Loughborough, UK).

2.2 Calcium Flux Measurements and analysis

[Ca^{2+}]i was determined using the method developed by Grynkiewicz et al. based on the use of the Ca^{2+}- sensitive fluorescent dyes (Grynkiewicz et al., 1985). The membrane permeable derivative of the ratiometric calcium indicator Fura-2, the acetoxymethylester (AM) form of Fura-2, Fura-2-AM (Sigma-Aldrich) was the dye of choice. Fura-2-AM was loaded into cells at a final concentration of 2µM.

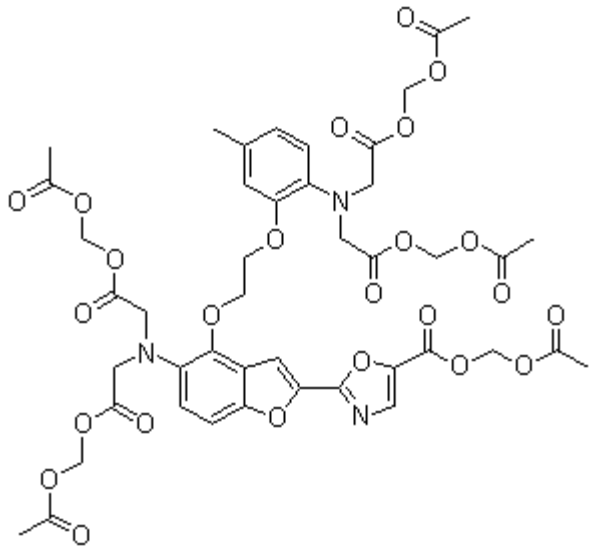

Figure 2.1 Structure of Fura-2-acetoxymethyl ester, Fura-2-AM. Image obtained from http://www.chemblink.com/products/108964-32-5.htm.

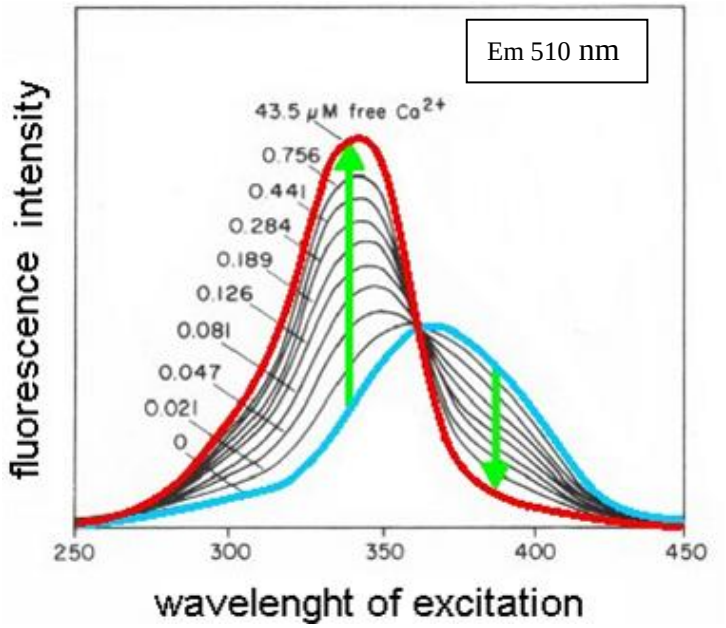

Figure 2.2 Intensity of fluorescence of Fura-2-AM plotted versus excitation wavelength for different calcium concentrations. In the absence of calcium Fura-2-AM maximum excitation wavelength is at 380 nm (blue peak) whereas at high concentrations of the ion its excitation wavelength occurs at 340 nm (red peak). The fluorescence intensity is measured at an emission wavelength of 510 nm. Image obtained from http://www.bphys.uni-linz.ac.at/bioph/res/icg/fura.html.

This assay is based on the property of Fura-2AM to change its fluorescence emission intensity at 510 nm upon binding to calcium ions. In the absence of calcium Fura-2-AM maximum fluorescence measured at 510 nm occurs upon excitation at 380 nm whereas at high concentrations of the ion its maximum fluorescence emission at 510 nm occurs upon excitation at 340 nm.

Cells were harvested with 2 mM EDTA/PBS after 5 min incubation at 37 °C and washed twice in calcium buffer (148 mM NaCl, 5 mM KCl, 2.5 mM CaCl2, 10 mM Hepes, 1 mM glucose, pH 7.4). The washing step was as follows: cells were resuspended in calcium buffer, centrifuged at 182 g for 5 minutes in a Fisher Scientific accuSpin 1R centrifuge and the supernatant was discarded. Cells were next incubated with 2 μM Fura-2AM (Invitrogen) at 37 °C for 1 hour in the dark. A stock solution of fura-2-AM was initially made in DMSO to a final concentration of 1 mM and stored at -20 °C in the dark. Inhibitors were added during the incubation period. Cells were next washed three times in calcium flux buffer (unless stated otherwise) and re-suspended in

the same buffer at 2×10^6 cells/mL. Subsequently 100 µl were pipetted into black bottomed, 96 well plates (Fisher Scientific, UK) and placed in a plate reader. Chemokine-induced intracellular calcium mobilisation was determined by BMGlabtechFluostar OPTIMA fluorometer (BMG Labtech, Germany). The peak values of intracellular calcium ion concentration following the chemokine stimulation were determined by exciting cells successively at 340 nm and 380 nm and measuring the alternate resulting emission intensities at 510 nm. Samples were exited at 340 and 380 nm with an interval of 1.1 seconds and a ratiometric trace was recorded and plotted into a graph by calculating the ratio of 340 nm emission over 380 nm emission. In order to get a value representative of the concentration of intracellular calcium, the average of measurements prior to chemokine stimulation were subtracted from the maximal ratiometric measurement recorded after chemokine stimulation.

2.3 Immunocytochemistry

Cells were seeded onto sterile glass square coverslips kept in a six wells plate. In the case of CHO.CCR5, HEK.CCR5 and HeLa.RCR9 cells, following cell adherence, cells were maintained in 1.5 ml 5% (v/v) FCS- DMEM medium until 90% confluence. The medium was removed and replaced with serum free medium prior to the performance of the experiment. Cells were exposed to experimental treatments as described in materials and reagents. In the case of THP-1 cells, cells were incubated in complete RPMI medium until they had reached the adequate concentration and then they were re-suspended in serum free RPMI and treated with different compounds. Following treatment, cells followed different procedures depending on the type of stain as described below.

2.3.1 CCR5 stain

Cells were washed twice with 500 µl cold PBS. The washing step was as follows: cells were resuspended in PBS, centrifuged at 182 g for 5 minutes in a Fisher Scientific accuSpin 1R centrifuge and the supernatant was discarded. Next, cells were stained with the HEK/1/85a/7a antibody for 1 hour at 4°C.

After this time cells were washed again and incubated with the anti-rat TRITC or FITC secondary antibody at a concentration 1:1000 at 4°C for 1 h.

Cells were then washed twice in cold PBS, fixed in 4% paraformaldehyde for 10 minutes and mounted in glycerol onto slides.

2.3.2 $G\alpha_q$ stain

For the $G\alpha_{q/11}$ stain, CHO.CCR5 and HEK.CCR5 cells were grown on cover-slips overnight. Cells were washed twice in 0.1% Triton X-100 for 20 minutes to cause cell permeabilization and fixed in 100% methanol for 3 minutes at -20°C. Cells were then incubated for 20 minutes in blocking buffer (5% non-fat dry milk in PBS), washed twice by resuspending them in PBS and centrifuging them at 182 g for 5 minutes in a Fisher Scientific accuSpin 1R centrifuge, cells were incubated with the Anti-$G\alpha_{q/11}$ antibody (C-19) for 1 hour at a final concentration of 2 µg/ml at room temperature. After having washed twice by resuspending cells in PBS-Triton 0.1% and centrifuging them at 182 g for 5 minutes in a Fisher Scientific accuSpin 1R centrifuge, the secondary anti-rabbit FITC antibody was added at 1:250 dilution for 1 h at room temperature. Cells were consequently washed three times with PBS-Triton 0.1%, once with PBS as previously explained and mounted onto slides.

Slides were left to dry at room temperature and images were taken using the Zeiss Axiovision 2 system.

2.3.3 Caveolin stain

For the Caveolin stain, after cells were grown on cover-slips in six wells plate overnight, cells were fixed in 4% paraformaldehyde, resuspended in PBS and centrifuged at 182 g for 5 minutes to discard the supernatant. This step was repeated twice. Cells were next permeabilised by incubation in 1% Triton X-100/2% BSA in PBS for 10 minutes at room temperature. Cells were then washed twice with cold PBS and incubated with anti-Caveolin antibody at a concentration of 1:500 for 1 hour at room temperature, washed twice in cold PBS and incubated with the anti-rabbit FITC secondary antibody at a concentration of 1:250 and at room temperature for 1 hour. Cells

were washed three times in PBS and mounted onto slides as before. Slides were left to dry at room temperature and images were taken using the Zeiss Axiovision 2 system.

2.3.4 PKC Stain

HeLa.RC49 cells were harvested with 2mM EDTA/PBS and washed twice in PBS and cells were grown on cover-slips overnight. THP-1 cells were placed onto a cover-slip and left to dry in the laminar flow hood for five minutes. Next, acetone was carefully added to both HeLa.RC49 and THP-1 cells and cells were placed at -20 °C for five minutes. After this time cells were washed twice with ice-cold PBS and primary antibodies specific for the different PKC isoforms were added to the cells and incubated at 37 °C for 1 h. After 1 hour cells were washed twice in PBS as previously described and incubated with anti-mouse FITC secondary antibody at 37 °C for 1 h. The nuclei were stained by the addition of mounting medium with DAPI (fluoro-gel mounting medium, Interchim). Slides were left to dry at room temperature and images were taken using an inverted Leica DMII fluorescence microscope

Table 2.1 Primary antibodies used in immunofluorescence

Antibody	Host	Manufacturer	Dilution/ concentration
Anti-CCR5 HEK/1/85a/7a	Rat	Gift from J. A. Mckeating, Reading	Undiluted
Anti-Caveolin	Rabbit	Santa Cruz	1:500
Anti Gq (C-19):sc-392	Rabbit	Santa Cruz	2µg/ml
Anti PKCα,βγ (MC5):sc-80	Mouse	Santa Cruz	1:200
Anti PKCα (H7) sc.8397	Mouse	Santa Cruz	1:100
Anti PKCε (E-50):sc-1681	Mouse	Santa Cruz	1:50
Anti-PKCζ (H-1): sc-17781	Mouse	Santa Cruz	1:100

Table 2.2 Secondary Antibodies used in immunofluorescence

Antibody	Manufacturer	Dilution
Anti Rat FITC conjugated IgG	Sigma- Aldrich	1:1000
Anti Rat TRITC conjugated IgG	Sigma- Aldrich	1:200
Anti-Mouse FITC conjugated IgG	Sigma-Aldrich	1:1000

2.4 Flow cytometry analysis

CHO.CCR5 and HEK.CCR5 were harvested with 2 mM EDTA/PBS and washed twice in PBS before the primary antibody was added. THP-1 cells were re-suspended in RPMI medium at a concentration of $2x10^6$ per ml and washed twice; cells were resuspended in PBS, centrifuged at 182 g for 5 minutes in the Fisher Scientific accuSpin 1R centrifuge and the supernatant was discarded. Cells were then incubated with

different inhibitors or left untreated (control) as previously described. After washing twice with PBS, the primary antibody was added. For CCR5 stain, cells were incubated with undiluted HEK/1/85a/7a stock antibody or left untreated as control for 1 h at 4˚ C to prevent internalisation. After this incubation period cells were washed twice in ice-cold PBS to remove the excess of CCR5 antibody. Cells were then incubated with the fluorescently labelled secondary antibody (anti-rat FITC) at 1:1000 dilution in fat-reduced milk powder (Marvel) 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. Data was analysed using Expo32 software.

2.5 Chemokine inhibition of forskolin-stimulated cAMP accumulation

The Promega cAMP-Glo™ assay was used to measure cAMP concentration within CHO.CCR5 cells. Briefly, the assay created to measure $G\alpha_i$ or $G\alpha_s$ activation, is based on the principle that cyclic AMP (cAMP) stimulates protein kinase A (PKA) holoenzyme activity, decreasing, thereby, the available ATP. The assay includes a luciferase enzyme able to emit light proportionally to ATP formation and it, thus, leads to decreased light production upon cAMP formation. The bioluminescence produced by the enzyme is inversely proportional to the amount of cAMP.

Cells were seeded out at 25,000 cells per well in a 96 well plate overnight on poly-L-lysine treated plates. Cells were next washed twice; cells were resuspended in PBS, centrifuged at 182 g for 5 minutes in the Fisher Scientific accuSpin 1R centrifuge and the supernatant was discarded Cells were then incubated with PBS in the presence of phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (1 mM) and inhibitors. Cells were stimulated with forskolin (10 μM) and varying concentrations of chemokine for 30 min at 37 °C. Cells were then lysed for 15 min and cAMP levels were determined following the manufacturer's guidelines with a BMG labtechPolarstar luminescence plate reader (BMG Labtech, Germany).

2.6 Chemotaxis

For chemotaxis experiments, CTX Plates (5μm pore plates) (NeuroprobeInc, USA) were used. The bottom of the plate was blocked by adding 30μl/well of blocking

buffer (1% BSA in RPMI). THP-1 cells were span down for five minutes at 182 g and resuspended in working buffer (0.1% BSA in RPMI). Cells were treated with the required experimental reagents for 30 minutes to 1 hour at 37 °C. Cells were next washed twice in RPMI medium (resuspended in RPMI, centrifuged at 182 g for 5 minutes in the Fisher Scientific accuSpin 1R centrifuge and the supernatant was discarded) and resuspended at a concentration of 25 x 10^4 cells per well. Chemokines were prepared at different concentrations in working buffer and added to the bottom of the plate in a volume of 31 μl per well as per manufacturer's instructions and the membrane filter was secured on the top. Cells were carefully added in a volume of 20 μl/well with a pipette as per manufacturer's instructions. The plate was subsequently incubated in a humidified chamber for 5 hours at 37 °C and 5% CO2. After this period, the membrane was scrapped with a sheet of paper, separated from the bottom of the plate and cells that had migrated through the membrane towards the different chemokine concentrations were counted by re-suspending the solution in the wells and pipetting 10 μl/well into the haemocytometer chamber (Marienfeld, Germany). Data were done in duplicates and expressed as number of migrating cells as counted in the haemocytometer.

2.7 Cholesterol assays

2.7.1 Cholesterol modification

For cholesterol depletion two different approaches were used: total cellular cholesterol was depleted using methyl-β-cyclodextrin (MCD) whereas *de* novo cholesterol synthesis was inhibited by treatment with lovastatin or simvastatin, inhibitors of HMG-CoA reductase. Plasma membrane cholesterol was extracted by MCD. This molecule is a well-established tool that selectively and quickly extracts cholesterol from plasma membranes in preference to other lipids. These treatments reduce intracellular cholesterol without affecting cell viability within the time window selected for the experiments. Control cells were left untreated. For cholesterol depletion, 10 mM MCD was incubated with cells for 30 minutes at 37 °C and 5 % CO2. Cholesterol synthesis inhibition was achieved by incubating cells for 1-3 days with different concentrations of lovastatin and simvastatin.

2.7.2 Total cholesterol levels measurement

Changes in cholesterol levels after the addition of different inhibitors were determined using the Amplex Red cholesterol assay (Invitrogen) according to manufacturer's protocol. The Amplex Red Cholesterol Assay Kit offers a simple fluorometric method for the quantitation of cholesterol using a fluorometer.

This assay is based on an enzyme-coupled reaction that detects both free cholesterol and cholesteryl esters. Cholesteryl esters are hydrolysed by cholesterol esterase into cholesterol, which is then oxidized by cholesterol oxidase to produce H_2O_2 and the corresponding ketone product. The H_2O_2 is then detected by Amplex Red reagent thanks to the addition of HRP which enables the interaction with a 1:1 stoichiometry between H_2O_2 and the Amplex Red reagent to produce highly fluorescent resorufin. Cells were pre-incubated with cholesterol oxidase and horseradish peroxidase and Amplex red for 20 minutes in the absence of light at 37˚C and next they were excited at 530–560 nm and emission fluorescence was detected at 590 nm by BMG labtechPolarstar luminescence plate reader (BMG Labtech, Germany).

2.8 Small interfering RNA (siRNA) transfection

2.8.1 siRNA optimization technique

HeLa cells were incubated in PBS/EDTA for 10 minutes at 37°C and 5 % CO2, washed (resuspended in PBS, centrifuged at 182 g for 5 minutes in the Fisher Scientific accuSpin 1R centrifuge and the supernatant was discarded), re-suspended at $6x10^4$ per ml and seeded in 125 µl of DMEM medium in a clear bottom 96 wells plate. Next, different concentrations of transfection reagent and different transfection reagents as well as different concentrations of siRNA negative vector were mixed up as explained below and gently added to the wells. The Allstarts transfection control we used is a siRNA sequence with no homology to any known mammalian gene and which was fluorescently labelled on the 3' end of the sense strand with rhodamine making it possible to visualise siRNA transfection efficiency.

2.8.2 siRNA transfection

2.8.2.1 Chemical-based transfection

HeLa cells were incubated in 2 mM PBS/EDTA for 10 minutes at 37 °C, washed, re-suspended at $1x10^5$ cells per well and were seeded in a 24 wells plate in 500 µl of DMEM medium and incubated at 37°C and 5 % CO2. Lyophilised siRNA (Caveolin-1 siRNA) was re-suspended in RNase free water (Quiagen) to form a 20 µM concentration stock solution. For each well: shortly before transfection, siRNA was diluted into 100 µl of DMEM medium FCS free at concentrations varying between 1.7 and 60 nM. The solution was pipetted up and down. Next, 1.5 µl of INTERFErin (Polyplus, France) were added to the mixture and the solution was vortexed for ten seconds. The eppendorfs were left at room temperature for 10 minutes to allow the complexes to form and then 100µl were added drop-wise to each well containing the seeded cells. The plate was gently swirled and incubated at 37° C and 5% CO2 for 48 to 72 hours. After this period, cells were removed by PBS/EDTA. The same number of cells from each well was collected and Caveolin-1 expression was analysed by western blot.

Table 2.3 siRNA data

Gene name	**siRNA**	
Caveolin-1	Target sequence 5´-AAGCATCAACTTGCAGAAAGA-3´	
	Sense	5´-GCAUCAACUUGCAGAAAGATT-3´
	Antisense	5´-UCUUUCUGCAAGUUGAUGCTT-3´

2.9 Western Blot

2.9.1 Protein extraction

HeLa.RC49 cells were seeded onto 35 mm culture dishes and maintained until 90% confluency. HeLa.RC49 cells were then incubated in PBS/EDTA at 37°C, centrifuged at 182 g per 5 minutes and washed twice with ice cold PBS. Cells lysis was done by re-suspension of the pellet with lysis buffer (Tris-HCl 0.1 M, 20% glycerol,

10% SDS, pH 7.6) followed by sonication (ten pulses of 3 seconds at 60% amplitude and 0.8 pulse) or by direct addition of sample buffer (4% SDS, 0.02% bromophenol blue, 20% glycerol, 10% mercaptopropanodiol, 80 mM Tris, pH 6.8) followed by 5 minutes boiling at 95°C. Whole cell lysates were next centrifuged at 13230 g at 4°C for 10 minutes. The supernatant was then collected and the pellet of cell debris discarded.

2.9.2 Protein quantification

The protein concentration of each lysate was determined using the Protein A280 Method (NanoDropND-1000 UV-Vis Spectrophotometer, Labtech International, UK). In order to add the same amount of protein to each well, the total concentration of protein was estimated by measuring absorbance at 280 nm. Although at 280 nm it is possible that some nucleic acids absorbance is present, this concentration would be the same in all the samples and, therefore, it would not interfere with the results. 2 μl of total cells lysate was put in the nanodrop and the total concentration of protein for each sample was acquired. The same amount of protein was then added to each well by diluting the cell lysate with the adequate amount of sample buffer.

2.9.2.1 Sample preparation

A certain volume of a 20% (v/v) loading buffer solution (160 mM Tris, 4% (w/v) Sodium docecylsulphate (SDS), 30% (v/v) glycerol, 0.01% (w/v) bromophenol blue, 12% (v/v) β-mercaptoethanol, pH 6.8) was added to each sample. Protein samples with loading buffer were heated at 85°C for 5 minutes to ensure complete protein denaturation and cooled down on ice for 5 minutes. Finally, samples were spun at 13230 g in a Fisher Scientific accuSpin micro centrifuge for 15 minutes.

2.9.3 SDS-PAGE gel electrophoresis

Protein samples together with a broad range protein marker (Bio-Rad, UK) were loaded onto SDS-PAGE gels for electrophoresis. The running gel layer was prepared at 12% acrylamide (0.67155g Tris/SDS 2% pH 8.8, 13% acrylamide v/v, 0.1% ammonium persulphate w/v, 0.01% TEMED v/v) and samples were loaded onto a 4.5% acrylamide stacking gel, (0.1089g Tris/SDS 2% pH 8.8, 4.5% acrylamide v/v, 0.1% ammonium persulphate w/v, 0.01% TEMED v/v) which allows the proteins to stack together when

they enter the gel. Samples were then run at a constant voltage of 180 V at room temperature until the dye front reached the bottom of the gel.

2.9.4 Protein transfer

A nitrocellulose membrane, several filter papers and two sponges were incubated in transfer buffer solution (25 mM Tris, 192 mM glycine, 10% methanol, pH 8.3) at room temperature for 15 minutes prior to assembly in a semi-dry transfer blotter (Bio-Rad, UK). The layers were placed as follows in the base of the transfer blotter: sponge, filter papers, nitrocellulose membrane, gel, filter papers, and sponge. The proteins were left to be transferred onto the membrane for 45 minutes at 15 V. Following transfer, molecular weight marker could be seen on the membranes, indicating protein transfer was successful.

2.9.5 Immunoblotting and development

The nitrocellulose membrane was next blocked in 0.5% (v/v) Tween-20 in PBS (PBS-T) with 5% (w/v) fat-reduced milk powder (Marvel) blocking solution for 1 hour at room temperature with gentle agitation. The blocking solution was then removed and the membrane was incubated in blocking solution containing the primer antibody at the adequate dilution (see table 2.4) at 4°C overnight on a rotary wheel. The next morning, the antibody was removed from the membrane by washing it three times in 0.5% (v/v) Tween-20 in PBS (PBS-T) for 20 minutes at room temperature with gentle agitation and the membrane was incubated for one hour with the secondary antibody (see table 2.5). After this period, the secondary antibody was washed three times in 0.5% (v/v) Tween-20 in PBS (PBS-T) for 20 minutes at room temperature with gentle agitation. Protein detection was performed by the addition of the Pierce ECL reagents (ThermoScientific) according to the manufacturer's instructions. The ECL reagents were added in the same proportion, left in the dark for 1 minute at room temperature and then drained with a clean filter paper and inserted between two layers of cling film inside an X-Ray Cassette. In the dark, the chemiluminescence film CL-XPosure^{TM} Film (Thermo Scientific) was placed on the top of the cling film and the cassette was shut. Paper was left in contact with the cling film allowing the chemiluminescence reaction to happen for a varying amount of time and then the film was manually developed using Kodak GBX

Developer (Sigma Aldrich) according to manufacturer's instructions.

For the β-actin control, after membrane development, it was recovered from the cassette, washed twice for 5 minutes in 0.5% (v/v) Tween-20 in PBS (PBS-T) at room temperature with gentle agitation and then washed for 15 minutes with the stripping blot plus strong solution (Millipore, California, USA) to remove the antibodies and ECL reagents from the membrane. After this period, the membrane was washed again in 0.5% (v/v) Tween-20 in PBS (PBS-T) and was next blocked in 0.5% (v/v) Tween-20 in PBS (PBS-T) with 5% (w/v) fat-reduced milk powder blocking solution for 1 hour at room temperature with gentle agitation. Next, the membrane was incubated with the mouse anti-β-actin antibody at the concentrations indicated in table 2.4 for 1 hour. The antibody was next removed from the membrane by washing it three times in 0.5% (v/v) Tween-20 in PBS (PBS-T) at 20 minutes interval and membrane was incubated for one hour with the secondary antibody (see table 2.5) at room temperature. After this period secondary antibody was washed three times in 0.5% (v/v) Tween-20 in PBS (PBS-T) at 20 minutes interval. Protein detection was performed by the addition of the pierce ECL reagents (ThermoScientific, UK) as explained above.

Table 2.4 Primary Antibodies used for Western blot experiment

Antibody	Host	Manufacturer	Dilution
Anti-CCR5 HEK/1/85a/7a	Rat	Gift from J. A. Mckeating, Reading	1:100
Anti-Caveolin	Rabbit	Santa Cruz	1:500
Anti-β-actin	Mouse	Santa Cruz	1:50000

Table 2.5 Secondary Antibodies used for Western blot experiment

Antibody	Host	Manufacturer	Dilution
Anti-Rat IgG HorseradishPeroxidase	Rabbit	Sigma-Aldrich	1:10000
Anti-Rabbit IgGHorseradish Peroxidase	Goat	Sigma-Aldrich	1:10000
Anti-Mouse IgG Horseradish Peroxidase	Goat	Sigma-Aldrich	1:10000

2.10 Analysis of gene expression by quantitative real-time polymerase chain reaction (qRT-PCR)

2.10.1 Cell preparation and RNA extraction

$3x10^6$ THP-1 or HeLa.RC49 cells were centrifuged at 182 g for 5 minutes in the Fisher Scientific accuSpin 1R centrifuge and the pellet was used for RNA extraction using the RNeasy mini kit (Qiagen) as per manufacturer's instructions. The RNeasy technology allows isolating up to 100 μg of RNA longer than 200 nucleotides on a silica membrane. Briefly, cells were re-suspended in 350 μl of RLT buffer to get disrupted.

1% 14.3 Mβ-meracaptoethanol was added to the lysis buffer shortly before cells were re-suspended in it. Pellet was homogenized by pipetting the sample up and down a few times. Next, 1 volume of 70% ethanol, which provides the right binding conditions for the RNA to bind the membrane, was added to the homogenized lysate and mixed well by pipetting up and down. Subsequently, 700 µl of this solution were transferred to a RNeasy spin column placed in a RNAse and DNAse free 2 ml collection tube, and centrifuged at 10,000 rpm for 15 seconds. The flow-through was discarded. The next step was to add 700 µl of RW1 buffer to the RNeasy spin column and spin it down for 15 seconds at 10,000 rpm to wash the membrane. The flow through was discarded. The next step was to wash the membrane twice by adding 500 µl of RPE buffer to the spin column and centrifuging 15 seconds the first time and 2 minutes the second time. Lastly, the spin column was carefully transferred to a new 1.5 ml collection tube and 50 µl of RNase-free water were added to the membrane to elute the RNA. The sample was centrifuged at 10,000 rpm for 1 min and the eluate from this step was added again to the column to elute possible RNA left in the membrane. After centrifuging at 10,000 rpm a last time, the RNA solution obtained was frozen down at -70 °C until cDNA was formed.

2.10.2 RNA quantification

Total RNA was quantified using Nanodrop 1000 spectrophotometer by adding 1 µl of the RNA solution to the nanodrop plaque and selecting the program nucleic acid on the software of the computer. The purity of the sample was determined by analysing the ratio of absorbance at 280 nm over 260 nm. The closer this value is to 2 the purer the concentration of the sample RNA.

2.10.3 cDNA Synthesis

50 ng/µl of RNA was employed to synthesize cDNA by using Taqman Reverse Transcriptase Reagents (Applied Biosystems). 50 ng/µl were added to the right amount of RNase-free water to get 4.5 µl RNase-free water-RNA per sample. For one sample 2.5µl of $MgCl_2$, 1 µl of 10 x RT buffer, 1 µl of dNTP, 0.25 µl of RNase and 0.5 µl of Random hexamers were added together forming a reaction mixture (see table 2.6). Next, 5,5 µl of the reverse transcription mastermix was added for each sample. Samples were

run on PTC-100 Peltier Thermal Cycler for 10 minutes at 21°C, 15 minutes at 42°C, 5 minutes at 99 °C and 5 minutes at 4 °C. cDNA was then diluted with 15 µl H_2O.

2.10.4 PCR

For PCR, SYBR green (Sigma Aldrich) was used to quantify gene expression using the Qiagen's real time PCR cycler, the Rotor-GeneQ, as per manufacturer's instructions (Table 2.7). For one reaction, 10 µl SYBR green, 4 µl of H_2O and 1µl of CCR5 or 18S forward + reverse primers, were pipetted to the special tubes containing already 5 µl of the diluted cDNA from the cells, which were placed forming a circle in the Qiagen's real time PCR cycler. The reaction mixture was then heated for 1 cycle of 2 minutes at 95°C followed by 40 cycles of 15 seconds at 95°C, and 40 seconds at 60°C. Gene expression was quantified in relation to the house keeping gene 18s and fold change was calculated from the 0 values.

Table 2.6 RT step**.** Mastermix for cDNA synthesis

Number of Samples	MgCl2 (25µM)	10 x RT Buffer	dNTP	RT	RNase	Random Hexamers
1	2.5µl	1µl	1µl	0.25µl	0.25µl	0.5 µl

Table 2.7 PCR-step

Number of samples	cDNA	SYBR green PCR Mastermix	ddH20	Primers forward + Reverse (5µM)
1	5µl	10 µl	4 µl	1 µl

Table 2.8 Genes analysed by RT-PCR and primers used. (Invitrogene)

Gene name	**Primer**
CCR5	Forward 5'-TGC TAC TCG GGA ATC CTA AAA A- 3' Reverse 5'- AAG AAT TCC TGG AAG GTG TTC A-3'
18s	Forward 5'- GCA ATT ATT CCC CAT GAA CG-3' Reverse 5'- GGG ACT TAA TCA ACG CAA GC-3'

2.10.4.1 qRT-PCR data and statistical analysis

Results were analysed by the standard curve method. In this method, a standard curve is first created from an RNA sample of known concentration or an RNA sample which had been given arbitrary values. This curve is then used as a reference standard for extrapolating quantitative information for mRNA targets of unknown concentrations. The RNA used for fabricating this standard curve comes from the same sample than the one used to measure the target genes. Five standards were created from total cDNA. Samples were diluted five times following a serial dilution technique so that the more concentrated standard was given a value 1 and the more diluted one a value 1/16. By using this method, fold change in the target gene is calculated with the use of a reference gene. Changes in the average copy number in the experimental sample are divided by the average copy number in the control sample (reference gene), giving the fold change in the target gene. Statistical analyses were estimated by performing unpaired, two-tailed t-test on samples. Significant changes towards control cells are indicated by asterisks (*p < 0.05, **p < 0.01,*** p < 0.001) and were calculated in GraphPad Prism 5.

2.11 General analysis of data

Data were analysed using GraphPad Prism 5 (GraphPad Software, San Diego, CA). Concentration/response curves for CCL3 in calcium flux assays were fitted 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 or by performing unpaired, two-tailed t-test on samples if only two parameters were compared. Significant changes towards control cells are indicated by asterisks (*p < 0.05, **p < 0.01,*** p < 0.001). Similarly, log EC_{50} and efficacy values were calculated in GraphPad Prism 5.

CHAPTER 3- CHOLESTEROL IMPORTANCE IN CCR5 STABLY TRANSFECTED CELLS.

3.1 Introduction

In the past few years cholesterol in the membrane has become a central subject for research on cell signal transduction due to its importance in the regulation of numerous intracellular cascades. Cholesterol is a major component of the plasma membrane, being mostly situated in special regions called lipid rafts. These areas are rich in sphingolipids, glycosylphosphatidylinositol (GPI)-anchored proteins and prenylated or palmitoylated signalling molecules like some G protein subunits, GPCR and Caveolins (Pike, 2003). Lipid rafts have been demonstrated to play an essential role in signal transduction pathways, as will be explained below, but also in the entry of parasites (Fernandes et al., 2007; Lin and Rikihisa, 2003), toxins (Abrami et al., 2003; Orlandi and Fishman, 1998), bacteria (Abrami and van der Goot, 1999) and viruses into the cell (Nguyen and Taub, 2002a; Nguyen and Taub, 2004). They were initially described as areas in the plasma membrane that present a more ordered and less fluid structure in comparison to its surroundings and are characterised by their low density and resistance to non-ionic detergents like Triton X-100 at low temperature. This property allows isolation of lipid rafts by flotation in a sucrose gradient where they are distributed in the less dense fractions (Pike, 2003).

The exact role of lipid rafts in cell signalling and trafficking has not been fully characterised yet. One of the possible functions of lipid rafts has been suggested to be the compartmentalization of signalling molecules in the plasma membrane. According to this theory, lipid rafts would promote the accumulation of proteins so that they are more easily activated by phosphorylation of local kinases and so that they interact among them, increasing signal transduction (Simons and Toomre, 2000). Several examples of signalling pathways with different dependence on lipid rafts integrity will be analysed in this chapter.

Lipid rafts can be divided into two different groups: planar lipid rafts and caveolae (Ohkubo and Nakahata, 2007). Planar lipid rafts are cholesterol and sphingolipid rich regions of the membrane that concentrate numerous signalling proteins and lack the structural protein Caveolin. In contrast, caveolae gather all the properties of planar lipid rafts but can also be characterised by the high expression of the integral membrane protein Caveolin and by their property to form invaginations in the membrane (Williams and Lisanti, 2004a).

The main function of caveolae is to participate in endocytic pathways which transport molecules like cholesterol from the cell membrane to Golgi vesicles or other intracellular organelles, and vice versa (Hansen and Nichols, 2010).

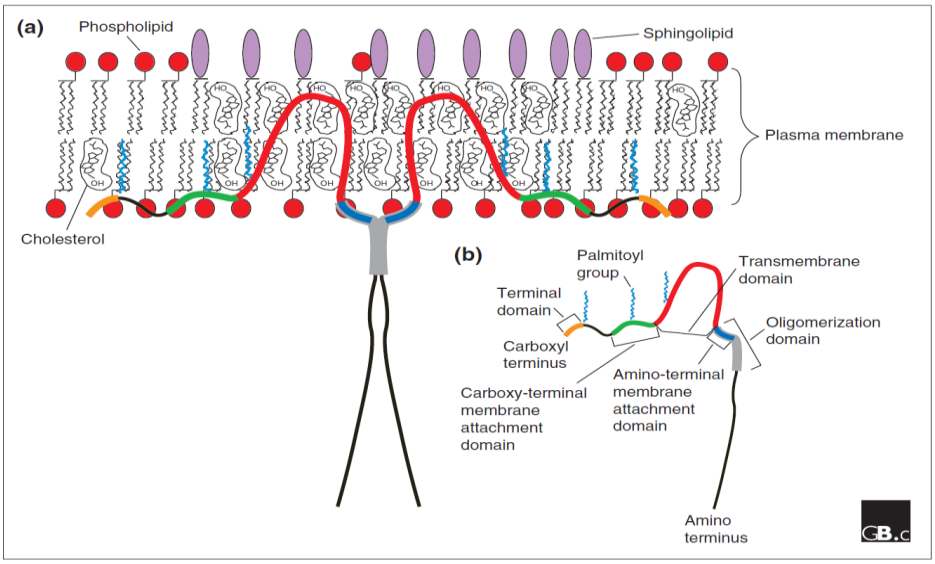

Figure 3.1 Caveolin-1 structure. a) Forming dimmers in the membrane. b) Schematic structure of Caveolin topography. From Williams et al. (Williams and Lisanti, 2004a).

Caveolins are 22-24 KDa proteins with a cytosolic N- and C-terminus that are situated in the cytosolic leaflet of the plasma membrane. The human Caveolin genes encodes for three different types: Caveolin-1, Caveolin-2 and Caveolin-3 although only Caveolin-1 has been shown to be essential for maintaining caveolae structure (Williams and Lisanti, 2004b). Caveolin-1 and Caveolin-2 are expressed in non-muscle and smooth muscle tissues and, according to Song et al. (Song et al., 1996), the presence of Caveolin-1 is needed for the expression of Caveolin-2. On the other hand, Caveolin-3 is only expressed in striated muscle cells (Song et al., 1996). These proteins are characterised by the expression of phosphorylation and palmitoylation sites as well as an

oligomerization domain (see Figure 3.1). Tyr 14 is Caveolin´s main phosphorylation site. This phosphorylation is triggered by many Caveolin activating molecules such as chemokines (Ge and Pachter, 2004) and is known to be essential for Caveolin participation in downstream pathways (Grande-Garcia and del Pozo, 2008).

Recent identification of Caveloin-1 as a regulator of several signalling mechanisms has increased research interest in its interactions with other proteins and role in the plasma membrane. For example, chemotaxis is highly dependent on Caveolin owing to its involvement in the activation of Rho GTP-ases-like proteins (Grande-Garcia and del Pozo, 2008). Caveolin has also recently been described as a tumour suppressor gene due to its role as a negative regulator of a variety of mitogenic signalling pathways (Engelman et al., 1998; Zhang et al., 2000). These studies have demonstrated its involvement in decreasing tumorigenicity, chemotaxis and cell growth. Furthermore, Zhang and colleagues also showed that Caveolin-1 expression is highly reduced or non-existent in a metastatic rat mammary adenocarcinoma cell line (MTLn3) and in the human mammary carcinoma cell lines (MCF-7 and T47D) (Engelman et al., 1998; Zhang et al., 2000).

In contrast to the above theories, more recent studies show that Caveolin-1 is positively involved in regulating cell migration, polarization and growth, while having a tumour promoter activity (Ge and Pachter, 2004; Gonzalez et al., 2004; Grande-Garcia and del Pozo, 2008; Park and Han, 2009). Consistent with the important role of Caveolin in cancer, this molecule has been shown to be expressed in many melanoma cell lines, being largely responsible for its aggressiveness (Felicetti et al., 2009). Further evidence supporting the theory that Caveolin is important for cancer progression is demonstrated by recent reports showing Caveolin-1 microvesicles being secreted by different tumours and correlating with their invasiveness (Tahir et al., 2008; Watanabe et al., 2009). For example, Caveolin-1 microvesicles can be found at high concentrations in prostate cancer and have been proposed as a marker for prostate cancer diagnosis (Tahir et al., 2008; Watanabe et al., 2009).

These studies provide reliable evidence that Caveolin can play opposing roles in cancer progression. It is then a priority to understand the factors that control Caveolin signalling mechanisms in order to understand in which circumstances it will act as a tumour suppression gene and in which it will act as an oncogene.

One of the characteristics of lipid rafts is that they are so small that they cannot be seen using a standard light microscope (Simons and Toomre, 2000). However, high resolution electron microscopy, EM, has been able to resolve caveolae and has allowed scientists to visualise caveolae disruption upon treatment with certain cholesterol modifying agents. The most common tools to disrupt lipid rafts are the cholesterol sequestrating agents filipin and nystatin, the cholesterol depleting agent methyl-β-cyclodextrin (MCD) and methods that perturb raft stability such as the addition of exogenous cholesterol, gangliosides or fatty acids (Simons and Toomre, 2000). Treatment of cells with MCD causes the flattening of the membrane and the destruction of caveolae which can be reversed by reloading cells with cholesterol (Figure 3.2).

The work in this thesis will focus on cholesterol sequestration and cholesterol depletion methods. Filipin and nystatin are antibiotics capable of complexing cholesterol in the plasma membrane. Filipin specifically binds 3-β-hydroxysterols in a 1:1 stoichiometry, forming large aggregates that lie parallel to and in the centre of the lipid bilayer (de Kruijff and Demel, 1974) whereas nystatin forms sterol dependent ion channels in the plasma membrane (Bolard, 1986). β-Cyclodextrins are cyclic oligosaccharides consisting of 7-β (1-4)- glucopyranose units whose external face is highly hydrophilic and the internal one highly hydrophobic; the latter being able to capture hydrophobic molecules like cholesterol (Christian et al., 1997). Among the different types of cyclodextrins available, methyl β-cyclodextrin (MCD), which has been chemically modified to improve solubility, complex formation and reduce toxicity, has been demonstrated to be the most efficient one at depleting cellular cholesterol for cell signalling experiments (Christian et al., 1997).

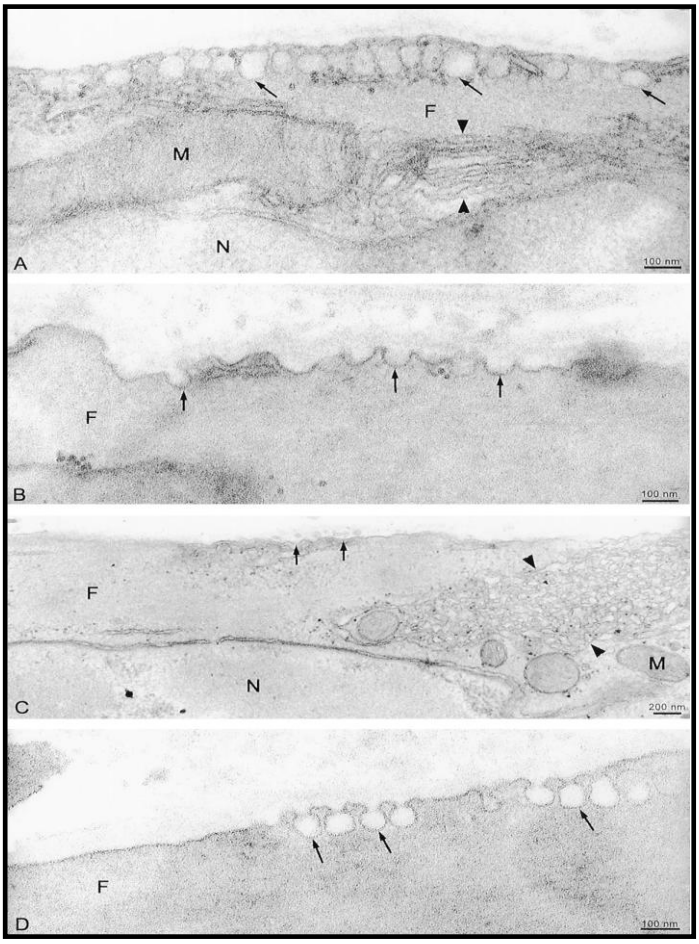

Figure 3.2 Micrographs (by electron microscopy [EM]) of the medial smooth muscle cells (SMCs) in tail arteries. A, Normal caveolae (arrows) are shown in an SMC from a control preparation. Arrow heads indicate Golgi apparatus; M, mitochondrion; N, nucleus; and F, filaments. B and C, cholesterol-depleted SMCs are shown. Caveolae are less numerous and, when present, are opened to a variable degree. D, Normal caveolae are seen after reloading with cholesterol. (Dreja et al., 2002).

Several studies have shown that CCR5 colocalises with lipid raft markers (Carter et al., 2009; Manes et al., 1999; Nguyen and Taub, 2002b; Popik et al., 2002), which leads to the supposition that the receptor is mainly distributed along these special areas of the membrane. Further evidence for CCR5 expression in lipid raft domains is supported by this receptor being post translationally palmitoylated, which has been shown to act as a cue for targeting to these cholesterol rich microdomains in the plasma membrane (Kraft et al., 2001; Percherancier et al., 2001; Venkatesan et al., 2003).

CCR5 partitioning to lipid rafts does not mean it locates in caveolae domains. In the present chapter the role of Caveolin-1 in CCR5 signalling and expression will be

analysed. Caveolin is known to regulate calcium signalling responses initiated by several receptors such as muscarinic receptors (Gosens et al., 2007; Kubale et al., 2007; Russo et al., 2009) and to bind to G proteins subunits modulating their activity. For instance, Caveolin-1 can promote G protein active conformation (Bhatnagar et al., 2004; Sengupta et al., 2008) and has also been shown to promote G protein inactive state (Kong et al., 2007). The factors involved in these opposing roles of Caveolin are yet to be identified. Considering all the information available regarding the role of caveolin in other receptors signalling, the lack of data regarding the CCR5-Caveolin relationship becomes evident.

The role of cholesterol in CCR5 signalling was also studied in detail. Considering that cholesterol is one of the main components of lipid rafts, it is expected that variations in its concentration would affect CCR5 signalling properties. Indeed, CCR5's conformation has been shown to be dependent on membrane cholesterol (Nguyen and Taub, 2002b). Cholesterol depletion with hydroxypropyl-β-cyclodextrin (BCD) or cholesterol oxidation with cholesterol oxidase promotes significant conformational changes that blocks CCL4 binding to the receptor and CCR5 signalling (Nguyen and Taub, 2003a).

Cholesterol has been shown to be essential for cell polarization and migration of neutrophils although cholesterol depletion had no effect on early chemoattractant signalling events such as G-protein activation, intracellular calcium flux or MAPK activation (Rose et al., 2008). These data show the different dependence on cholesterol of intracellular pathways activated by chemokine receptors. Understanding CCR5 signalling and internalisation processes are especially interesting for controlling HIV infection. It has been previously explained that the number of CCR5 receptors in the plasma membrane is essential for the initial binding of the virus and for HIV entry into cells (Lin et al., 2002). Additionally, some studies agree that CCR5 signalling is essential for viral replication (Alfano et al., 2000; Alfano et al., 1999; Lin et al., 2006; Wang and Oppenheim, 1999) although some others argue that CCR5 signalling, phosphorylation and internalisation processes are independent from HIV infection (Alkhatib et al., 1997; Amara et al., 2003; Aramori et al., 1997a). Nevertheless, it is accepted that cholesterol depletion with MCD impairs HIV infection, either due to alterations on CCR5 membrane expression or to CCR5 signalling inhibition (Carter et al., 2009; Liao et al., 2001; Manes et al., 2000; Viard et al., 2002; Vidricaire and

Tremblay, 2007; Vila-Coro et al., 2000; Weiner et al., 1992).

CCR5 can internalise through clathrin-coated pits and through caveolae (Mueller et al., 2002). Mueller et al. verified that CCR5 endocytosis was impaired by cells treatment with chlorpromazine and sucrose, known to block clathrin coated pit dependent internalisation, and by filipin and nystatin, which are responsible for caveolae disruption. Other chemokine receptors display the same tendency as CCR5 in terms of internalisation; CCR4 endocytosis is also dependent on both clathrin coated pits and caveolae (Mariani et al., 2004). However, there are chemokine receptors that exclusively internalise using either pathway. For instance, Cav-1 knockdown in astrocytes expressing CCR2 causes a complete impairment of receptor internalisation (Ge and Pachter, 2004) providing evidence that CCR2 internalisation is only dependent on caveolae pathways. On the contrary, the chemokine receptors CXCR1 and CXCR2 are just internalised by clathrin coated pits dependent pathways (Rose et al., 2004).

As it has been previously outlined, clathrin coated pits and caveolae inhibitors have been broadly used to analyse the internalisation pathways of different chemokine receptors, including CCR5. Nevertheless, the importance of clathrin-dependent pathways and cholesterol complexation with filipin and nystatin has not been characterised for CCR5-induced signal transduction. Thus, in this study the influence of sucrose, chlorpromazine, filipin and nystatin on CCR5-dependent intracellular events has been analysed. The importance of Caveolin-1 expression on CCR5 signalling has also been looked at. Also, the cholesterol depleting drug MCD has been used to deplete cellular cholesterol in order to analyse CCR5-induced calcium release, expression and internalisation in CCR5 stably transfected cells lacking this important membrane component.

3.2 Aims

The aim of this chapter is to understand how cholesterol in the membrane as well as structures like caveolae and clathrin coated pits affect CCR5 signalling and internalisation. CCR5 induced calcium mobilization from the ER and cAMP assays have been used as a measure of CCR5 activation. Immunofluorescence and flow cytometry experiments have been performed to measure CCR5 localization and expression levels. Finally, cholesterol assays to determine the amount of cholesterol depleted have

complemented the work. Considering the importance of CCR5 signalling and regulation for HIV infection and numerous inflammatory diseases it is clear that a perfect understanding of CCR5's environment and the factors that contribute to its stability are essential for the development of new therapies.

3.3 Results

3.3.1 Cholesterol depletion but not cholesterol complexation blocks CCL3 induced CCR5 signalling

3.3.1.1 Changes in intracellular calcium mobilization

Cells stably expressing CCR5 were treated with sucrose, filipin, nystatin and MCD and CCL3 capacity to induce calcium release was analysed. Intracellular calcium mobilization is one of the first events to occur after CCR5 stimulation and, therefore, can be easily used as a system to measure receptor activation. Here we analyse CCR5's ability to release calcium from ER stores in the presence of sucrose, filipin, nystatin and MCD.

In this chapter, the CCL3 EC50, 100 nM, has been used to perform experiments at a single concentration of chemokine.

The ability of CHO.CCR5 and HEK.CCR5 to initiate calcium signalling in response to CCL3 challenge was markedly reduced following treatment with MCD, but was similar to untreated cells following incubation with nystatin, filipin, or sucrose (Figure 3.3 and 3.4). The effects of cholesterol sequestration were very similar in both CHO.CCR5 and HEK.CCR5 which suggests that effects upon signalling are not cell line specific and function via a ubiquitous pathway. Repletion of cholesterol after MCD treatment had some recovering effects on CCR5 induced calcium release as observed in Figure 3.3 (CHO.CCR5) and Figure 3.4 (HEK.CCR5). Cholesterol depletion by MCD and cholesterol complexation with filipin and nystatin are known to disrupt caveolae. Therefore, the fact that cholesterol repletion abrogated the reductive effect on calcium release that MCD treatment had, and that filipin and nystatin had no effect on calcium signalling, indicates that the reduction in calcium increase of MCD is mainly due to cholesterol depletion and not to caveolae disruption. It has been demonstrated that

filipin, nystatin and sucrose block CCL3-dependent CCR5 internalisation which indicates that this receptor is endocytosed through caveolae dependent and independent pathways (Mueller et al., 2002). However, it seems that these pathways are not needed for CCR5 signalling. These findings were reiterated in a similar study into CCR3 signalling and endocytosis (Zimmermann and Rothenberg, 2003). It was shown that sucrose treatment was able to block receptor internalisation but had no effect on calcium release. However, this group also demonstrated that sucrose treatment completely abolished actin polymerization upon CCR3 stimulation, indicating that clathrin-dependent pathways were needed for some of the downstream signalling events. Similarly, Li et al. (Li and Nord, 2004) showed that Caveolin disruption with filipin in proximal tubule cells expressing the CD40 receptor abrogated the signalling mechanisms normally induced by this receptor. These are, therefore, two examples where clathrin and caveolae pathways, respectively, were needed not only for receptor internalisation but for receptor signalling as well. According to the data obtained in this section it seems that neither of these pathways is required for CCR5-induced signalling.

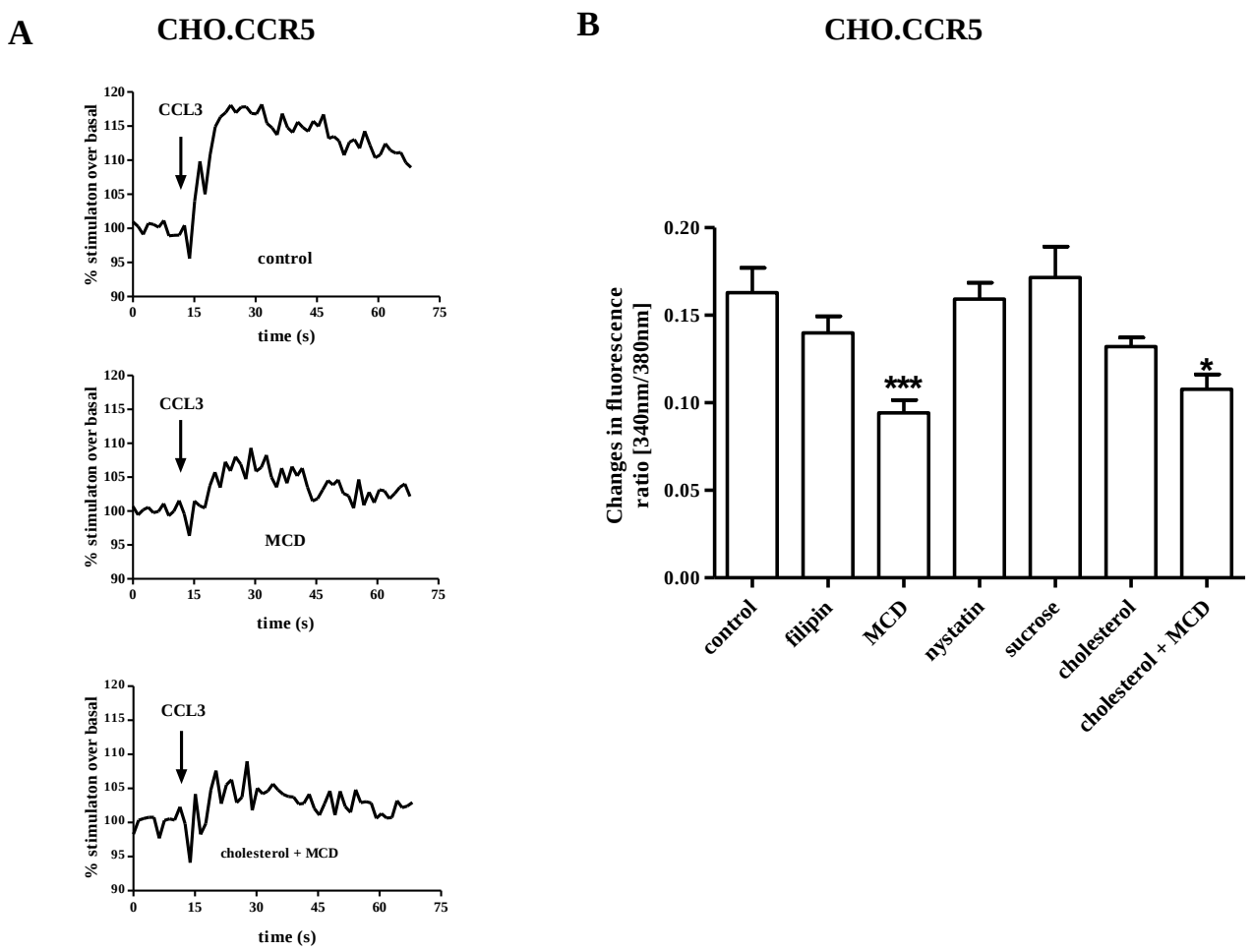

Figure 3.3 Changes in intracellular Ca^{2+} in CHO.CCR5 cells in the presence of inhibitors. The intracellular calcium ion concentration was determined in CHO.CCR5 cells following stimulation by chemokines as described in the materials and methods section. Cells were incubated with MCD (10 mM), cholesterol (2 mM), both, filipin (5 µg/ml), nystatin (50 µg/ml) and sucrose (0.4 M) for 1 h before cells were stimulated with 100 nM CCL3. A) shows single traces in real time in the presence or absence of inhibitors, B) shows cells pre-treated with different inhibitors or vehicle (control), significant changes to control data are shown by asterisks (* $p \leq 0.05$, *** $p \leq 0.001$). Data are expressed as fluorescence ratio and represent mean ± SEM from at least four independent experiments. (Cardaba et al., 2008).

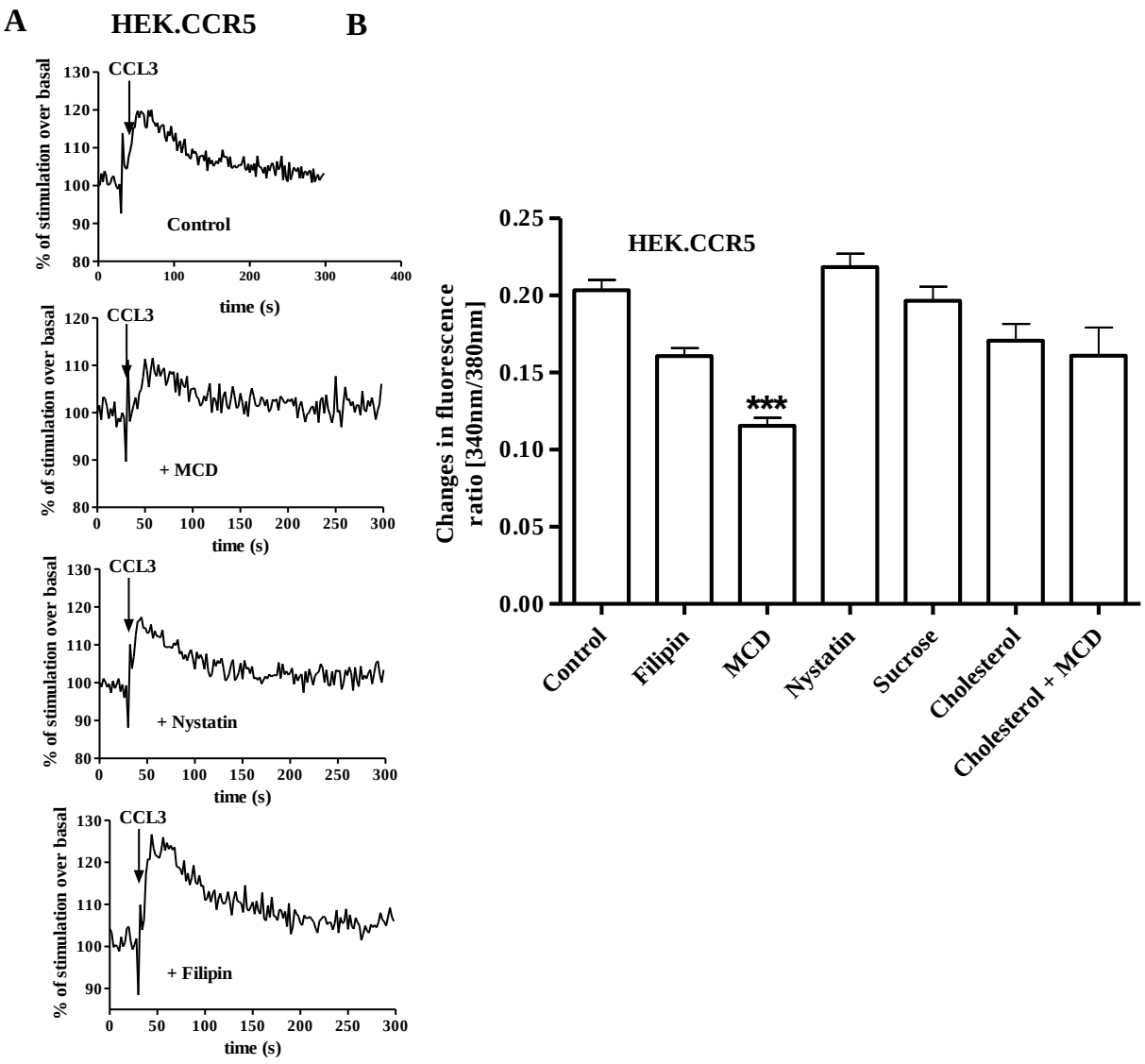

Figure 3.4 Changes in intracellular Ca^{2+} in HEK.CCR5 cells in the presence of inhibitors. The intracellular calcium ion concentration was determined in HEK.CCR5 cells following stimulation by chemokines as described in the materials and methods section. Cells were incubated with MCD (10 mM), cholesterol (2 mM), both, filipin (5 µg/ml), nystatin (50 µg/ml) and sucrose (0.4 M) for 1 h before cells were stimulated with 100 nM CCL3. A) shows single traces in real time in the presence or absence of inhibitors, B) shows cells pre-treated with different inhibitors or vehicle (control); significant changes to control data are shown by asterisks (*** $p \leq 0.001$). Data are expressed as fluorescence ratio and represent mean ± SEM from at least four independent experiments. (Cardaba et al., 2008).

The next aim of this chapter was to investigate whether the effect observed upon MCD treatment was chemokine-dose dependent and its effect on CCL3 potency. Dose response curves were created in MCD treated HEK.CCR5 cells (Figure 3.5). Figure shows that MCD treatment decreases $LogEC_{50}$ from -7.59 in control cells to -6.24 in MCD treated cells without affecting the predicted efficacy (pE) significantly. When MCD + cholesterol were added to HEK.CCR5 cells it was found that CCL3 potency increased the $LogEC_{50}$ to -7.267 but it also lowered CCL3 efficacy.

To investigate whether these results were chemokine dependent, HEK.CCR5 cells were treated with MCD or cholesterol + MCD and stimulated with the chemokines CCL5, CCL3L1 and the CCR5 specific ligand CCL4. CCL3L1 is an isoform of CCL3 which has a proline instead of a serine in its N-terminus and has been shown to be much more potent for CCR5 binding and activation (Mueller et al., 2006). It can be observed (Figure 3.6) that the three chemokines follow the same trend as CCL3. MCD significantly blocks calcium release upon CCL5 and CCL3L1 stimulation.

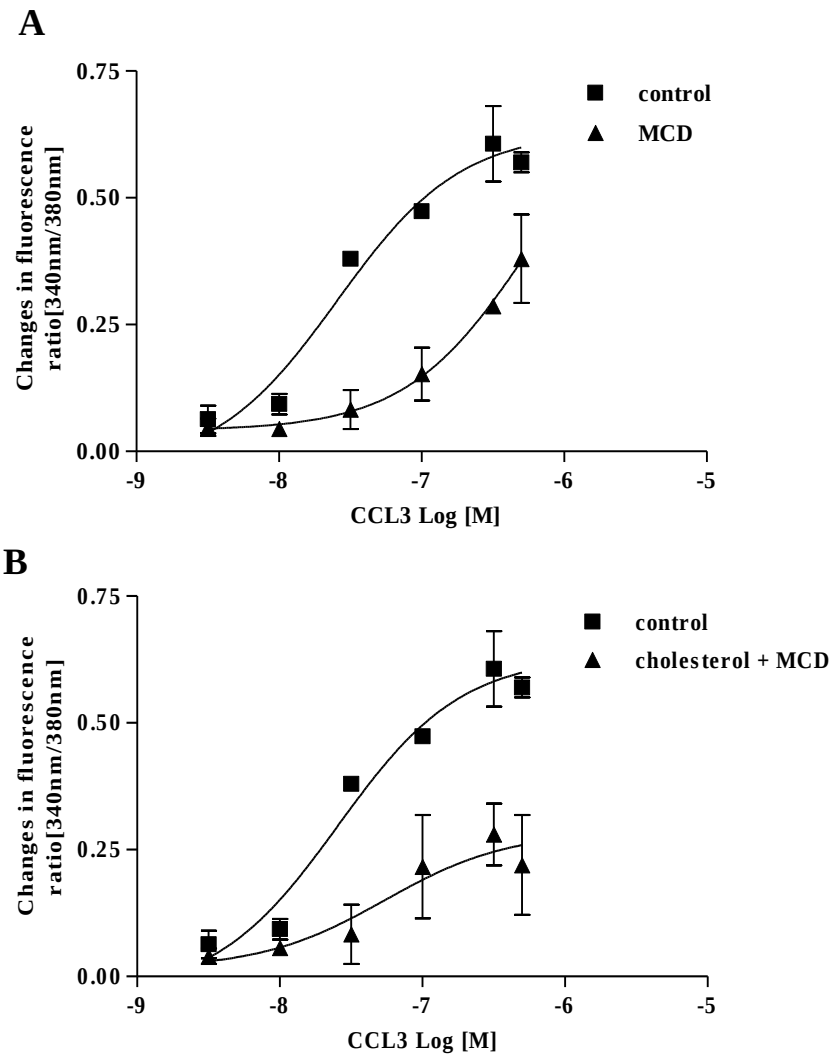

Figure 3.5 MCD effects on dose response relationships for release of intracellular Ca2+. The intracellular calcium ion concentration was determined in HEK.CCR5 cells following stimulation by chemokines as described in the materials and methods section. Cells were incubated with MCD (10 mM), cholesterol (2 mM) or both for 1 h before cells were stimulated with CCL3 at different concentrations. A) Shows control cells and MCD treated cells, B) shows control cells and cells treated with MCD plus cholesterol. Data represent mean ± SEM from at least three independent experiments. (Cardaba et al., 2008).

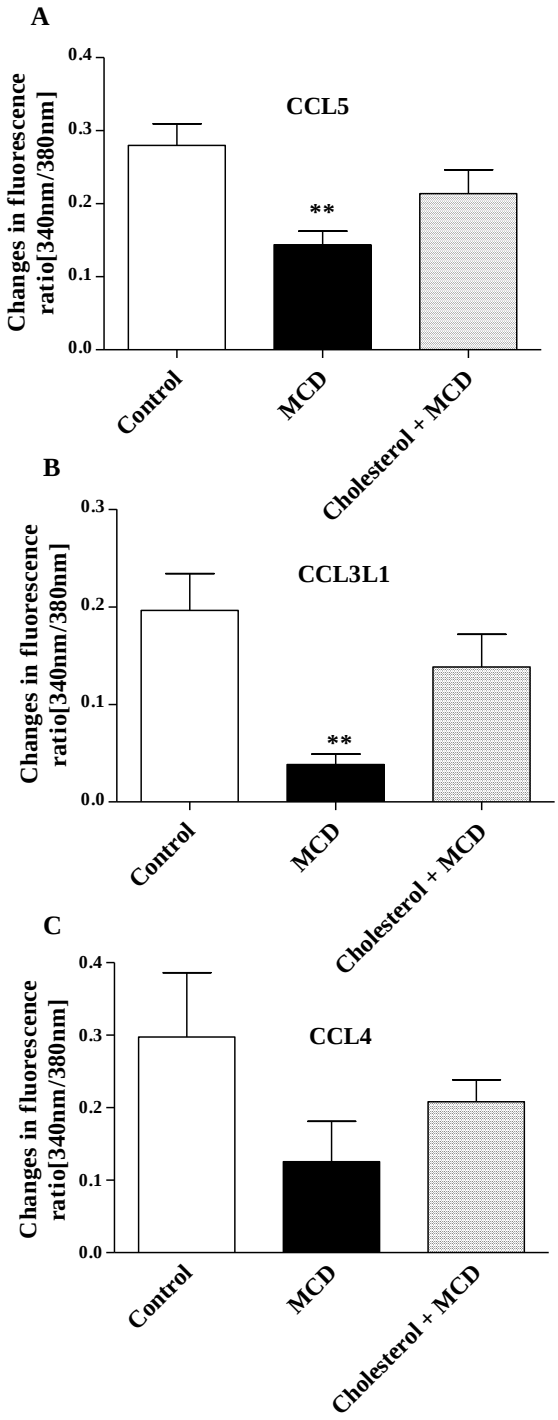

Figure 3.6 Effect of MCD on release of calcium initiated by a variety of chemokines. The intracellular calcium ion concentration was determined in HEK.CCR5 cells following stimulation by chemokines as described in the materials and methods section. Cells were incubated with MCD (10 mM), cholesterol (2 mM) or both for 1 h before cells were stimulated with 100 nM CCL3. Cells were incubated with different inhibitors for 1 h before stimulation with A) CCL5, B) CCL3L1 or C) CCL4 at 100 nM. Data are expressed as fluorescence ratio and represent mean ± SEM from at least four independent experiments. Significant changes to control are shown by asterisks (**≤0.01). (Cardaba et al., 2008).

3.3.1.2 Inhibition of cAMP accumulation

CCR5 is mainly coupled to $G\alpha_i$ proteins, which inhibit AC formation of cAMP (Oppermann, 2004). It has been shown that cholesterol depletion blocks calcium mobilization, and thereby we now wanted to know whether this effect was due to a change in the receptor's conformation, as previously shown by others, and a consequent alteration of its coupling to $G\alpha_i$ proteins. Activation of CCR5 with a ligand reduces cAMP cellular levels due to inhibition of AC. In this occasion, MCD, nystatin, filipin and sucrose were used to analyse their effect on inhibition of cAMP accumulation. Figure 3.7 illustrates that MCD treatment of cells leads to a complete abrogation of CCL3 blockage of cAMP accumulation. MCD treatment results in a flat line, indicating no inhibition or activation of forskolin-stimulated cAMP accumulation. This result indicates that MCD reduces the ability of CCR5 to signal through $G\alpha_i$ proteins and, since coupling to a $G\alpha_s$ protein would cause cAMP activation, it also shows that MCD treatment does not promote the signalling through $G\alpha_s$ proteins.

On the contrary, cholesterol complexation with filipin only led to a slight change in CCL3 potency and nystatin caused a significant reduction of CCL3 potency having no effect on the efficacy. Interestingly, cholesterol addition had similar effects to nystatin treatment, shifting the curve to the right and lowering CCL3 potency but not changing the efficacy of CCL3. Considering these results it seems that, as it happened in calcium mobilization assays, MCD treatment but not filipin or nystatin treatments, blocks CCR5-dependent inhibition of AC. Taken together these data show that cholesterol in the membrane is essential for the normal signalling of CCR5 through $G\alpha_i$ proteins which results in a reduced calcium mobilization and in inhibition of cAMP formation.

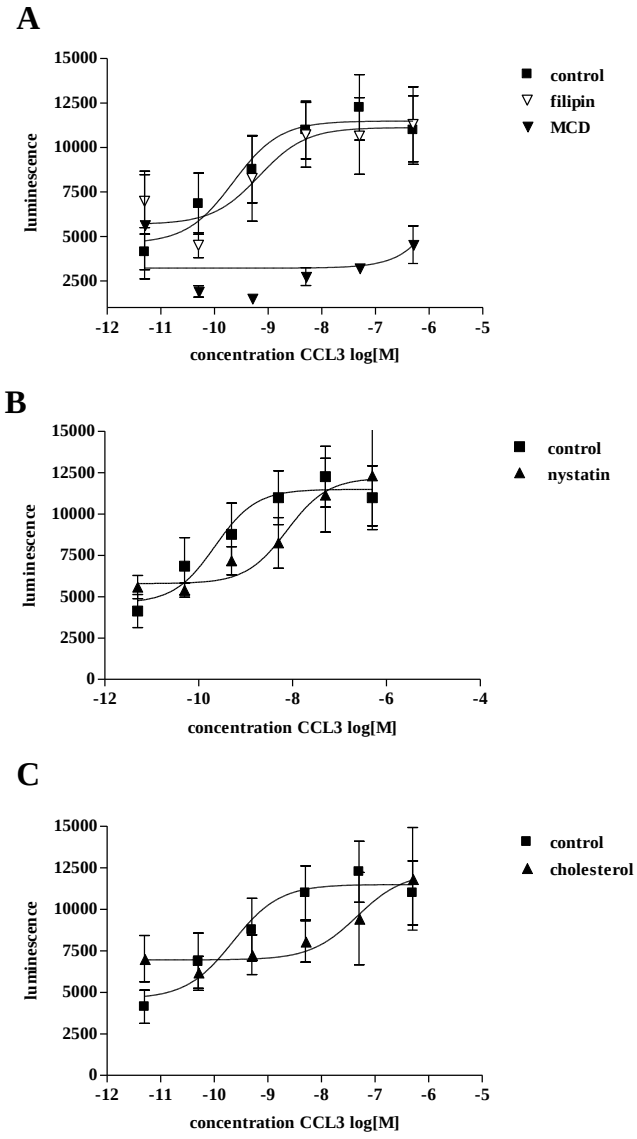

Figure 3.7 Chemokine inhibition of forskolin-stimulated cAMP accumulation in CHO.CCR5 cells. CHO.CCR5 cells were pre-treated with MCD (10 mM), cholesterol (2 mM), filipin (5 µg/ml) and nystatin (50 µg/ml) for 1 h before when stated and then stimulated with forskolin and 100 nM CCL3. The Promega cAMP-Glo™ assay kit was used according to the manufacturer's instructions to measure cAMP concentrations as described in materials and methods. A) shows control cells and cells treated with filipin and MCD, respectively, B) shows control cells and cells treated with nystatin, C) shows control cells and cells treated with cholesterol. Data are mean ± SEM from four or more separate experiments performed in duplicate. (Cardaba et al., 2008).

3.3.2 Cholesterol depletion with MCD promotes CCR5 signalling through a PTX-independent G protein.

The results shown above demonstrate that cholesterol depletion with MCD, but not cholesterol complexation with filipin or nystatin, blocks CCR5 signalling as measured by calcium release and inhibition of cAMP formation. It is widely accepted that receptors coupled to $G\alpha_i$ proteins like CCR5 stimulate PLC and, therefore, release calcium to the cytosol through the signalling initiated by the βγ subunit. However, inhibition of forskolin-stimulated cAMP accumulation happens through the activation of the $G\alpha_i$ subunit. In this section the G α subunit responsible for CCR5 signalling upon cholesterol depletion has been investigated. While measuring cAMP accumulation inhibition, $G\alpha_i$ activity is being analysed whereas calcium release experiments could potentially be dependent on any Gα subunit activation. In order to study this further, cells were treated with PTX for 2 hours and after this time changes in calcium mobilization were analysed. As shown in Figure 3.8, PTX treatment of HEK.CCR5 cells completely inhibits calcium release upon CCL3 stimulation, suggesting an exclusive role of $G\alpha_i$ in CCR5-dependent calcium release. Interestingly, it was found that MCD treatment of cells abrogates the inhibitory effect PTX had on calcium release. PTX was unable to completely block the signalling upon MCD treatment of HEK.CCR5.

It is possible that MCD, by changing the membrane microdomains the receptor is in, promotes a change in CCR5 conformation and maybe G proteins redistribute in the membrane, leading to the receptor coupling a different PTX-resistant G protein. Moreover, the fact that cAMP accumulation is not increased upon cholesterol depletion discards the possibility of CCR5 coupling a $G\alpha_s$.

Altogether it seems that MCD completely abrogates the blockage of cAMP accumulation whereas it allows some calcium signalling to occur after treatment of cells with PTX. It is accepted that CCR5 can couple to G proteins different from $G\alpha_i$ (Mueller and Strange, 2004a) and, therefore, it is a possibility that the difference in signalling observed between the two read-out systems used is due to the measurement of a different G protein activity in both assays.

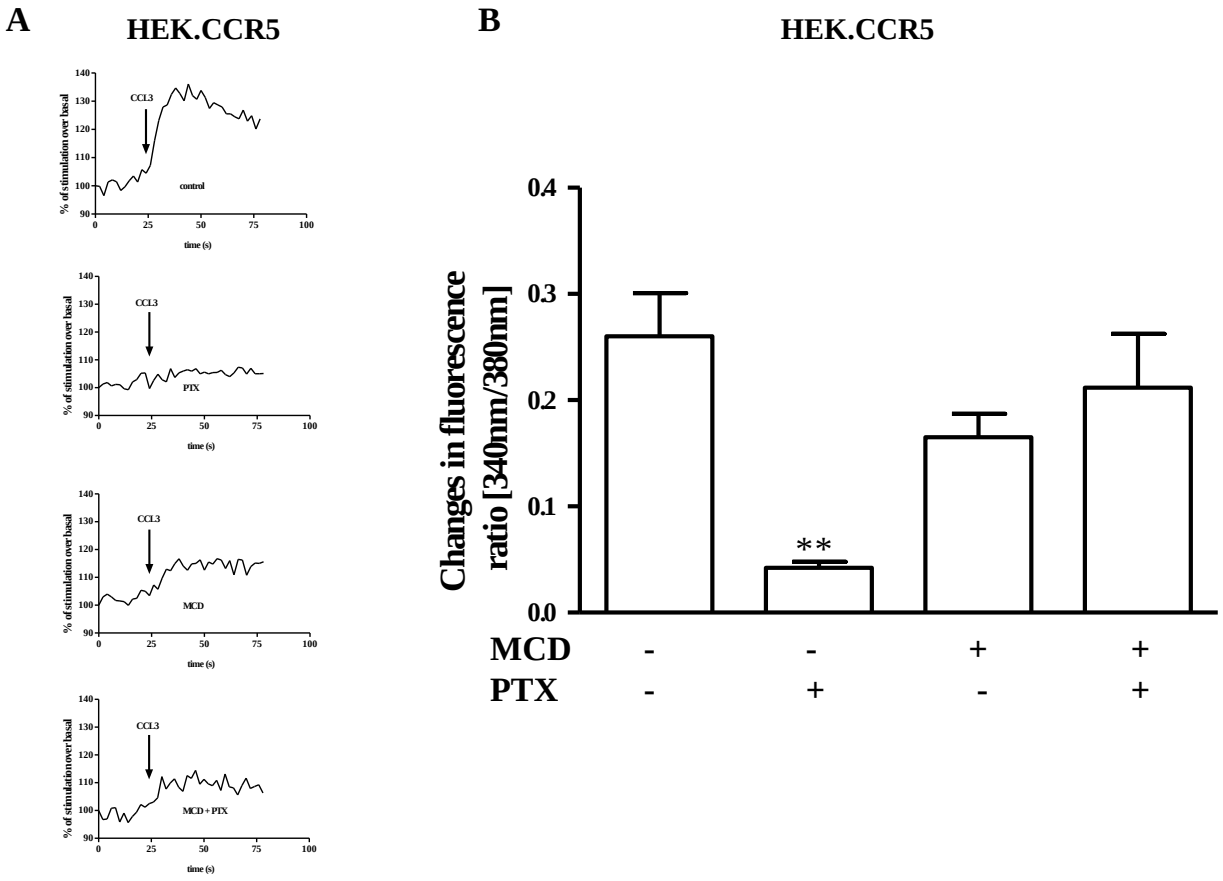

Figure 3.8 Release of intracellular calcium becomes PTX-independent after depletion of cholesterol. The intracellular calcium ion concentration was determined in HEK.CCR5 cells following stimulation by chemokines as described in the materials and methods section. Cells were incubated with PTX (0.1 µg/ml) and MCD (10 mM) for 2 h, when indicated, before cells were stimulated with CCL3. A) shows single traces in real time in the presence or absence of inhibitors, B) shows cells pre-treated with different inhibitors or vehicle (control), significant changes to control data are shown mean ± SEM from at least four independent experiments. (Cardaba et al., 2008).

The findings that HEK.CCR5 cells treated with MCD lead to calcium fluxes through a PTX-independent G protein and a detailed literature search prompted us to think that the signal observed after MCD treatment could be due to CCR5 coupling to $G\alpha_q$. Thus, we wanted to analyse possible variations in $G\alpha_q$ expression after MCD treatment of cells. Fig 3.9 shows that $G\alpha_q$ is situated at the edges of the cell and that neither MCD treatment nor ligand binding interfere with its membrane expression or location. It is noteworthy that $G\alpha_q$ immunofluorescence experiments upon inhibitors treatments would only allow us to see a possible change in its expression. It is possible though, that lipid rafts disruption by MCD treatment affects $G\alpha_q$ interactions with other rafts proteins such as Caveolin or CCR5, inducing a change in the intensity and duration

of CCR5 calcium signalling without altering $G\alpha_q$ membrane expression.

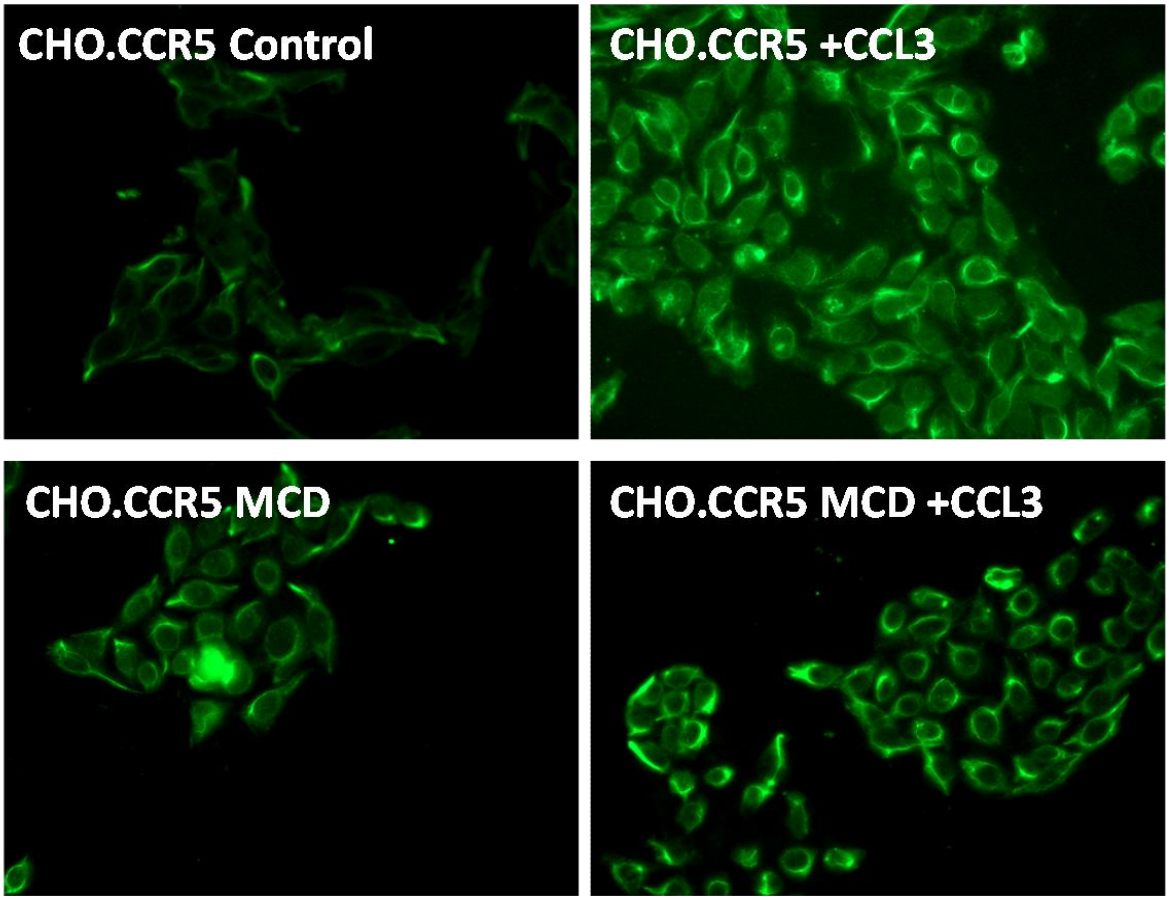

Figure 3.9 $G\alpha_q$ stain in CHO.CCR5 cells. Cells treated with MCD (10 mM for 1 h) or left untreated (control) were stimulated with CCL3 200 nM for 30 minutes or left untreated. Then cells were then permeabilized with 0.1% triton x-100 in PBS, fixed in methanol, blocked with 5% non-fat milk in PBS and incubated with the anti Gαq/11 rabbit antibody (C-19). Stain was performed with the secondary anti rabbit FITC antibody. Pictures were taken using the Zeiss Axiovision 2 system.

3.3.3 Is Caveolin-1 needed for CCR5 signalling?

Similarly to MCD, filipin and nystatin are able to disrupt caveolae. However, these two inhibitors do not affect caveolae in the same way as MCD (Awasthi-Kalia et al., 2001). Bearing in mind that filipin and nystatin do not affect CCR5-induced calcium signalling and MCD does, we hypothesised that different modulation of caveolae structure may be behind the distinct effects observed. Caveolin-1 is the main component of caveolae and, therefore, it could be speculated that inhibitors of the caveolae pathway

would somehow affect the expression and distribution of Caveolin-1. Therefore, the aim of this section is to determine the role of Caveolin-1 expression on CCR5 signalling. To start with, the effects of filipin and MCD on Caveolin-1 expression were analysed. It can be appreciated (Figure 3.9) that filipin disturbs Caveolin-1 organization whereas MCD does not seem to affect it. Filipin treatment appears to remove all Caveolin-1 from the plasma membrane redistributing it to other locations in the cytosol.

The results commented above indicate that Caveolin-1 might have an essential role in CCR5 internalisation since filipin, known to inhibit CCR5 down-regulation, completely disrupts Caveolin-1 expression. Likewise, MCD treatment was able to block calcium release and cAMP accumulation inhibition but had no effect on Caveolin-1 expression, indicating a possible independent role for Caveolin-1 in CCR5-related signalling events. Considering that filipin has no effect on CCR5-induced calcium release and that it completely removes Caveolin-1 from the plasma membrane, the next step was to confirm that Caveolin-1 was not needed for CCL3 ability to trigger calcium release from ER stores.

Small interfering RNA (siRNA) was used to knockdown Caveolin-1 in HeLa.RC49 cells. Although for most of the experiments done so far HEK.CCR5 and CHO.CCR5 cells were used, HeLa.RC49 cells were chosen to perform these experiments because Caveolin-1 siRNA had been validated in this cell line. HeLa.RC49 cells had been previously used to test the effects of MCD on CCR5 induced calcium release and the same results as in CHO.CCR5 and HEK.CCR5 cells were obtained, making it suitable for the present experiment.

Figure 3.10 illustrates that a complete knockdown of Caveolin-1 does not affect CCL3 induced calcium mobilization in HeLa.RC49 cells. These data is consistent with the fact that filipin, which reduces Caveolin-1 expression does not block calcium mobilization. On the other hand, MCD, which has been shown not to affect Caveolin-1 distribution or expression, does block calcium responses induced by CCR5 activation. Taken together the results analysed in this section indicate that removal of Caveolin-1 through filipin treatment or siRNA knockdown cannot be linked to a reduction in CCR5 signalling. It seems that alterations in the content of cellular cholesterol or the act of extracting cholesterol itself are responsible for a decrease in CCR5 signalling and that Caveolin-1 or caveolae do not play a key part in these processes.

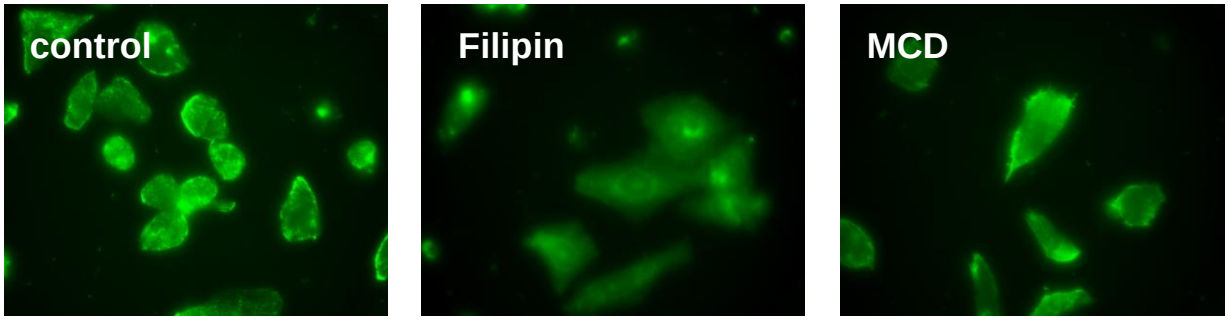

Figure 3.10 Effects of inhibitors on Caveolin-1 localisation in the membrane. CHO.CCR5 cells were grown on coverslips overnight and incubated in medium without serum for 1 h in the presence of 5µg/ml filipin and 10 mM MCD when indicated. Cells were washed and a stain was performed using anti-Caveolin-1 antibody and anti-rabbit-FITC. Pictures were taken using the Zeiss Axiovision 2 system. Data show representative cells. (Cardaba et al., 2008).

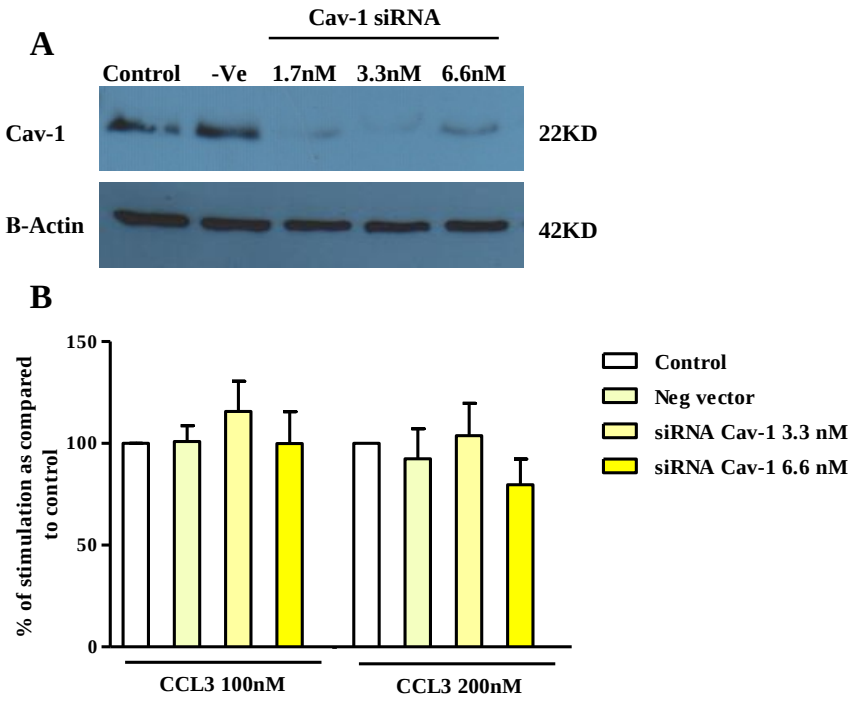

Figure 3.11 Caveolin-1 is not essential for calcium responses upon CCR5 stimulation. HeLa.RCR9 cells were transiently transfected with siRNA for Caveolin-1 by chemical transfection. After 2 days, Caveolin-1 expression levels were measured by western blot (A). Figure B shows intracellular calcium mobilization upon CCL3 stimulation in Caveolin-1 knock-down cells. Data shows mean ± SEM of 3 to 5 independent experiments.

3.3.4 CCR5 expression and internalisation dependence on cholesterol

In this chapter MCD's effect on CCR5 signalling has been analysed. MCD reduces the ability of CCR5 to signal through $G\alpha_i$ proteins. However, the effects of MCD on CCR5 expression and internalisation have not been studied so far. Taking into account that cholesterol depletion causes an almost complete blockage of CCR5 signalling, it is important to analyse whether this effect was caused by alterations in CCR5 expression in the plasma membrane. Thus, the aims of this section were to analyse the number of CCR5 molecules left in the plasma membrane upon MCD treatment as well as to determine if the internalisation rate of CCR5 is affected by cholesterol depletion.

Detection of CCR5 surface expression by immunofluorescence microscopy (Figure 3.12 A), or antibody labelling followed by flow cytometry (Figure 3.12 B) showed that MCD treatment of cells does not have significant effects on CCR5 expression. Interestingly, Carter's group (Carter et al., 2009) have demonstrated that treatment of macrophages with MCD for 1 hour caused a 100% loss in the number of CCR5 molecules in the plasma membrane. The reasons behind this lack of agreement might be related to functional differences between macrophages, CHO.CCR5 and HEK.CCR5 cells

The consequences of cholesterol depletion in CCR5 internalisation upon MCD treatment were next investigated. It appears that MCD affects CCR5 dependent calcium response independently of caveolae. MCD has been shown to block the formation of clathrin coated pits vesicles and to inhibit the internalisation of the transferring receptor, known to internalise exclusively through clathrin-dependent pathways (Rodal et al., 1999). Similarly, this molecule is widely known to block caveolae dependent internalisation which seems normal considering the clear effect it has in flattening these cave-like structures (Feng et al., 2009; Hong et al., 2009). However, when CCR5 internalisation experiments were here performed it was found that MCD treatment of cells does not alter the endocytosis rate of the receptor. Figure 3.13 shows the effect of MCD and cholesterol in CCL3 induced CCR5 internalisation. In MCD treated cells (F), stimulation with CCL3 causes a loss of receptor expression similar to that observed in control cells (B). Neither addition of cholesterol on its own nor MCD with cholesterol have any effect on CCR5 internalisation (B nor H, respectively). These results indicate that MCD removal of cholesterol is not involved in CCR5 internalisation. The reason why cholesterol depletion of cells does not affect CCR5 endocytic pathways is completely unidentified. Cholesterol depletion has been shown to block clathrin coated pits and caveolae internalisation and since CCR5 uses either endocytic pathway it may be necessary to perform new internalisation experiments using more accurate methods to clearly identify if CCR5-endocytosis is dependent on cholesterol. Flow cytometry experiments would represent a more precise mode of measuring the number of CCR5 molecules left in the plasma membrane upon ligand stimulation in cholesterol depleted membranes.

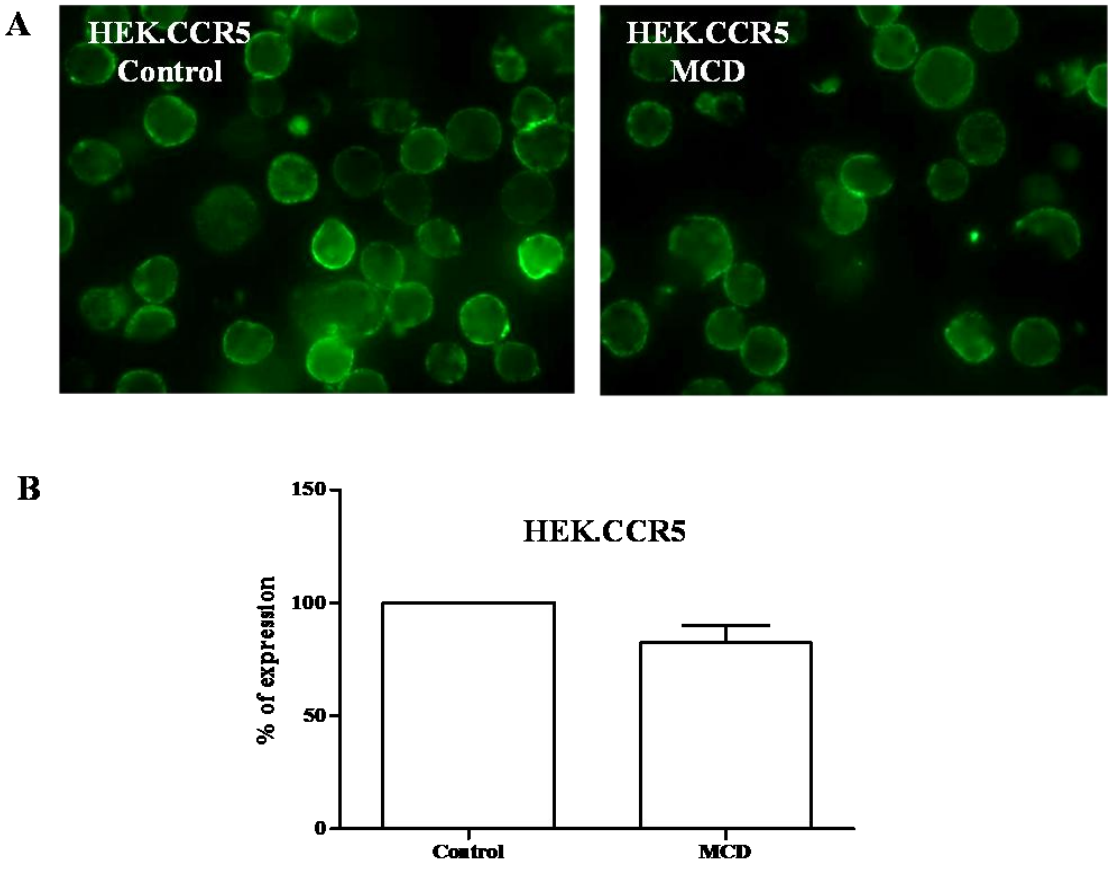

Figure 3.12 CCR5 expression levels are not significantly affected upon cholesterol depletion. HEK.CCR5 cells were treated with 10 Mm MCD for 1 h, labelled with the anti.CCR5 HEK/1/85a/7a antibody and stained with the anti-rat-FITC secondary antibody. A) Shows Immunofluorescence experiments in HEK.CCR5 cells labelled with the anti.CCR5 HEK/1/85a/7a antibody and stained with the anti-rat-FITC secondary antibody. B) Flow cytometry analysis of CCR5 expression in HEK.CCR5 cells after MCD treatment for 1 h. Cells were labelled with the anti.CCR5 HEK/1/85a/7a antibody and stained with the anti-rat-FITC secondary antibody. Pictures were taken using the Zeiss Axiovision 2 system. Data shown represents mean ± SEM of at least 3 independent experiments for B and a representative picture for A.

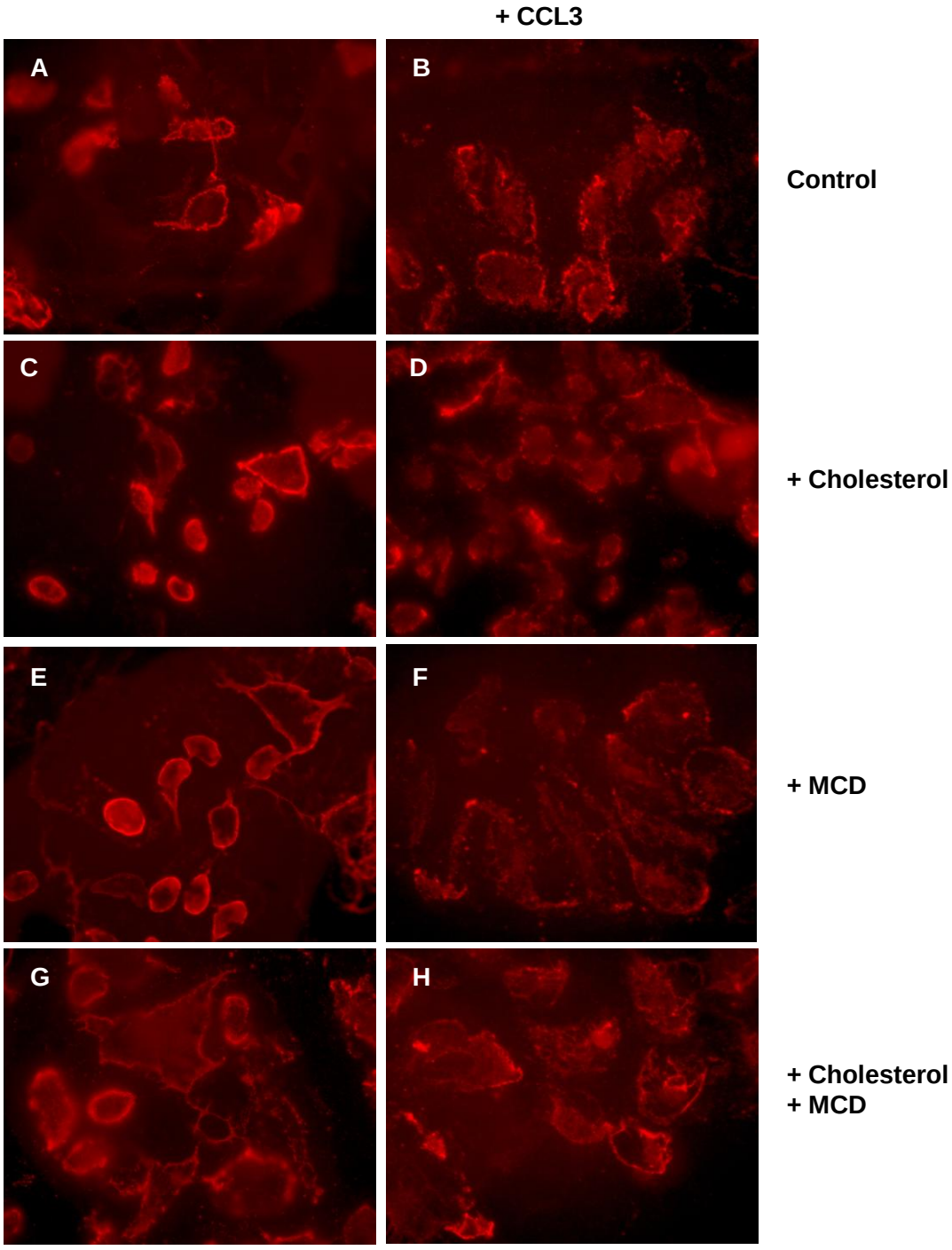
+ CCL3
A
B
Control
C
D
+ Cholesterol
E
F
+ MCD
G
H
+ Cholesterol
+ MCD

Figure 3.13 Effects of inhibitors on CCR5 internalisation. CHO.CCR5 cells were grown on coverslips overnight, incubated in medium without serum for 1 h in the presence of MCD (10 mM), cholesterol (2 mM), or both inhibitors when indicated, and then incubated with 100 nM chemokines or vehicle control for 45 min [a) control , b) CCL3 c) cholesterol, d) cholesterol+CCL3, e) MCD, f) MCD+CCL3,g) cholesterol+MCD, h) cholesterol+MCD+CCL3]. Cells were washed and a stain was performed using anti-CCR5 HEK/1/85a/7a antibody and anti-rat-TRITC. Pictures were taken using the Zeiss Axiovision 2 system. Data show representative cells from three independent experiments with similar findings (Cardaba et al., 2008).

3.4 Discussion.

Understanding the mechanisms that regulate CCR5 signalling and internalisation has been considered top priority since this receptor was discovered to be essential for HIV infection. The involvement of CCR5 in inflammatory diseases like atherosclerosis or RA is a further reason for studying this receptor's function.

In the current chapter the role of some compounds involved in the blockage of CCR5 internalisation has been studied in the signal triggered by this receptor. For this, the effect of inhibitors responsible for the blockage of clathrin coated pits and caveolae-dependent internalisation pathways have been analysed on intracellular calcium mobilization initiated by CCR5 activation. To date there are no references in the literature concerning the effect of these molecules on CCR5 signal transduction. Experimental data from this study provides proof that the inhibitors of clathrin-dependent pathways, sucrose and chlorpromazine, do not interfere with CCR5-induced calcium release. In addition to these results it has been demonstrated that disruption of lipid rafts with filipin and nystatin has no effect on CCR5-dependent calcium mobilization. Interestingly, it has been reported that MCD depletion of cholesterol significantly reduces the ability of the receptor to produce calcium fluxes, with cholesterol repletion after MCD treatment able to recover the signal to some extent. MCD treatment caused a huge reduction of CCL3 potency while it did not affect the predicted efficacy. Additionally, when ligand specificity was investigated in this study, it could be determined that the effects observed upon MCD treatment were not chemokine specific. It was observed that cholesterol depletion-dependent blockage of CCR5 signalling was also present when CCR5 was challenged with the chemokines

CCL5 and CCL3-L1.The results presented here are in accordance with previous findings where cholesterol depletion has been widely reported to cause blockage of chemokine receptors signal transduction (Monastyrskaya et al., 2005; Nguyen and Taub, 2002b; Pike and Casey, 2002; Rahangdale et al., 2006).

CCR5 has mainly been shown to signal through $G\alpha_i$ proteins although it has also been reported to be able to interact with other G proteins (Mueller and Strange, 2004a; Oppermann, 2004). The present report has confirmed that CCR5-signalling through $G\alpha_i$ increases calcium release and causes inhibition of cAMP formation (Cardaba et al., 2008). When cells were treated with the cholesterol depleting agent MCD, it became apparent that cAMP production is neither activated nor blocked by CCR5 stimulation. These data might indicate that MCD promotes the association of CCR5 with a Gα subunit different from $G\alpha_i$ or $G\alpha_s$. Therefore, it could be suggested that cholesterol removal induces the coupling of CCR5 to $G\alpha_{q/11}$ or $G\alpha_{12/13}$, all of them known not to be involved in regulating AC (Fields and Casey, 1997).

On the other hand, it is essential to highlight the fact that PTX blocks CCR5 confirms that Gαi is not involved but does not necessarily mean that another G protein is. CCR5 has been shown able to activate Janus Kinase 2 (JAK2) independently of G proteins (Mueller and Strange, 2004a), which indicates that cholesterol depletion by MCD could promote G protein-independent pathways. Therefore, further experiments should be performed to understand the involvement of a PTX-resistant G protein in cholesterol depleted cells.

In order to confirm a possible CCR5 signalling through $G\alpha_{q/11}$ or $G\alpha_{12/13}$subunits, HEK.CCR5 control and MCD-treated cells were treated with pertussis toxin (PTX), a toxin that blocks all signalling from $G\alpha_i$. As was expected, cholesterol depletion promoted the binding of CCR5 to a PTX independent G protein or causes the activation of G protein-independent pathways. Numerous studies have shown that cholesterol depletion could cause the movement of a receptor to a different domain of the plasma membrane, potentially allowing the coupling to a different G protein (Cuschieri, 2004; Huang et al., 2007; Xu et al., 2006). However, no studies were found showing CCR5 translocation out of lipid rafts or re-association with a distinct G protein after lipid rafts disruption. Thus, this might be the first time that cholesterol modulation of the plasma membrane is believed to interfere with CCR5-G proteins association.

CCR5 signalling entirely depends on the type of G protein it couples to. As it has been mentioned, CCR5 coupling to $G\alpha_i$ proteins causes inhibition of cAMP formation whereas coupling of the receptor to a different G protein does not affect the levels of this important second messenger. Furthermore, findings obtained in this study indicate that the association of the receptor with a protein different from $G\alpha_i$ produces weaker calcium responses than these initiated by CCR5stimulation of $G\alpha_i$ which can obviously alter many processes in the cell. Altogether these data estimate that modulation of cholesterol levels might have important consequences in cell signalling initiated by alterations in the coupling of CCR5 to its heterotrimeric partner.

Interestingly, it was shown that MCD-dependent effect on calcium mobilization and cAMP events was not related to a decrease in CCR5 expression in the plasma membrane. These results indicate that MCD affects some of the pathways involved in CCR5 signal transduction without having an influence on the number of molecules available for ligand activation.

The role of Caveolin-1 on CCR5 signalling has been analysed in this report. As indicated, caveolae disrupting agents different from MCD did not alter CCR5 signalling, which gives some indication of the weak role caveolae have on CCR5-dependent signal transduction. Small interfering RNA (siRNA) represents a more accurate technique to deplete the plasma membrane from caveolae's most basic component, Caveolin-1. Accordingly, siRNA was used to reduce Caveolin-1 levels and study CCR5-dependent calcium signalling in HeLa.RC49 cells. It was observed that Caveolin-1 siRNA transfected cells have similar ability to release calcium upon CCL3 stimulation when compared with cells transfected with the scrambled oligomer sequence. Immunofluorescence experiments showing that MCD, which is capable of blocking calcium events, had no apparent effect in Caveolin-1 distribution or expression in the cell further support this results. Interestingly, filipin, known to disrupt CCR5 internalisation, could remove Caveolin-1 expression from the surface. These findings suggest that although Caveolin-1 is essential for receptor internalisation, it has a weak role in CCR5-related signal transduction pathways.

Along the same lines, MCD could be shown not to have any effect in CCL3 induced CCR5 internalisation which once more supports the idea that MCD removal of

cellular cholesterol might not affect Caveolin-1 expression and, therefore, does not influence this endocytic pathway. Several studies have shown the importance of cholesterol for caveolae dependent internalisation pathways (Feng et al., 2009; Hong et al., 2009). For instance, it has been reported that β-arrestin- and clathrin-dependent endocytosis require membrane cholesterol for LPA1 lysophosphatidic acid receptors (Urs et al., 2005). It is also accepted that MCD clearly impairs the formation of chathrin-coated endocytic vesicles (Rodal et al., 1999). Considering these data, it would be expected that MCD treatment of cells impaired CCR5 dependent internalisation due to either pathway. However, the results revealed in this chapter show that CCL3-induced internalisation of the receptor was not altered by cholesterol depletion. These findings are based on immunofluorescence experiments where the remaining number of CCR5 receptors upon CCL3 stimulation was resolute by sight, pointing out the need of further experiments where the exact number of receptor molecules in the cell surface after CCL3 stimulation in MCD and control cells could be determined.

Also, the fact that cholesterol depletion does not affect Caveolin-1 expression in the cell membrane, strengthens the possibility that cholesterol depletion changes CCR5 conformation and promoting its interaction with $G\alpha_q$ proteins that are stable after cholesterol depletion due to its binding to Caveolin-1 (Oh and Schnitzer, 2001).

The interest in Caveolin-1 importance in CCR5 signalling partly lies in the role Caveolin plays in signal transduction pathways initiated by other receptors. For instance, it is known that Caveolin determines cell growth and migration by acting as a scaffolding protein. Ge et al. showed inhibition of MCP-1 induced chemotaxis, receptor internalisation and calcium signalling of astrocytes when cells were successfully transfected with Caveolin-1 siRNA (Ge and Pachter, 2004). Besides, an interesting study reveals that Caveolin-1 is required for optimal calcium responses in human airway smooth muscle, being this attributed to the fact that caveolae invaginations shorten the distance between the receptor and the ER, in such a way that the signalling turns more efficient (Prakash et al., 2007).

In disagreement with these reports, showing a main role for Caveolin-1 on calcium signalling, the current study does not prove Caveolin-1 to be involved in calcium mobilization triggered by CCR5 activation. However, there is a possibility that this protein affects some of the other signalling pathways initiated by CCR5 activation.

Overall, this report has provided wide evidences that cholesterol plays an essential role in CCR5 signal transduction while having no apparent effect on its expression or internalisation. What is more, an increase in membrane fluidity caused by cholesterol depletion is responsible for the switch of CCR5-association from $G\alpha_i$ to a PTX-independent G protein or to a G protein-independent pathway which triggers completely different signalling pathways to the original G protein.

Cholesterol levels in the body change depending on the diet, age and can also be highly influenced by cholesterol lowering drugs. Hence, the fact that cholesterol content in membranes can have such a dramatic effect on the signalling of CCR5 by reducing the amount of calcium released and by diverting its signalling to an AC independent pathway, could have important repercussions in CCR5-derived cell signalling mechanisms.

CHAPTER 4- THE MONOCYTIC CELL LINE THP-1 NEEDS CHOLESTEROL DEPLETION FOR OPTIMAL SIGNALLING

4.1 Introduction

Human monocytes have a few characteristics that make them less than ideal for research purposes. Two of the more important ones are that they are difficult to obtain in large numbers and when extracted from different patients they do not represent a homogenous group of cells. Monocytes are derived from haematopoietic stem cells. Pluripotent stem cells from myeloid stem cells form the colony-forming unit for granulocytes-monocytes (CFU-GM), which is the precursor of monocytes, macrophages and granulocytes. CFU-GM differentiates into monoblasts which, in turn, differentiate into pro-monocytes, the immediate precursors of monocytes (see Figure 4.1).

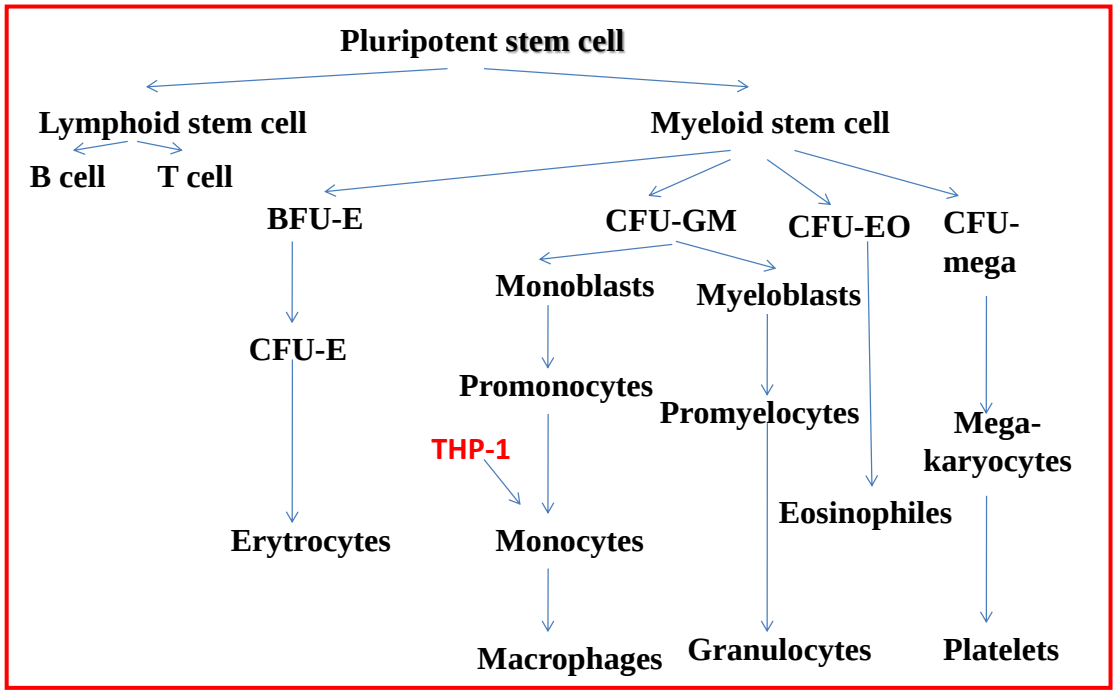

Figure 4.1 Haematopoietic cell differentiation. THP-1 cells are in red. Arrows indicate their differentiation potential. Diagram adapted from Auwerx et al. (Auwerx, 1991).

THP-1 is a human monocytic cell line that was obtained from the peripheral blood of a 1 year old human male with acute monocytic leukaemia (Tsuchiya et al., 1980). THP-1 cells are promonocytes that are very similar to human monocytes. They share with them morphology, secretory products, oncogene expression, membrane antigens expression and some genes involved in the metabolism of lipid derivatives (Auwerx, 1991). THP-1 cells can be differentiated into macrophages by treatment with the phorbol ester PMA (Tsuchiya et al., 1980) or with 1,25-dihydroxyvitamin D3 (Schwende et al., 1996). This process involves a series of morphological and physiological changes in the cell such as loss of round shape, adherence to the tissue culture plates, nucleus homogenous shape loss, and increase in phagocytic vacuoles in the cytoplasm (Auwerx, 1991). It is also well known that changes in certain oncogenes expression, essential for cellular regulation, are brought about by THP-1 differentiation into macrophages (Gowda et al., 1986). Due to these properties THP-1 cells have been extensively used in research as a close model to human monocytes (Hiraoka et al., 2004; Tian et al., 2008;Vaddi and Newton, 1994).

In addition to its widespread application on monocytes research, THP-1 cells represent a good model to study the secretion of certain proteins. This cell line has been shown to secrete cytokines and peptides hormones in a similar manner to macrophages. The most studied cytokine secreted by THP-1 is IL-1, which is produced upon numerous stimuli. Other particularities of differentiated THP-1 cells are that they express the same genes involved in lipid metabolism than macrophages, and share with them the capacity to be easily loaded up with lipids (Auwerx, 1991). Owing to these characteristics, THP-1 cells become foam cells in the same way that macrophages do in atherosclerotic lesions, making them an excellent model for the study of cholesterol derived pathologies. This is something very relevant to the study being described in this chapter since cholesterol modulation of THP-1 cells will give some indication of how cholesterol loading of macrophages could affect the signalling properties of these cells.

Atherosclerosis is a complex process with a variable etiology generally initiated by an excess of oxidised cholesterol formation that damages wall arteries. One of the main characteristics of this pathology is the recruitment of monocytes and T lymphocytes to the inflammation site by the chemokines released by damaged endothelial cells. Once in contact with the lesion cells, monocytes differentiate into macrophages and start absorbing the excess of cholesterol until they lyse and leave deposits of cholesterol in the artery (Glass et al., 2006). It is known that chemokine receptors play a key role in directing both monocytes and T cells to the inflammation site and that artificial modulation of some of these receptors can regulate the atherosclerotic process (Phillips et al., 2005). Thus, it is essential to understand how cholesterol modulation affects the signalling of chemokine receptors in environments where an excess of cholesterol governs the signalling scene.

THP-1 cells mainly express CCR1, CCR2 (Phillips, Lutz et al. 2005) and CCR5 (Cardaba and Mueller, 2009) chemokine receptors and, therefore, represent a good model to study how chemokine signalling networks interact in a natural system. Cell signalling experiments performed in stably transfected cells only expressing one type of receptor would fail to represent the signalling mechanisms that naturally occur in a living organism. In the previous chapter two cell lines stably expressing the chemokine receptor CCR5 were used to study the signalling characteristics of this receptor in different situations. Special interest was put on how cholesterol modulation altered CCR5 dependent signal transduction pathways. Now the results obtained with these cell

lines will be verified in a cell line closely related to human monocytes, endogenously expressing CCR5. As monocytes are known to accumulate in an environment where cholesterol levels are often elevated, especially in certain pathologies, this section will focus on the effects cholesterol modulating drugs have on CCR5 signal transduction in the monocytic cell line THP-1.

4.2 Aim

In the current study THP-1 cells have been used to compare the results obtained with CHO and HEK cells exogenously expressing CCR5 with a cell line naturally expressing the receptor. The consequences of lipid rafts disruptions in CCR5-induced signalling responses like intracellular calcium mobilization and chemotaxis assays will be investigated. Finally, a possible mechanism through which MCD affects CCR5 signalling in this cell line will be discussed.

4.3 Results

4.3.1 Cholesterol depletion but not complexation increases intracellular calcium mobilization in THP-1 cells.

Cholesterol is thought to be essential for the correct signalling of many GPCR (Monastyrskaya et al., 2005; Sooksawate and Simmonds, 2001). CCR5 has also been demonstrated to need membrane cholesterol to keep the ability to stimulate intracellular pathways (Cardaba and Mueller, 2009; Nguyen and Taub, 2002b; Nguyen and Taub, 2003a, b)

In the previous chapter it has been shown that cholesterol extraction with MCD but not cholesterol sequestration with other drugs had an inhibitory effect on CCR5 signalling. In this chapter the effects of lipid raft disruption were investigated in a cell line that naturally expresses CCR5 as well as other chemokine receptors. Considering that GPCRs share the intracellular machinery that enables signal transduction, THP-1 cells represents a more physiological system to study chemokine receptors signalling.

In this chapter, CCL3 has been used at a concentration of 200 nM in most of the

experiments contrary to the 100 nM used in CHO.CCR5 and HEK.CCR5 cells as the optimal signalling of THP-1 cells is at higher concentrations than the stably transfected cells.

When cholesterol was depleted from THP-1 cells and calcium signalling was analysed surprising results were obtained. CCL3 activation of CCR5 in untreated cells causes the expected increase in calcium mobilization (Figure 4.2). Interestingly, when cells were treated with MCD this increase in calcium mobilization was far more pronounced (Figure 4.2 A, B, C). To assess whether this increase in receptor signalling was due to caveolae disruption we used the cholesterol sequestering drugs filipin and nystatin, also known to disrupt lipid rafts and caveolae. Contrary to MCD treatment, filipin and nystatin only caused a slight increase in calcium mobilization (Figure 4.2 D). These results indicate that MCD extraction of cholesterol but not cholesterol complexation by filipin or nystatin is responsible for the increase in intracellular calcium release.

Dose responses performed in our laboratory indicated that the EC50 of CCL3 in MCD-treated THP-1 cells was 200 n M. It is important to note that the concentration of CCL3 used in this chapter doubles the one used in chapter 3, where the EC50 of CCL3 was 100 nM. The reason behind this difference is that MCD-treated THP-1 cells respond better to higher concentrations of chemokine. Interestingly, the basal signal of THP-1 untreated cells is much lower than that of CHO.CCR5 or HEK.CCR5 cells, which might be due to differences in the number of CCR5 molecules expressed on these cell lines.

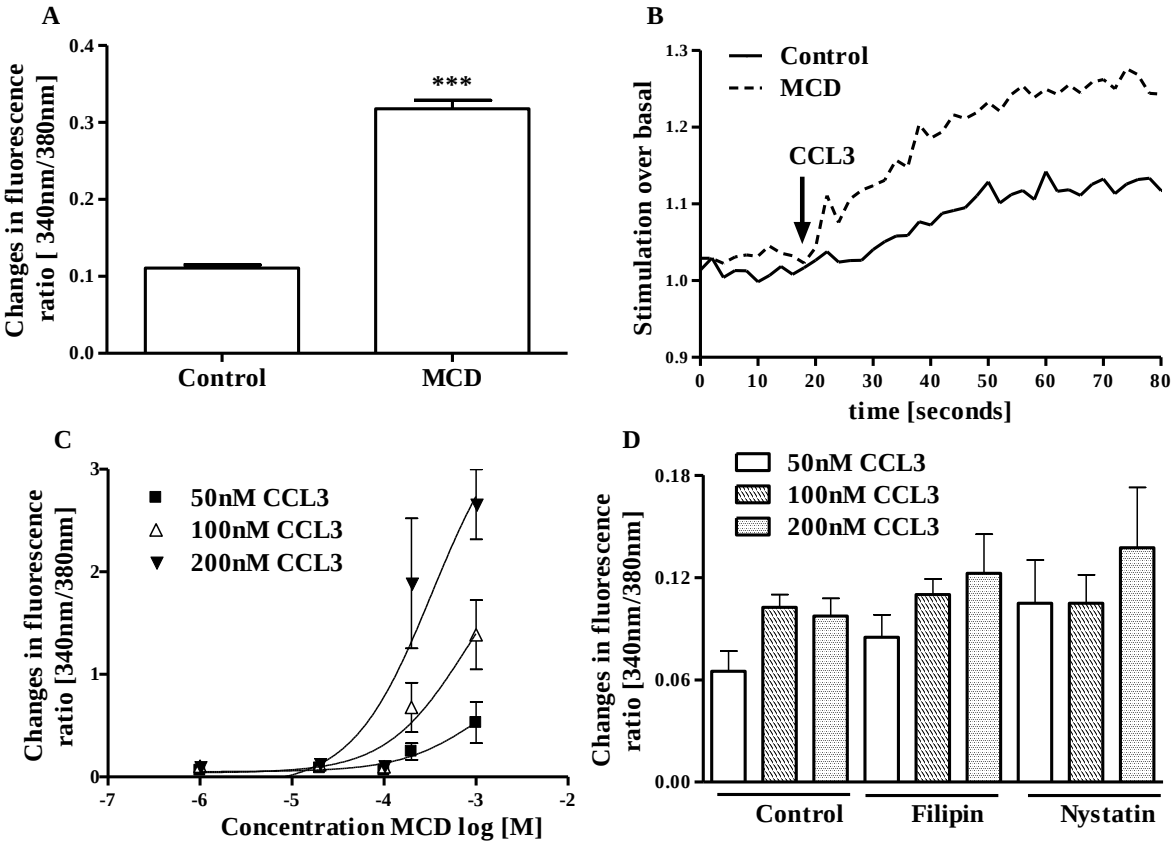

Figure 4.2 Changes in intracellular calcium in THP-1 cells in the presence of inhibitors. A) Cells were treated with MCD and challenged with 200 nM CCL3. B) Shows real traces of THP-1 cells treated with 10 mM MCD and stimulated with 200 nM CCL3. C) Shows concentration response curve for THP-1 cells treated with 10 mM MCD. D) Cells were treated with filipin (5 µg/ml), nystatin (50 µg/ml) or vehicle (control) and challenged with different concentrations of CCL3. Significant changes towards control cells are indicated by asterisks (***$p < 0.001$). 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 ± SEM from at least three independent experiments for the bar charts and a representative tracer for the calcium flux. (Cardaba and Mueller, 2009)

4.3.2 Analysis of cholesterol levels after MCD and filipin treatment

In order to understand the reasons why MCD but not filipin and nystatin had such an enhancing effect on signal transduction as measured by calcium release, experiments measuring the amount of cholesterol left in the membrane after MCD, filipin or nystatin treatment were performed. An enzymatic assay was used to analyse the total concentration of cellular cholesterol and it was found that only MCD treatment could

reduce cellular cholesterol considerably. Whereas MCD depletes 70% of total cellular cholesterol, filipin does not affect cholesterol concentration at all (Figure 4.3). Therefore, it can be suggested that THP-1 cells have a higher ability to release calcium to the cytosol when cholesterol has been moved away from the membrane. MCD and filipin, both are known to disrupt lipid rafts and, therefore, the fact that these drugs have different effects on THP-1 signalling points at the possibility that the increase in signalling observed in cholesterol depleted cells occurs due to the reduced cholesterol contents and is not related to lipid rafts disruption. Nevertheless, it could also be considered that MCD, by extracting cholesterol from the cell without forming part of it like filipin, has a different effect on CCR5-induced calcium signalling. Actually, it has been reported that MCD-dependent cholesterol depletion causes increases in calcium release whereas filipin treatment has the opposite effect (Qin et al., 2006) which supports the different data here obtained in filipin and MCD-treated THP-1 cells.

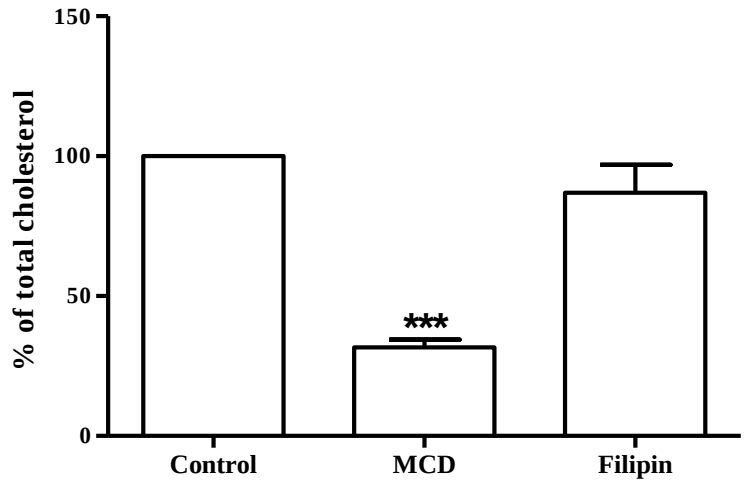

Figure 4.3 Effects of MCD and filipin on cholesterol contents in THP-1 cells. Cells were incubated in the presence of MCD (10 mM) and filipin (5 µg/ml) for 1h prior to cholesterol assay performance. Data represent mean ± SEM from at least three independent experiments. Significant changes towards control cells are indicated by asterisks (***$p < 0.001$). (Cardaba and Mueller, 2009).

4.3.3 The effects observed in MCD treated THP-1 cells are CCR5 specific and cholesterol dependent.

MCD treatment of cells has a drastic effect on releasing intracellular calcium in response to CCL3. Considering that a natural increase in cytosolic calcium concentration triggers many signalling pathways, this vast increase in calcium mobilization could have important effects in the ability of the cell to transmit certain signals. Consequently, it was next sought to understand the exact mechanism behind this increase in CCR5 signalling. For this, cells were treated with inhibitors specific for different enzymes known to be involved in calcium signalling responses activated upon CCR5 activation. In addition to this, the possibility that signal transduction responses triggered by other chemokine receptors had a role on the effect here described was also investigated by stimulating cells with the CCR5-specific chemokine CCL4. With the purpose of ascertaining that calcium was being released from ER stores due to IP3 production triggered by CCR5 activation, the source of calcium origin was looked at as well. Additionally, the effects of cholesterol loading of THP-1 cells were studied to understand if the effects of MCD were completely dependent on its ability to deplete cellular cholesterol.

4.3.3.1 Cholesterol depletion causes increase in intracellular calcium released from internal stores.

The increase in calcium release upon cholesterol depletion was such that the question of whether calcium came from ER stores or from the extracellular medium arose. CCR5 has only been shown to release calcium from internal stores but considering the massive response obtained, the origin of calcium release upon CCR5 stimulation was investigated. When THP-1 cells were treated with MCD and challenged with CCL3 in the absence of calcium in the medium (Figure 4.4) it could still be observed a huge increase in CCR5 activation. This shows that, as expected, MCD treatment of cells stimulates the release of calcium from intracellular stores and not calcium entry from the extracellular medium.

These findings have some relevance as they indicate that the effect of cholesterol loss from the cell is highly related to CCR5 dependent signalling pathways and not to modifications in plasma membrane channels allowing the entry of extracellular calcium.

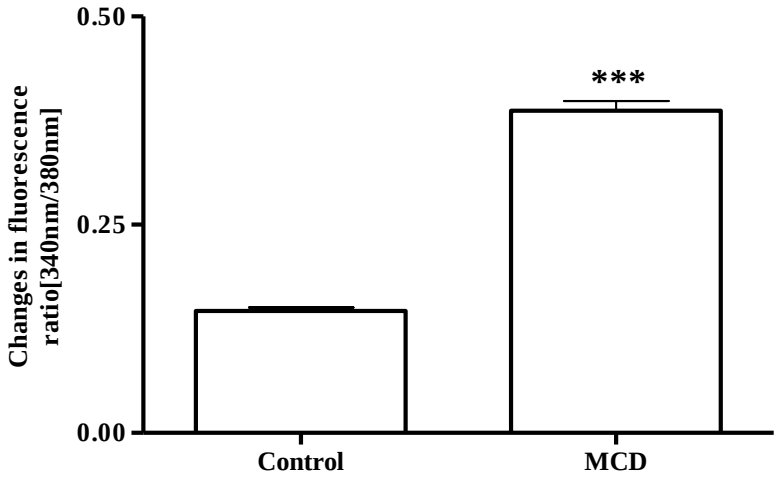

Figure 4.4 The increase in calcium release observed is not dependent on extracellular calcium. THP-1 cells were treated with 10 mM MCD and stimulated with 200 nM CCL3 in calcium free buffer. Data represent mean ± SEM from at least three independent experiments for the bar charts and a representative tracer for the calcium flux. Significant changes towards control cells are indicated by asterisks (***$p < 0.001$).

4.3.3.2 Does the CCR5 specific chemokine CCL4 also enhance calcium release?

As it has been previously mentioned, THP-1 cells express CCR5 receptor together with other chemokine receptors that could potentially signal upon CCL3 stimulation. For instance the chemokine receptor CCR1 has been shown to be strongly activated upon CCL3 stimulation (Murphy et al., 2000). In order to understand if the effects observed upon CCL3 stimulation were exclusively due to CCR5 activation, THP-1 cells were treated with the CCR5-specific chemokine CCL4 (Murphy et al., 2000). Interestingly it can be observed (Figure 4.5) that CCL4 induction of CCR5 signalling, although also increased, is not as pronounced as when the receptor is stimulated by CCL3.

These data suggest that although calcium release responses in THP-1 cholesterol depleted cells are dependent on CCR5 signalling, this effect might also be boosted by other chemokine receptors such as CCR1.

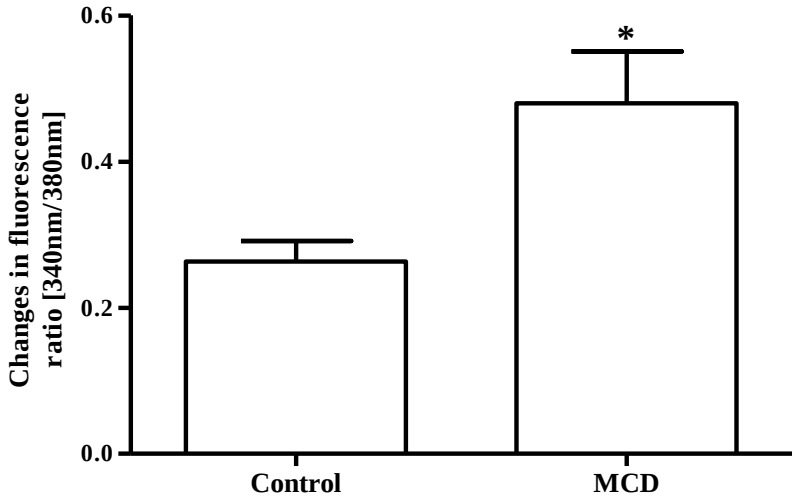

Figure 4.5 Intracellular calcium release in cholesterol depleted THP-1 cells challenged with 200 nM CCL4. Cells were treated with 10 mM MCD or left untreated (vehicle). Data represent mean ± SEM from at least three independent experiments for the bar charts and a representative tracer for the calcium flux. Significant changes towards control cells are indicated by asterisks (*$p < 0.05$).

4.3.3.3 MCD-treated THP-1 cells signalling can be blocked with PLC, PI3K and IP3R inhibitors, all enzymes involved in CCR5 signalling.

We next tried to understand whether the effects observed upon cholesterol depletion were related to CCR5 signalling by blocking some of the enzymes known to be involved in CCR5-dependent calcium release. Before studying in depth CCR5 signalling in THP-1 cells, experiments were completed analysing the enzymes involved in calcium release upon CCR5 stimulation in CHO.CCR5, HeLa.RC49 and HEK.CCR5 cells. We demonstrated that blocking PLC and PI3-K completely abrogate calcium fluxes initiated by CCR5 (see Figure 4.6 and 4.7). Since PLC is the enzyme responsible for IP3 production it seems clear that its inhibition should impair any signalling mechanism dependent on this second messenger. The role of PI3K is less apparent. However, PLC activation has been shown to be dependent on PI3K activity (Bony, Roche et al. 2001) and this may explain why PI3K blockage abrogates calcium signalling. Therefore, these findings provide strong evidence that PLC and PI3K act as a scaffold that helps transduce CCR5 signalling. Moreover, the importance of these enzymes on CCR5 signalling has been reported previously (Chuang et al., 2009; Harmon and Ratner, 2008; Huang et al., 2009a; Shideman et al., 2006).

In the next experiment therefore, we used the PLC inhibitor U73122 (Bleasdale et

al., 1990), the PI3K inhibitor Ly294002 (Vlahos et al., 1994) and 2-APB, an IP3R antagonist (Bootman et al., 2002) and blocker of store-operated calcium channels (SOCs) (Iwasaki et al., 2001), to verify that CCR5 signalling is directly involved in the effect of MCD in THP-1 cells.

Two other inhibitors were added to the ones described above as we thought they could shed some light on the mechanisms behind calcium signalling increase. Mastoparan is a peptide toxin obtained from wasp venom that acts as an amphiphilic G protein activator that binds to the PTX-sensitive proteins G_i and G_o (Higashijima et al., 1990; Higashijima et al., 1988). It also binds with high affinity to calmodulin and inhibits the sarcoplasmic reticulum Ca^{2+}-ATPase (Longland et al., 1999). The exact effect of mastoparan on chemokine receptors signalling is not clear. It has been shown to stimulate G proteins activity by promoting GTP binding to the $G\alpha_i$ subunit but at the same time it is known to stimulate the GTP-ase activity of G proteins which would result in a shorten of the G protein lifespan (Higashijima et al., 1990).

The Rho-kinase inhibitor Y23672 was also used to try to define the mechanism through which MCD may enhance calcium release in THP-1 cells. Rho-kinases are important enzymes for actin polymerization and cytoskeleton rearrangement and its activation has been shown to be dependent on membrane cholesterol levels before (Qin et al., 2006). Additionally, MCD treatment is known to increase tyrosine-dependent phosphorylations and the activation of the Ras-ERK MAP kinase pathway (Kabouridis, 2006) which gives some indication of the involvement of ras proteins on MCD-dependent pathways. Consequently, a Rho-kinase inhibitor was used to analyse if blocking this enzyme had any effect in MCD-induced increase in calcium signalling.

Figure 4.8 shows that all the inhibitors used but the rho inhibitor Y27632, are able to abrogate the increasing effect on calcium release upon MCD treatment. These results indicate that cholesterol depletion increases the ability of the ligand to induce calcium mobilization through the axis G proteins-PLC-IP3. When rho-kinases (rock) are blocked, a significant increase in intracellular calcium mobilization can be observed. The reason why this happened is completely unknown and no reports showing similar results could be found in the literature.

Therefore, more research on this subject would be needed to understand how blockage of rho-kinases (rock) proteins can lead to an increase in calcium release in cholesterol depleted monocytes.

From this experiment it can be concluded that the enhancement of calcium release observed in MCD-dependent lipid rafts disrupted cells could be returned to basal levels by inhibiting PLC, PI3K, by blocking the IP3R or by modulating G proteins conformation with mastoparan. These results indicate that cholesterol depletion directly modulate chemokine receptors dependent intracellular cascades. Some studies have shown that MCD treatment of cells resulted in phosphorylation and activation of PLC (Kabouridis, 2006) which would support the results obtained in this section. This increase in PLC activity could directly enhance the amount of calcium released due to an increase of IP3 formation and there is the possibility that MCD not only promotes the activation of PLC but also of PI3K or IP3R.

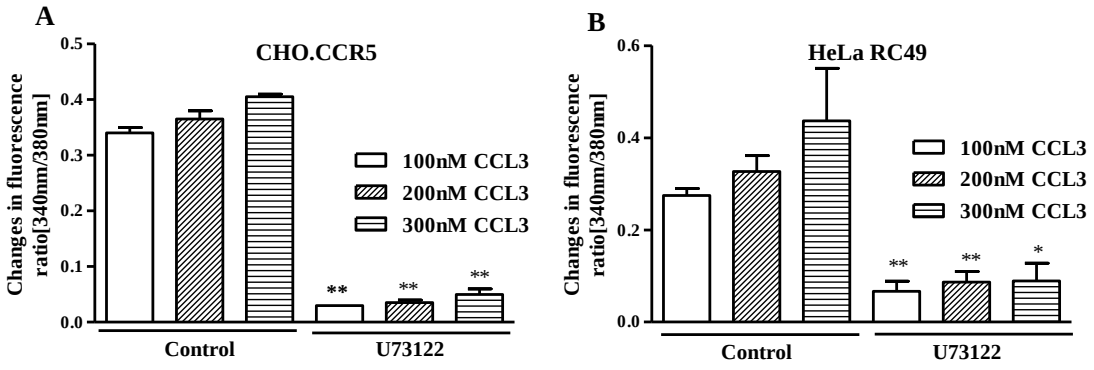

Figure 4.6 PLC activity is needed for CCR5 dependent calcium signalling. Cells were treated with the PLC inhibitor 10 µM U73122 for 30 minutes or left untreated (vehicle) and were stimulated with different concentrations of CCL3. Graph shows calcium flux assay for CHO.CCR5 (A) of HeLa.RC49 (B) cells. Data represent mean ± SEM from at least three independent experiments for the bar charts and a representative tracer for the calcium flux. Significant changes towards control cells are indicated by asterisks (*$p < 0.05$, **$p < 0.01$).

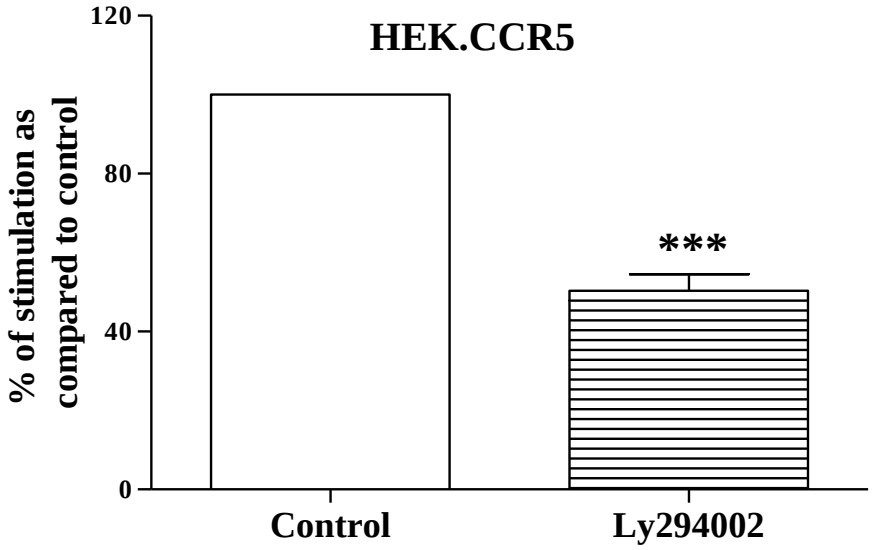

Figure 4.7 PI3-K activity is needed for CCR5 dependent calcium signalling. HEK.CCR5 cells were treated with the PI3-K inhibitor Ly294002 (20 µM) for 30 minutes or left untreated (vehicle) and were stimulated with 200 nM CCL3 prior to performance of a calcium assay. Data represent mean ± SEM from at least three independent experiments for the bar charts and a representative tracer for the calcium flux. Significant changes towards control cells are indicated by asterisks (***$p < 0.001$).

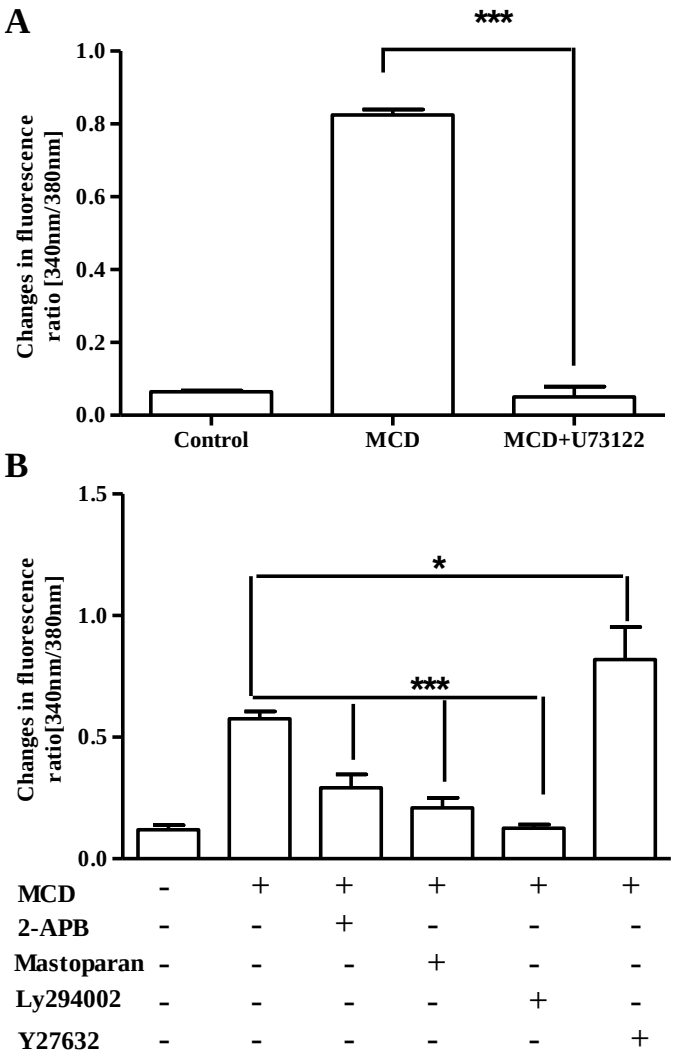

Figure 4.8 Inhibitors of the main enzymes involved in calcium release bring calcium release back to normal levels after cholesterol depletion. A) THP-1 cells were treated with 10 mM MCD, MCD and the PLC inhibitor, U73122 (10 µM) or left untreated (control) before cells were stimulated with 200 nM CCL3. B) THP-1 cells were treated with MCD and MCD plus 2-APB (20 µM), mastoparan (10 µM), LY294002 (20 µM) and Y27632 (10 µM), before they were challenged with 200nM CCL3. Data represent mean ± SEM from at least three independent experiments for the bar charts and a representative tracer for the calcium flux. Significant changes towards control cells are indicated by asterisks (*$p < 0.05$, ***$p < 0.001$).

4.3.3.4 Cholesterol repletion of cells abrogates the increase in signalling.

In this section the possibility that loading cholesterol back to the cell could reverse the effect of cholesterol depletion was analysed. Cells were treated with MCD, MCD and cholesterol or left untreated and calcium mobilization upon CCL3 stimulation of the receptor was measured as before. Figure 4.9 shows that cells treatment with MCD and

cholesterol brings MCD's signalling back to basal levels, corroborating the hypothesis that it is the loss of cellular cholesterol what causes the increase in calcium mobilization.

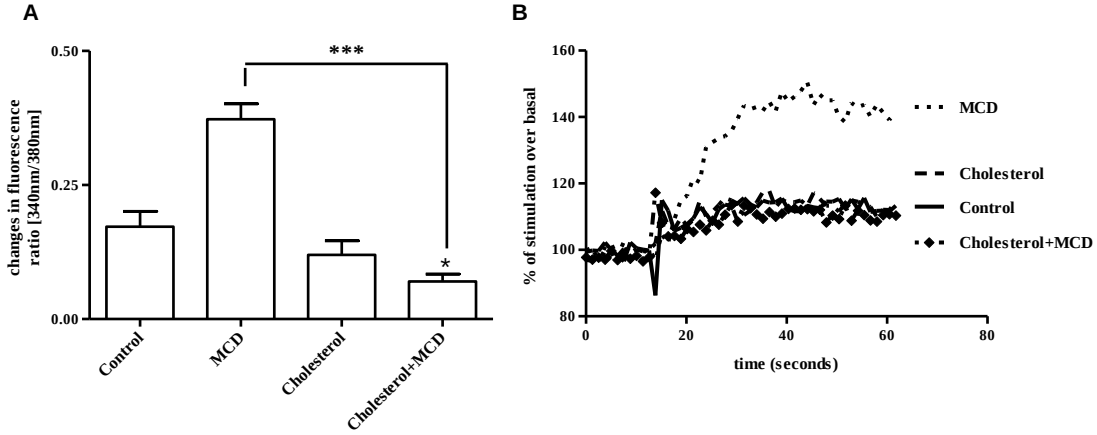

Figure 4.9 Cholesterol repletion of cells reverses the increasing effect of MCD on calcium release. A) THP-1 cells were treated with 10 mM MCD, 2 mM cholesterol or MCD plus cholesterol before receptor activation with 100 nM CCL3. B) Single calcium traces of THP-1 cells from A. Data represent mean ± SEM from at least three independent experiments for the bar charts and a representative tracer for the calcium flux. Significant changes towards control cells are indicated by asterisks (*p < 0.05, ***p < 0.001). (Cardaba and Mueller, 2009)

4.3.4 MCD slightly increases CCR5 expression on THP-1 cells.

One of the possibilities considered when a decrease in cholesterol levels was observed to cause a loss of CCR5 signalling in CHO.CCR5 and HEK.CCR5 cells was that MCD, by disrupting lipid rafts, was impairing CCR5 expression in the plasma membrane. However, when cholesterol was subtracted from CHO.CCR5 and HEK.CCR5 cells no alterations in receptor expression were observed (chapter 3). Now the effect of MCD treatment was studied on THP-1 cells to see whether changes in CCR5 receptor expression could explain the increase in CCR5 dependent signalling. CCR5 expression was measured by flow cytometry upon 30 minutes treatment with MCD. Interestingly, it was found that cholesterol depletion slightly increased CCR5 membrane expression in THP-1 cells (Figure 4.10). Nevertheless, this increase was not significant and cannot account for the high enhancement of calcium release observed in THP-1 cells upon cholesterol depletion. It is worth noting that the THP-1 cells used in this study do not consist of a uniform population of CCR5 expressing cells. As can be

seen in Figure 4.10 B, THP-1 cells can be divided into a group of cells highly expressing CCR5 receptors and a group expressing low amounts of it. This might be due to cells being in a different maturation or differentiation state. It is actually known that chemokine receptors expression can vary along the differentiation state of THP-1 cells. For instance, it was shown that this cell line lost CCR2 expression upon PMA treatment whereas the levels of CCR1 remained unaltered (Phillips et al., 2005).

A study done by Nguyen et al. (Nguyen and Taub, 2002b) demonstrated that cholesterol depletion of CEM-NKR-CCR5 cells changed the affinity of the chemokine CCL4 for CCR5 and they demonstrated that this was due to changes in CCR5 receptor conformation after cholesterol depletion. It could be hypothesised that this effect is slightly different in THP-1 cells and cholesterol depletion changes CCR5 conformation so that it is more easily recognised by the antibody used in this study. Another possibility would be that lipid rafts disruption increases the number of CCR5 receptors being recruited to the plasma membrane although considering that CCR5 is specifically targeted to lipid rafts, this does not seem very likely.

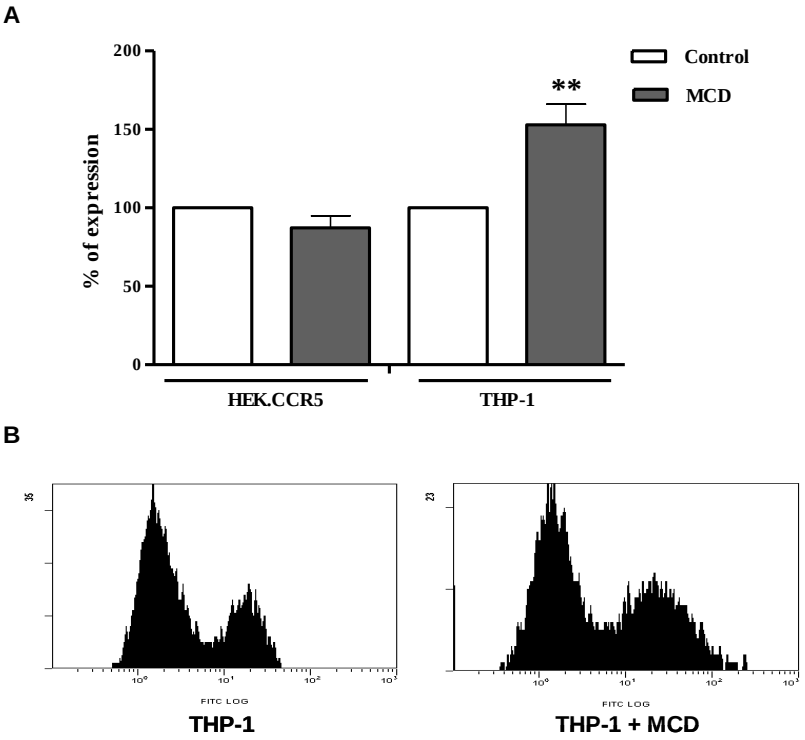

Figure 4.10 MCD treatment of cells increases CCR5 expression in THP-1 cells. A) Flow cytometry analysis of HEK.CCR5 and THP-1 cells after treatment with 10 mM MCD for 1 h. Cells were stained with an anti-CCR5 antibody for 1 h and the corresponding FITC-conjugated secondary antibody for 1h and analysed using flow cytometry, data represents percentage of Geo Mean fluorescence compared to vehicle treated (control) cells. B) Shows flow cytometry histograms analysing CCR5 expression on control THP-1 cells or MCD-treated cells. Data represent mean ± SEM from at least three independent experiments for the bar charts and a representative tracer for the calcium flux. Significant changes towards control cells are indicated by asterisks (**$p < 0.01$). (Cardaba and Mueller, 2009).

4.3.5 MCD depletion of cholesterol promotes the coupling of the receptor to a PTX independent G protein.

In the previous chapter it was demonstrated that cholesterol depletion in HEK.CCR5 cells causes the coupling of the receptor to a PTX-resistant G protein. Therefore, the G protein subunit involved in calcium release signalling in THP-1 cells was also investigated. In normal conditions THP-1 cells seem to couple to $G\alpha_i$ proteins since treatment of cells with PTX significantly blocks calcium release to the cytosol (see Figure 4.11). In the next experiments, THP-1 cells were treated with MCD for 30 min, with PTX for 18 hours or with both substances simultaneously prior to calcium flux measurements. Figure 4.11 shows that the effect of PTX after 2 hours treatment with the toxin is abrogated by treatment of cells with MCD. These findings may be interpreted as

MCD promoting the coupling of CCR5 to a PTX-insensitive G protein. These data could help clarifying why cholesterol depletion from this cell line causes a big increase in intracellular calcium mobilization. In HEK.CCR5 cells, the calcium response in cholesterol depleted cells was still lower than in untreated cells. The fact that in THP-1 cells MCD treatment results in a higher response might be simply due to a more effective CCR5-PTX-independent-G protein association in cholesterol depleted cells due to differences between both cell lines that have not been yet characterised.

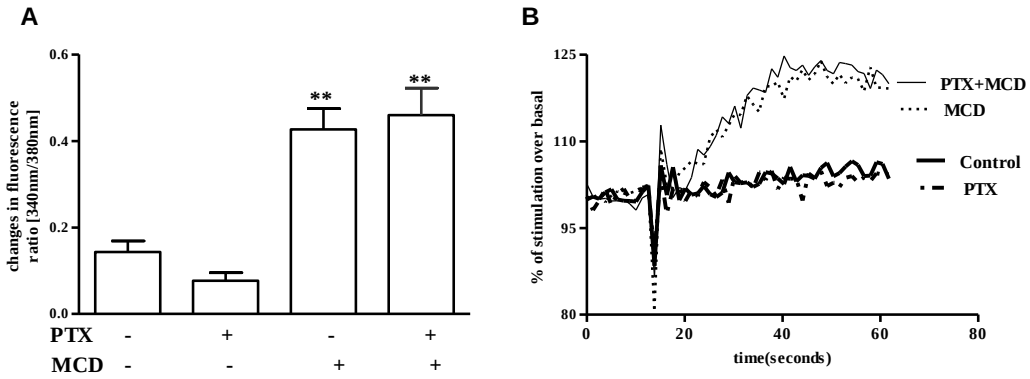

Figure 4.11 Effect of MCD on PTX treatment of THP-1 cells. A) Cells were treated with 1 µg/ml PTX or vehicle for two hours and 10 mM MCD was added 1 h before calcium flux was induced with 100 nM CCL3. B) Single calcium trace of THP-1 cells treated with PTX and/or MCD before addition of 100 nM CCL3. Significant changes towards control cells are indicated by asterisks (**p < 0.01). Data show mean ± SEM of at least 3 experiments in the case of the bar charts and a representative tracer for the calcium flux. (Cardaba and Mueller, 2009).

4.3.6 Is chemotaxis also increased in cholesterol depleted THP-1 cells?

One of the main roles of chemokine receptors is to direct white blood cells to inflammation sites following a chemokine gradient. The fact that calcium responses were so much enhanced in cholesterol depleted THP-1 cells lead to the study of other signalling responses in this cell line. Special interest was put on cell migration responses due to the role of CCR5 in chemotaxis in diverse human pathologies. There are

numerous reports giving evidence that CCR5 directs migration of numerous types of cancer cells and studies done on rheumatoid arthritis patients show that CCR5 is involved in the excessive number of immune cells recruited to the synovial tissue. In addition, CCR5 has been shown to recruit T cells from the blood and brain barrier and to cause an excess of inflammation that contributes to the development of diseases such as Alzheimer disease (Man et al., 2007). Therefore, the aim of this study was to analyse if, similarly to the increase observed in calcium release responses, CCR5 dependent chemotaxis in cholesterol depleted THP-1 cells was also over-stimulated. An increase in this pathway as significant as the increase observed in calcium mobilization could boost cell migration in situations where it is already excessive.

Currently there are no reports giving evidence of the connection between calcium release and chemotaxis for CCR5. The chemokine concentration needed to initiate calcium fluxes is different from the concentration required for optimal cell migration (Maghazachi and Al-Aoukaty, 1998) which indicates that these processes cannot be interconnected. However, numerous studies have discovered that both actions have common upstream enzymes like PLC or PI3K, and this opens the possibility that cell migration requires previous calcium signalling events.

In this section it could be shown that calcium mobilization and cell migration responses initiated by CCR5 are not interconnected. Figure 4.12 illustrates that MCD, far from increasing THP-1 cell migration upon CCL3 exposure, almost completely abrogates this process.

No studies have been found in the literature reporting that one specific treatment or stimulus can lead to a dramatic increase in calcium release and to a blockage of chemotaxis for CCR5 or other chemokine receptors. Therefore, this study provides an interesting piece of knowledge for further research into how both pathways are activated.

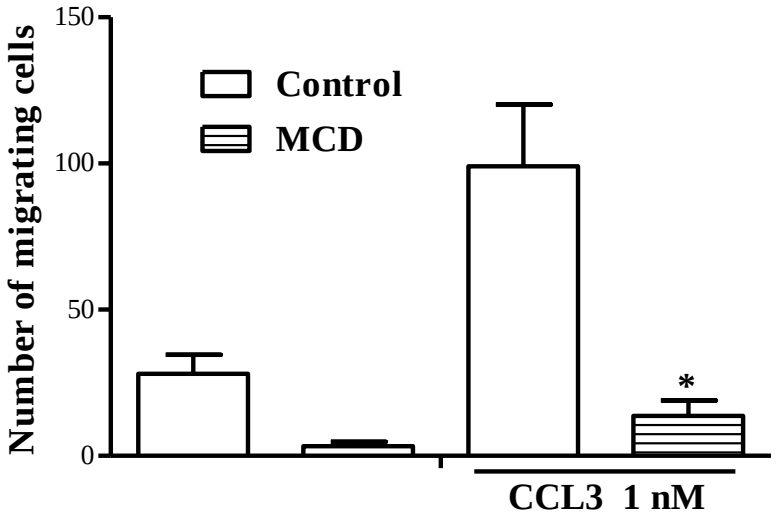

Figure 4.12 MCD treatment of THP-1 cells blocks chemotaxis upon treatment with the chemokine CCL3. THP-1 cells were treated with 10 mM MCD for 30 min or left untreated (control) prior to the performance of the chemotaxis assay. Data shown represent mean ± SEM of 3 independent experiments.

4.4 Discussion

Membrane lipid microdomains differentially regulate intracellular signalling events in THP-1 cells, which endogenously express CCR5, and cells exogenously expressing the receptor. It has been demonstrated that an important loss of cholesterol promoted by MCD treatment is needed for optimal calcium signalling responses in this monocytic cell line (Cardaba and Mueller, 2009). On the other hand it has been shown that chemotaxis processes require intact lipid rafts as shown by the fact that MCD abrogates cell migration upon CCL3.

There is extensive evidence that MCD affects intracellular signalling pathways in different ways depending both on the cell type and the signalling mechanism itself. For example, Tuluc et al. (Tuluc et al., 2003) show that while cholesterol depletion with MCD in human neutrophils has an inhibitory effect on calcium release induced by the IL-8 binding to its receptors CXCR1 and CXCR2, this phenomenon has no effect on calcium release induced by formyl-Met-Leu-Phe (fMLP) binding to the formyl peptide receptor-like 1 (FPRL1). These data indicate that lipid rafts integrity plays different roles

depending on the receptor reliance on these microdomains. Consequently, it can be said that the same cells can trigger varying responses depending on the type of stimuli. In their study, Tuluc et al. also demonstrated that MCD blocks Erk phosphorylation in stimulated neutrophils while increasing phosphorylation of p38 MAPK in unstimulated cells. Altogether, the report of Tuluc and co-workers provides strong evidence about the differing roles played by cholesterol depletion in the stimulation of distinct receptors in the same cell line. On the other hand, the research described in this thesis provides evidence of the same stimuli causing opposite reactions in different cell lines. The fact that MCD can modulate cellular signalling in an opposite way upon stimulation of a same receptor in two different cell types has been reported before (Chen et al., 2007).

Chapter 3 has reviewed many studies highlighting an inhibitory role for cholesterol depletion in GPCRs signalling. Although MCD treatment of cells has generally been shown to have an inhibitory effect on cell signalling, there are numerous examples of receptor signalling enhancement brought about by lipid raft disruption. For instance, stimulation of the epidermal growth factor (EGF) receptor in cholesterol depleted cells produces a significant increase in MAPK activity and enhances receptor dimerization and autophosphorylation (Chen and Resh, 2002; Furuchi and Anderson, 1998; Pike and Casey, 2002; Westover et al., 2003). In B cells, MCD treatment also increases calcium release through the activation of the B cell receptor (BCR) (Awasthi-Kalia et al., 2001). Another exciting study showing that cholesterol depletion could activate certain signalling pathways was performed by Kabouridis et al. (Kabouridis, 2006). This group shows that MCD treatment of T cell is responsible for PLC phosphorylation and recruitment to the membrane as well as for the activation of other important intracellular pathways.

In this chapter the effect of MCD has been compared to that of filipin, another lipid raft disrupting agent, to understand whether MCD modulates CCR5 signalling in a mechanism exclusively dependent on lipid raft disruption or whether there are other factors involved. It has been demonstrated that filipin treatment slightly increases calcium release on THP-1 cells and that treatment with this molecule is unable to reduce total cellular cholesterol. On the contrary, MCD treatment produces a loss of 70% of total cholesterol, giving an indication that the signal transduction enhancement observed in cholesterol depleted cells is more likely to be due to a decrease in cholesterol concentrations than to lipid rafts disruption.

As it has been outlined, MCD increases calcium release in B cells through activation of the B cell receptor (BCR) (Awasthi-Kalia et al., 2001). It is of interest to highlight that the same group also analysed the effects of filipin treatment on calcium signalling in B cells and demonstrated that it inhibits calcium mobilization. In an attempt to understand the difference between these two substances, they found that MCD has a larger capacity to disrupt lipid rafts than filipin. They state that the ability of MCD to expulse molecules like $G\alpha_i$ and $G\beta\gamma$ subunits from lipid rafts and to prevent the B cell receptor from partitioning to these membrane areas plays a major role in the effects observed. It is important to note that MCD has also been shown to induce $G\alpha_i$ partition to non-raft microdomains in THP-1 cells (Kabouridis, 2006) which gives an indication of what could be happening in the present study. Awasthi-Kalia et al. hypothesises that lipid rafts serve as inhibitory regions for the signalling of the B cell receptor. When in this study filipin was used to disrupt lipid rafts, only a slight increase in intracellular calcium mobilization was appreciated, representing another example of MCD and filipin having different effects on the signalling of a receptor.

The loss of $G\beta\gamma$ subunit from lipid rafts reported after MCD treatment (Awasthi-Kalia et al., 2001) may explain a stop of signal transduction through this subunit. Considering that calcium fluxes initiated by CCR5 coupling to a $G\alpha_i$ protein are triggered by the $\beta\gamma$ subunit, it may be expected that, in THP-1 cells, the lack of this subunit in lipid rafts promotes the coupling of the receptor to a different G protein subunit.

The situation described above would as well provide an explanation for a reduction in THP-1 chemotactic responses. It is essential to understand that chemotactic responses are initiated by the coupling of a chemokine receptor to $G\alpha_i$ proteins as shown by the fact that PTX completely abrogates this response (Thelen, 2001). Consequently, MCD promotion of CCR5-$G\alpha_i$ dissociation and subsequent coupling of the receptor to a different G protein would explain the lack of cell migration. However, as it has been reported above, MCD could be having other effects in the cell, which could be hiding the dependence of cell migration on calcium release and further experiments should be done to find out the exact role of MCD on cell migration.

In the current study, it has been shown that cholesterol depletion of THP-1 cells

might promote the association of CCR5 with a PTX-independent G protein. From this evidence it could be inferred that the increase in calcium release observed in THP-1 cells is due to the coupling of the receptor to a Gα subunit which enables a stronger activation of PLC. In support of this claim, there is evidence that $G\alpha_q$ has the ability to stimulate PLC not only through the βγ subunit but also through the Gα subunit (Taylor et al., 1991). This finding would mean double activation of PLC, which is likely to cause higher levels of IP3 and an enhancement of calcium mobilization from the ER (see Figure 4.13). Considering that $G\alpha_i$ can only activate PLC through the βγ subunit, it may be expected that this new association caused the observed increase in calcium release. Nevertheless, in chapter 3 it was stated that MCD treatment of CCR5 stably transfected cell lines caused the association of CCR5 to a PTX resistant G protein, which had a reduced ability to cause calcium mobilization as compared to untreated cells. A hypothetical explanation for the differing results is that CCR5 couples to a PTX-independent G proteins after cholesterol depletion in both cell types but, for unknown reasons, this coupling would have a reducing effect on CCR5 signalling in CHO.CCR5 and HEK.CCR5,whereas in THP-1 it would cause the observed increase in calcium release.

It is also important to mention that, as it has been highlighted in Chapter 3, there is also the possibility that MCD promotes CCR5 signalling through G protein-independent pathways. Therefore, it cannot be concluded that MCD promotes CCR5 signalling through a PTX-resistant G protein until this has been proven.

A possible disparity in the amount of cholesterol that these cells present under basal conditions cannot be ruled out as one of the causes for the different signalling here observed. It has been reported that THP-1 cells might concentrate high amounts of cholesterol due to a high uptake and a slow hydrolysis (Kritharides et al., 1998) which could account for the increase in receptor signalling observed in cholesterol depleted THP-1 cells but not on other cell types.

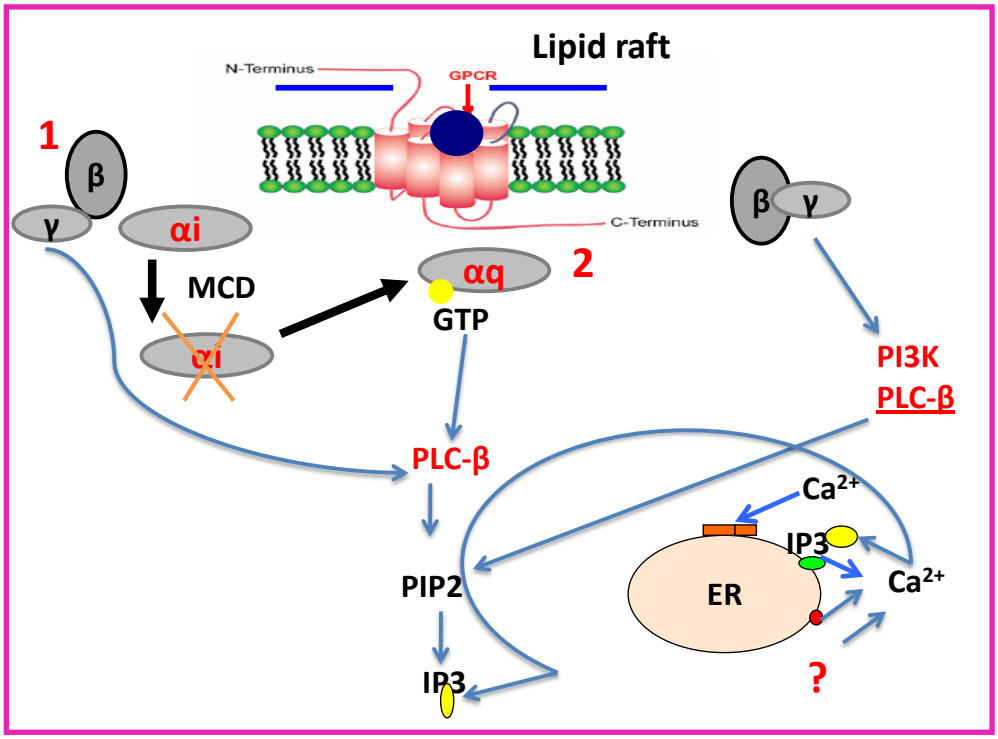

Figure 4.13 Diagram representing a hypothetical signalling mechanism upon cholesterol depletion of THP-1 cells. MCD promotes the coupling of the receptor to a PTX-resistant G protein such as $G\alpha_q$ which stimulates PLC through the Gα and the Gβγ subunit. (MCD promotes pathway 2). PLC double activation produces the second messenger IP3 which releases calcium from ER stores through the IP3R. The question mark indicates the possibility of other uncharacterised mechanisms being involved in the extra calcium released upon CCL3 stimulation of cholesterol depleted THP-1 cells.

Another possible scenario would be that under normal conditions in THP-1 cells, CCR5 is located in lipid rafts and moves out of them upon ligand stimulation. Thus, lipid raft disruption by MCD would extra activate the receptor by creating the same environment conditions than ligand activation. There are numerous studies showing that ligand stimulation promotes the migration of certain chemokine receptors out of lipid rafts (Huang et al., 2007; Rybin et al., 2000). Furthermore, some reports claim that cholesterol in the membrane acts as an inhibitor for the signalling of certain molecules. This is the case of the β_2 adrenergic receptor (Rybin et al., 2000) whose signalling is well activated upon cholesterol depletion.

In the case of CCR5, its location upon chemokine stimulation has yet to be investigated. CCR5 is targeted to lipid rafts but it might migrate out of them upon ligand stimulation. There is a possibility that this happens only in monocytic cells which would

explain the different effects cholesterol depletion has on CCR5 endogenously and CCR5 artificially expressing cells.

CCR5 forms a heterodimer with the chemokine receptor CCR2. A study performed by Mellado et al. (Mellado et al., 2001) shows that dimerization of these two receptors leads to completely different signalling pathways activation compared to when the receptors were signalling independently from each other. It was demonstrated that CCR2-CCR5 association promoted signalling responses through the PTX-resistant protein $G\alpha_{q/11}$. Along these lines, lipid rafts disruption is believed to cause the loss of many proteins from these regions, whereas other proteins are not affected. There is a chance that, contrary to $G\alpha_i$ and $G\beta\gamma$ subunits, $G\alpha_{q/11}$ is unaffected by lipid rafts disruption after MCD treatment. Altogether, these results set out the possibility that lipid rafts removal in THP-1 cells promotes signalling through a PTX-resistant protein. Additionally, MCD-induction of CCR5 association with other chemokine receptors like CCR2 in THP-1 cells cannot be ruled out.

As discussed in the previous chapter, Caveolin-1 does not seem to be involved in CCR5-dependent signalling in CHO.CCR5 and HEK.CCR5 cells. Nevertheless, the studies described below show that it might be reasonable to analyse the function of this key caveolae-structural protein in the enhancement of calcium signalling in THP-1. Several reports have shown that under basal conditions, Caveolin-1 is associated with the GDP-bound state of $G\alpha_i$ in lipid rafts in THP-1 cells and other cell systems (Cuschieri, 2004; Huang et al., 2007; Xu et al., 2006). Of interest, all studies agree that the association between Caveolin-1 and $G\alpha_i$ has inhibitory effects on the signalling induced by different receptors, which could be reversed by treatment of cells with MCD. Furthermore, these studies prove that the reason why MCD could abrogate this effect is that it dramatically reduces the coupling between Caveolin-1 and $G\alpha_i$ leading the latter to the stimulation of diverse responses initiated by specific ligands.

In this chapter it has been demonstrated that the increase of calcium release observed in cholesterol depleted THP-1 cells is dependent on PLC, PI3K and IP3R since inhibition of these molecules abrogates the MCD-dependent increased calcium signalling. This is very interesting since it excludes the possibility of an unspecific effect of MCD treatment in THP-1 cells. In addition to this, cholesterol repletion could bring the excess of signalling caused by MCD back to basal levels. Also, the fact that CCL4

stimulation of THP-1 cells follows the same trend (although the effect is not as marked as upon CCL3 stimulation) in CCR5 signalling in cholesterol depleted cells indicates that CCR5 is directly involved in the phenomenon described. However, the involvement of other chemokine receptors like CCR1 cannot be discarded since CCL4 causes a less intense response in calcium mobilization when compared to the more promiscuous chemokine CCL3.

The present report has found that THP-1 cells might behave differently to other cell lines upon cholesterol depletion by MCD. Whereas before it was reported that cholesterol depletion blocks CCR5-induced signalling pathways in CHO.CCR5, HEK.CCR5 cells and HeLa.RC49 cells, it is now demonstrated that the monocytic cells THP-1 react by highly increasing calcium release to the cytosol upon CCL3 treatment. Nevertheless, these results might require further experiments in order to understand if the effects observed among the different cell lines are really opposing. It is interesting to note that THP-1 cells signal significantly less than the CCR5 stably transfected cell lines tested and that, also, MCD increases CCR5 expression in these cells. Altogether, these two characteristics could account for the increase in calcium release observed upon chemokine stimulation.

Interestingly, other signalling processes like chemotaxis are not increased upon cholesterol depletion, highlighting a crucial result, the non-dependence of cell migration on calcium release for CCR5 signalling. This fact has been previously reported for other cell systems but this is the first time this conclusion has been reached for CCR5. An interesting study performed in PLCβ knock-down mice shows that calcium responses are suppressed in neutrophils extracted from these animals, whereas chemotaxis processes are not altered (Thelen, 2001), which indicates that calcium fluxes are not required to trigger cell migration.

Cholesterol enrichment of cells has negative effects on cell signalling as it reduces calcium fluxes and cell migration: Nguyen et al. (Nguyen et al., 2004) analysed the effects of cholesterol enrichment of T cells and found that cholesterol loading blocks calcium release and chemotactic responses. It is known that T cells get richer in cholesterol with aging (Douziech et al., 2002; Fulop et al., 2006; Fulop et al., 2001; Larbi et al., 2004a; Larbi et al., 2004b; Napier et al., 2005) and, therefore, it has been suggested that this excess in cholesterol may be responsible for the alterations in

immune responses characteristic of aging. Actually, the same groups also showed that an increase in cholesterol is responsible for a decrease in certain signalling pathways and that MCD treatment of these cells could rescue the normal signalling intensity.

On the other hand, cholesterol depletion from cells has generally been shown to block signalling responses induced by chemokine receptors, and it has been suggested that there exists an optimal cholesterol concentration able to stabilise lipid raft structure that should be maintained for correct signalling (Nguyen et al., 2004; Nguyen and Taub, 2004). For instance, Nguyes's group studied the effect of cholesterol depletion with MCD on CCR5 signalling upon stimulation with CCL4. Contrary to the findings here described, they demonstrated that calcium mobilization is impaired when CEM-NKR-CCR5 cells are treated with this cholesterol depleting agent (Nguyen and Taub, 2002a, b). This group demonstrated that the loss in calcium release was due to decrease in CCL4 binding to the receptor due to changes in CCR5 conformation upon cholesterol depletion.

We now here suggest that MCD might be affecting the intracellular proteins CCR5 interacts with and, therefore, modulating CCR5-derived signalling pathways. We base this theory on the fact that PTX is no longer able to block CCR5-induced calcium release after MCD-treatment. Contrary to Nguyen's opinion, we have shown that an optimal concentration of cholesterol is not always required. Here, an almost complete lack of cholesterol, which is the base of a more fluid membrane, can increase calcium mobilization upon CCR5 stimulation in THP-1 cells.

The differences between both studies might once more be related to the fact that Nguyen et al. used CCR5-transfected T cells whereas we have used THP-1 cells, naturally expressing the receptor. It is likely that the increase in calcium signalling observed in THP-1 cells is due to an increase in CCR5 surface expression upon MCD treatment, which might not occur in CEM-NKR-CCR5 cells.

These results may be useful for certain pathologies where cholesterol levels are being artificially modified regardless the effects in chemokine receptors signalling. For example, the use of statins to lower plasma cholesterol could have important effects in monocytes signalling pathways. If statins treatment had the same increasing effect on CCR5 induced calcium release as MCD, this could have important effects in the immune

responses of the high percentage of the population that is currently under statins treatment. Consequently, the effect of statins on CCR5 signalling will be investigated in detail in this thesis.

The use of MCD as a possible treatment for the altered T cells functions in an aged population cannot be considered because lipid rafts disruption alters the localization and signalling of a large number of proteins (Larbi et al., 2004b) and not all these changes are likely to have a positive effect on T cells-related immune response. On the other hand, research focusing on MCD is currently going on given that MCD´s disruption of lipid rafts has been shown to induce apoptosis of cancer cells (Li et al., 2006b). This drug has already been suggested as a topical anti-HIV agent (Khanna et al., 2002) and, considering the data highlighted above, it appears that researchers begin to weight up its use for other pathologies. The information presented in this study, especially the two main effects of MCD on THP-1 cells, enhancement of calcium release and inhibition of cell migration, would need to be carefully measured in the case that this drug is finally accepted for further trials.

White blood cells increasing calcium-derived signalling pathways or decreasing their migratory rate in cholesterol poor membranes can have crucial implications especially considering cholesterol level fluctuations in the body. In humans a rise in cholesterol serum is likely to occur with aging and certain diseases and this might affect the signalling behaviour of receptors such as CCR5 in vivo.

CHAPTER 5 - ANALYSIS OF INTRACELLULAR CALCIUM CHANNELS. HOW ARE CALCIUM RESPONSES ORIGINATED?

5.1 Introduction

Being involved in almost all cellular processes that take place in a cell, calcium is considered one of the most versatile second messengers that exist. It has the important task of transmitting extracellular information into cells to regulate the way they behave (Berridge et al., 2000). Thus, intracellular calcium homeostasis should be tightly regulated in order to avoid the development of unexpected processes.

Calcium regulates key functions on a great number of physiological processes from birth to death. In mammals the first lot of calcium fluxes occurs during the fertilisation process and is essential for the embryo to start dividing into daughter cells. After this initial role, calcium oscillations continue to contribute to cells differentiation and embryo formation (Berridge et al., 1998). Besides, calcium signalling is crucial for skeletal, smooth and cardiac muscle contraction and for cell proliferation as shown by the fact that calcium is in the centre of some of the newest chemotherapy approaches (Florea and Busselberg, 2009). This ion is also essential for transmitting neuronal signals and for the processes of learning and memories creation (Berridge, Bootman et al. 1998). Furthermore, high concentrations of calcium in the cytosol are known to control cell death and apoptosis (Verkhratsky, 2007) as will be discussed later.

The mechanism connecting CCR5 stimulation with an increase in intracellular calcium has been explained in the introduction of this thesis (look at introduction for references). Briefly, CCR5 stimulation promotes the liberation of IP3 from PIP2 by the action of PLC on PIP2. IP3 binds to its receptor, IP3R, in the ER, what causes calcium mobilization from stores in the ER. Calcium release from the ER can occur through stimulation of the IP3R or RyR. This increase in intracellular calcium triggers the opening of other calcium channels situated in the plasma membrane like voltage-operated calcium channels (VOCs) in a mechanism called capacitative calcium entry

(CCE) or store operated calcium entry (SOCE). This process increases even more the concentration of calcium ions in the cytosol and activates an ER calcium ATP-ase pump called SERCA which allows the refill of ER stores.

IP_3 was identified as a product of a membrane inositol lipid able to trigger important signalling cascades and since then it has been considered the main intracellular calcium releasing messenger (Berridge and Irvine, 1984). A few years later the IP3R was found in the ER and classified as a calcium channel and an IP3 binding transmembrane protein (Streb et al, 1983). The IP3R is composed of 4 subunits similarly to other calcium channels proteins. The channel region is located in the C terminus of the receptor whereas the IP3 binding site is located in the N-terminus. Upon IP3 binding, the receptor suffers important conformational changes that allow the formation of the pore channel and release calcium into the cytosol by regulating the openness of the calcium channel (Streb et al, 1983). IP3Rs are stimulated by the second messengers IP3 and calcium and can be blocked by caffeine (Sei et al., 2001) and heparin (Ehrlich and Watras, 1988). Many different kinases, PKC among them, can phosphorylate the IP3R. Actually it is considered that PKC phosphorylation of IP3R could represent a feedback mechanism in order to stop calcium release processes (Vanderheyden et al., 2009).

RyRs are a second type of receptors that upon certain stimuli open calcium channels that release calcium to the cytosol. They are formed by four subunits leaving a channel pore in the middle and are mainly expressed in the Endoplasmic Reticulum (ER) and in the sarcoplasmic reticulum (SR) membrane (Mackrill, 2010).

There are two main types of RyR, RyR1 and RyR2. RyR 1 is mainly expressed in the SR terminal cisternae of skeletal muscle and has a key role in muscle contraction. Some defects in this receptor’s function or conformation are linked with many pathologies. For instance, it was found that transgenic mice lacking RyR1 died perinatally due to problems derived from diaphragm contraction (Mickelson, Gallant et al. 1988). Another common and serious pathology, malignant hyperthermia (MH), characterised by hyperthermia, an unexplained increased in the amount of expired

Carbon dioxide, muscle rigidity, acidosis and hyperkalemia, is caused by defects in the gene encoding for RyR1 that creates an imbalance in calcium homeostasis (Mackrill; Mickelson et al., 1988).

RyR 2 is expressed at the highest levels in cells from the heart and any defects on the receptor are associated with arrhythmias and other cardio pathologies (Thomas et al.2010) as well as with epilepsy and neuron degeneration (Mackrill, 2010; Stutzmann et al., 2006).

RyRs can be stimulated by ryanodine, by the second messenger cyclic adenosine diphosphate ribose (cADPR) (Prakash et al., 1998) and by caffeine (Bhat et al., 1997) and are known to be blocked by high concentrations of ryanodine and ruthenium red (Ozawa, 2001). The same compound, ryanodine, locks the RyRs at half-open state at nanomolar concentrations, and fully closes them at micromolar concentration (Wingertzahn and Ochs, 2001).

In 1990, Wakui et al. analysed the role played by calcium ions in the cytosol in the stimulation of calcium release from ER stores. According to their experiments, calcium is able to stimulate calcium release from a calcium channel that is not sensitive to its blockage by heparin clearly pointing at the RyR (Wakui et al., 1990). It is confusing to understand the connection between IP3 and RyR receptors. Taking into account Wakui's view that calcium release from ER stores only stimulates RyR, would mean that the calcium released through RyR has no effect on IP3R. Nevertheless, some researchers have shown that this mechanism does not always occur. Gerasimenko et al.(Gerasimenko et al., 2003), claim that RyR stimulation dependent calcium signalling was not affected by modulation of IP3R, which according to them indicates that IP3R and RyR can be activated independently of each other.

On the other hand, there are studies showing that RyR and IP3R signalling are interconnected. Cancela et al. (Cancela, Gerasimenko et al. 2000), showed that inhibition of the ryanodine receptor by high concentrations of ryanodine could block IP3-dependent calcium release from the IP3R. Similarly, they found that micromolar concentrations of caffeine, which blocks IP3R, produced complete inhibition of calcium mobilization upon RyR agonists. According to this study, calcium release due to RyR inhibition would reduce the amount of calcium released through IP3R and vice versa. Overall there seems to be a lack of agreement in the mechanisms that link these two receptors.

Table 5.1 Agonists and antagonists of ER and acidic vesicles membrane proteins.

	IP3R	RyR	Ca2+ ATP-ase	H+ ATP-ase	Refs
IP3	Agonist				(Barritt, 1999)
Ryanodine		Agonist (10 µM) Antagonist (100 µM)			(Wingertzahn and Ochs, 2001) (Ozawa, 2001).
sADPR		Agonist			(Prakash et al., 1998)
NAADP		Agonist			(Dammermann and Guse, 2005)
Caffeine	Antagonist (>20 mM)	Agonist (<20 mM)			(Bhat et al., 1997) (Sei et al., 2001) (Wakui et al., 1990)
Heparin	Antagonist				(Ehrlich and Watras, 1988)
Ruthenium red	Antagonist				(Ozawa, 2001).
Thapsigargin			Antagonist		(Thastrup et al., 1990)
Bafilomycin				Antagonist	(Furuchi et al., 1993)
Calcium	Agonist?	Agonist			(Wakui et al., 1990)

ATP-ases, are enzymes that hydrolyse ATP to ADP and produce free energy that is used to transport different molecules against their concentration gradient (Vangheluwe et al., 2005). In this chapter two of these ATP-ases will be analysed in more detail. The ER calcium ATP-ase (SERCA) and the H+ ATP-ase situated in acidic organelles. SERCA transports calcium ions to the lumen of the ER or sarcoplasmic reticulum (Inesi et al., 2005) whereas the H+ ATP-ase has the role of keeping a low PH in acidic organelles (Furuchi et al., 1993).

In 1989 Thapstrup et al. (Thastrup et al., 1990) found the mechanism of action of thapsigargin, a tumour-promoting molecule, which had been widely used to empty ER stores. They demonstrated that thapsigargin blocks SERCA and produces the leakage of calcium from ER stores, emptying them and impairing further stimulation of calcium channels in this organelle.

As explained, calcium signalling is essential for a wide array of cellular mechanisms and alterations on these processes can lead to abnormal conditions. A key process for calcium homeostasis is the one regulated by the mitochondria, where calcium released by the ER is normally accepted by this organelle to be later returned to the ER. However, in situations where the levels of calcium are excessive, the mitochondrial metabolism becomes altered which can cause the activation of apoptosis processes (Duchen, 2000)

Compounds directly modifying calcium balance (Luciani et al., 2009; Martikainen et al., 1991) and many genes encoding for proteins that change intracellular calcium concentrations (Baffy et al., 1993; Okamoto et al., 2000; Pan et al., 2000) play key roles in apoptotic processes, highlighting the importance of intracellular calcium responses upon extracellular stimuli. For instance, Pan et al. (Pan, Damron et al. 2000) demonstrated that sustained depletion of ER calcium stores through RyR activation led to apoptosis of RyR transfected CHO cells. Another clear illustration that emptying ER stores can lead to apoptosis is represented by the inhibition of SERCA with thapsigargin, which leads to continued release of calcium from ER stores and triggers cellular programmed cell death (Denmeade and Isaacs, 2005; Sohoel et al., 2006).

In mammalian cells calcium stores are mainly situated in the ER but other organelles such as the nucleus, mitochondrion or acidic granules such as lysosomes, endosomes or exocytic vesicles can also serve as calcium reserves (Verkhratsky, 2007). Actually, it has been shown that in pancreatic acinar cells, IP3 was able to release calcium not only from the ER but also from acidic stores (Gerasimenko et al., 2006). Bafilomycin-A1, a macrolide antibiotic isolated from the fermentation of Streptomyces spp, which selectively inhibits the vacuolar-type proton translocating ATP-ases, was shown to decrease the amount of calcium ions released upon IP3 stimulation. Considering that bafilomycin-A1 treatment of cells blocks the acidification of acidic vesicles and prevents any calcium ions from being released from these cellular compartments (Furuchi et al., 1993), this group concluded that acidic vesicles were used as calcium stores as well as ER stores.

It is essential to emphasise that the involvements of proteins like RyR, SERCA or H+ATP-ases in CCR5 signalling has not been studied. There seems to be a lack of knowledge of the exact mechanism coupling chemokine receptors with intracellular

calcium responses.

Although IP3 production, binding to IP3R and calcium release to the cytosol upon ligand binding, are processes generally accepted for all GPCR leading to PLC activation, so far not many studies have characterised these cascades for CCR5. In the introduction of this thesis we have reported that calcium responses could be initiated by the IP3, the cADPR and the S1P pathways, highlighting the possibility that some of the calcium fluxes triggered by CCR5 activation could be dependent on cADPR or S1P.

Accordingly, it was demonstrated that RANTES-induced calcium mobilisation could be partially inhibited by both, molecules blocking RyR and IP3R (Inngjerdingen et al., 1999), showing a role for both receptors in this chemokine-induced calcium responses. The authors of this study suggest that Rantes stimulates both, the IP3R and the cADPR pathways. The idea that RANTES can evoke calcium signals through the stimulation of the second messenger cADPR has been demonstrated before (Partida-Sanchez et al., 2004; Shideman et al., 2006). Therefore, it appears that calcium release upon CCR5 receptor activation could in part be triggered by cADPR binding to RyR.

This work has intended to analyse how modulation of some of the proteins involved in the uptake and release of calcium in ER stores with specific inhibitors or through changes in the fluidity of the ER membrane, could affect CCR5 signalling as measured by calcium release. In order to understand if CCR5-induced calcium release was dependent on acidic reserves, the effect of the H+ ATP-ase pump situated in lysosomes and acidic vesicles was also analysed.

5.2 Aims

Although a few studies have claimed a role for the second messenger cADPR, calcium responses originated upon CCR5 stimulation are mainly reported to be due to IP3 binding IP3R. In this thesis we have previously shown that an IP3R antagonist was able to suppress CCR5 signalling and that extracellular calcium was not needed for CCR5-induced calcium fluxes, which points at intracellular calcium stores as the source of this second messenger. Yet, the mechanism responsible for calcium release to the cytosol through the IP3R upon CCR5 stimulation may be influenced by other factors that have not been characterised. Therefore, the aim of this chapter is to study in detail

how modulation of other membrane proteins situated near the IP3R affect its activation. Special interest has been put on studying these processes in cholesterol depleted THP-1 cells, seeking to understand the unsolved mechanism leading to the increase in calcium signalling in THP-1 cells lacking cholesterol described in the preceding chapter.

5.3 Result

5.3.1 CCR5-induced calcium responses come from ER stores.

In the first experiment we have determined the origin of calcium mobilization. HEK.CCR5, CHO.CCR5 and THP-1 cells were treated with the SERCA inhibitor thapsigargin before measuring calcium mobilization. Thapsigargin only blocks responses coming from the ER and, therefore, this experiment would provide information about the possible implication of other organelles in the calcium fluxes observed upon CCR5 stimulation. Figure 5.1 shows that 30 minutes treatment of cells with thapsigargin was enough to block calcium signalling upon CCL3 stimulation in the three cell lines studied, indicating that CCR5-dependent calcium release has its origins in ER stores.

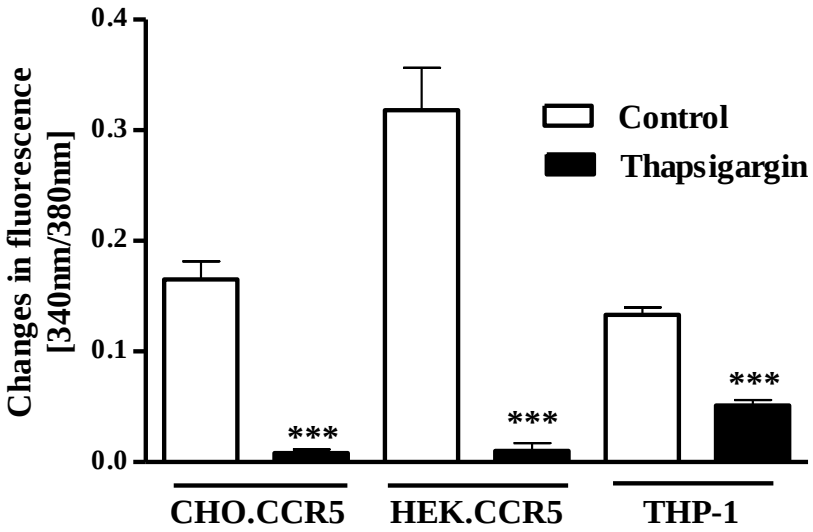

Figure 5.1 Thapsigargin treatment blocks calcium mobilisation. CHO.CCR5, HEK.CCR5 and THP-1 cells treated with 1μM of TG or left untreated (control) and were stimulated with CCL3 200 nM and calcium flux was measured. Significant changes towards control cells are indicated by asterisks (***$p < 0.001$). The points plotted are the mean and SEM of 3-5 independent experiments.

5.3.2 Stimulation of RyR leads to an increase in IP3R activity upon CCL3 stimulation.

RyR can release calcium to the cytosol upon stimulation with different compounds. Caffeine and ryanodine have been shown to act as agonists of this receptor. Caffeine can act as an IP3R antagonist at high concentrations or as RyR agonist at low concentration (Bhat et al., 1997; Sei et al., 2001).

Here, how stimulation of RyR by caffeine could affect IP_3-induced calcium release in HEK.CCR5, CHO.CCR5 and THP-1 cells was analysed. It can be observed that caffeine increased calcium release in CCR5-stimulated CHO.CCR5 (Figure 5.2 A) and HEK.CCR5 cells (Figure 5.2 B). This indicates that either calcium released from RyR stimulates the IP3R consequently increasing calcium mobilization or that CCL3 activate RyR and, therefore, an agonist of this receptor enhances the response. Nevertheless, it can be appreciated that caffeine has a different effect in THP-1 cells (Figure 5.2 C) than in CHO.CCR5 or HEK.CCR5 cells. Whereas caffeine significantly increases calcium signalling responses in CHO.CCR5 and HEK.CCR5 cells confirming its role as a RyR agonist, it reduces them in THP-1 cells, suggesting that in this cell line caffeine works

by blocking IP3R, maybe due to a higher affinity of this molecule for the IP3R in THP-1 cells. The different ability of caffeine in promoting calcium release raises the possibility that there may be a slight variance in the function of IP3R and RyR among these cell lines.

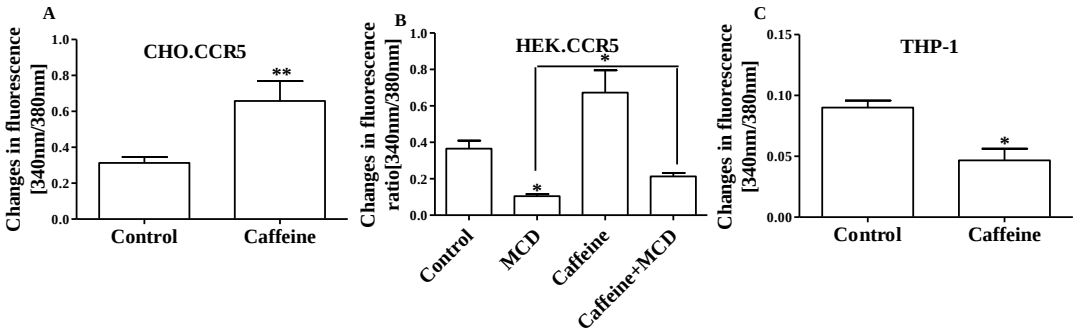

Figure 5.2 Effects of RyR stimulation with 10 mM caffeine prior to 200 nM CCL3 addition to the CHO.CCR5 (A), HEK.CCR5 (B) and THP-1 cells (C). Cells were pre-treated with caffeine for 30 minutes. In B, HEK.CCR5 cells were also treated with 10 mM MCD and MCD plus caffeine for 30 min prior to chemokine challenge of the receptor. Significant changes from control cells are indicated by asterisks (*$p < 0.05$, **$p < 0.01$). The points plotted are the mean and SEM of 3-5 independent experiments.

To ascertain that THP-1 behaviour upon RyR stimulation was different from the effect observed in stably transfected cells, low concentrations of ryanodine, another RyR agonist, were next used to observe the consequences of RyR stimulation on IP3R-dependent signalling. We could observe that, contrary to the effects of caffeine on THP-1 cells, ryanodine stimulation of RyR significantly increased intracellular calcium mobilization through CCR5 stimulation in all cell lines used (Figure 5.3).

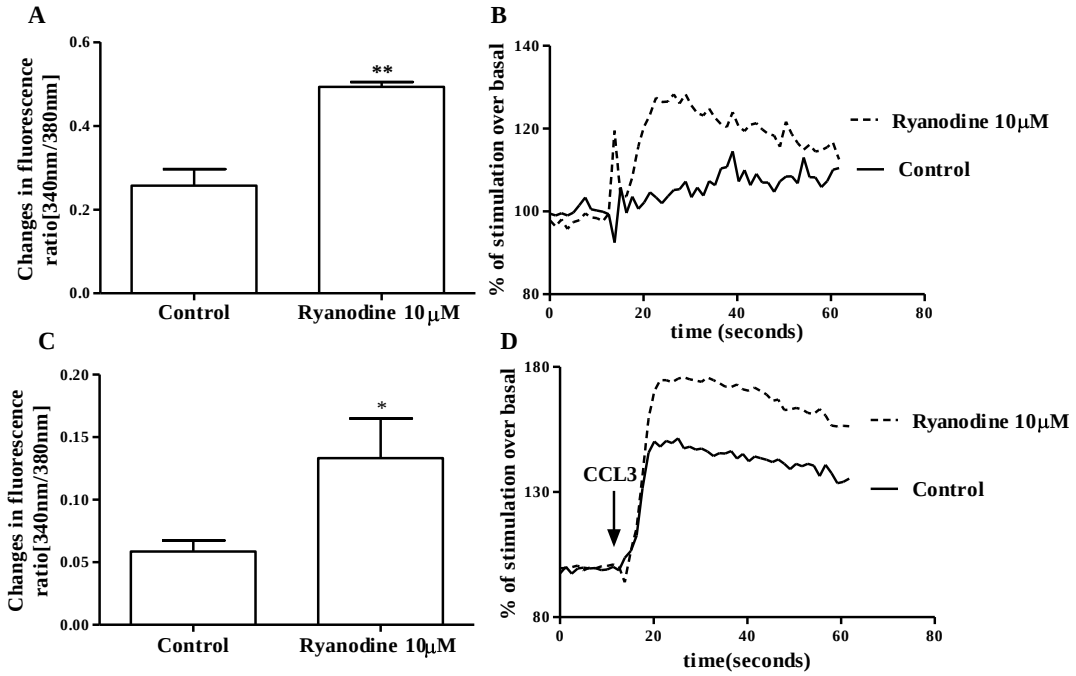

Figure 5.3 Effects of RyR stimulation with 10 μM ryanodine prior to 200 nM CCL3 addition to cells. A) Shows bar chart of HEK.CCR5 cells treated with ryanodine 10 μM or left untreated. B) Shows real traces of calcium flux in HEK.CCR5 cells. C) Shows THP-1 cells. D) Shows CHO.CCR5 cells. Significant changes towards control cells are indicated by asterisks (*$p < 0.05$). The points plotted are the mean and S.E.M of 3-5 independent experiments for the bar chart and a representative trace of at least 3 independent experiments for the real time calcium traces. (Cardaba and Mueller, 2009).

Interestingly, ryanodine enhances CCR5 signalling in stably transfected cells and in THP-1 cells which indicates that stimulation of RyR has a positive effect in the response triggered by CCR5 activation. We suggest that part of the calcium signalling observed upon CCL3 activation of the receptor is due to the generation of second messengers able to activate not only IP3R but also RyR.

5.3.3 Blockage of lysosomes H+ ATP-ase increases calcium release.

Although thapsigargin's complete blockage of calcium release upon CCR5 stimulation clearly pointed at ER stores as the source of calcium, another proton pump inhibitor was tried to rule out the possibility that other organelles were also involved in calcium mobilization. The proton pump situated in lysosomes and acidic vesicles and

responsible for keeping an acidic environment, was next targeted with bafilomycin-A1, a macrolide antibiotic derived from Streptomyces griseus which serves as a proton pump blocker (Furuchi et al., 1993). Blockage of this pump alters calcium responses coming from acidic stores. However, as Figure 5.4 shows, when we treated CHO.CCR5 cells with bafilomycin-A1 and stimulated them with a CCR5 ligand, a significant enhancement on calcium occurred.

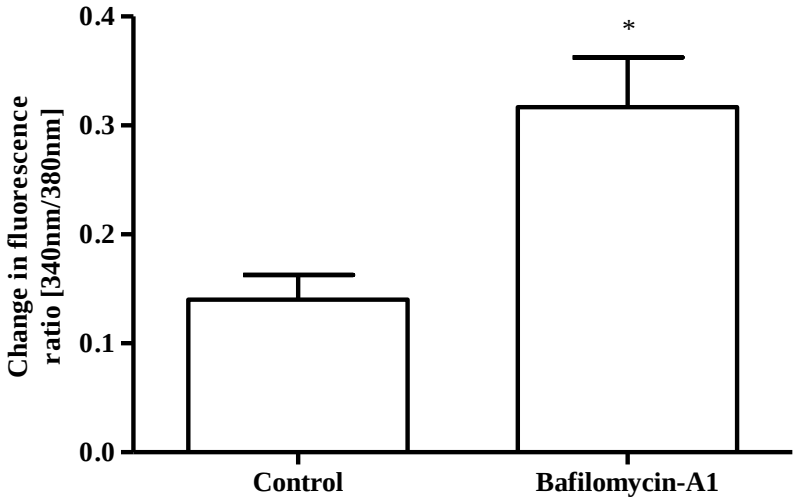

Figure 5.4 Blockage of the H+ATP-ase of acidic granules in CHO.CCR5 cells produces an increase in intracellular calcium mobilization. Cells were treated with 100 µM bafilomycin and stimulated with 200 nM CCL3. Graph shows changes in fluorescence ratio [340 nm/380 nm]. Significant changes towards control cells are indicated by asterisks (*$p < 0.05$). Data represent mean ± SEM from at least three

5.3.4 Effect of cholesterol depletion of THP-1 cells in ER calcium channels.

In this chapter we have shown how modulating some receptors and pumps in the ER membrane and in the lysosomes membrane can affect CCR5-dependent calcium release. In this next set of experiments it is sought to investigate whether the same effects can be observed in the calcium responses occurring in cholesterol depleted THP-1 cells. With this purpose, THP-1 cells were treated with MCD and subsequently treated with thapsigargin, caffeine, ryanodine or bafilomycin-A1. Special attention was put on understanding if any of these molecules were behind the ability of these cells to increase its signal responses upon CCR5 activation in the absence of cholesterol.

5.3.4.1 Cholesterol depletion of THP-1 cells abrogates the inhibitory effect of thapsigargin in calcium release.

Here THP-1 cells were treated with MCD and with MCD and thapsigargin to investigate if the unexpected effects in calcium signalling observed in cholesterol depleted cells (Chapter 4) were related to changes in intracellular calcium channels.

Thapsigargin was expected to block calcium release as it had been observed before. Nevertheless, it could be appreciated that thapsigargin treatment of THP-1 cells lacking cellular cholesterol was unable to reduce calcium mobilization upon CCR5 treatment (Figure 5.5 A and B). In an attempt to understand thapsigargin's effect in MCD-treated THP-1 cells, the experiment was repeated in calcium free serum to exclude the possibility that MCD in the presence of thapsigargin was promoting calcium entry from the extracellular medium. Indeed, the same results were obtained (Figure 5.5 D) when a calcium free buffer was used, indicating that calcium origin was the intracellular stores and that somehow, thapsigargin was not able to block SERCA after cholesterol depletion. Additionally, the same data was obtained when the CCR5-specific chemokine CCL4 was used to trigger calcium responses (Figure 5.5 C), highlighting the CCR5-specificity of this process.

It is interesting to observe that thapsigargin's capacity to block the calcium ATP-ase, SERCA, is lost in cholesterol depleted membranes. This could be due to calcium being released from different calcium stores in the absence of cholesterol or what is more likely, due to changes in SERCA pump conformation in a more fluid membrane which would alter its affinity for this inhibitory molecule as shown by the lack of effect of thapsigargin. Nevertheless, since not enough data has been obtained regarding this mechanism, only a hypothesis can be made.

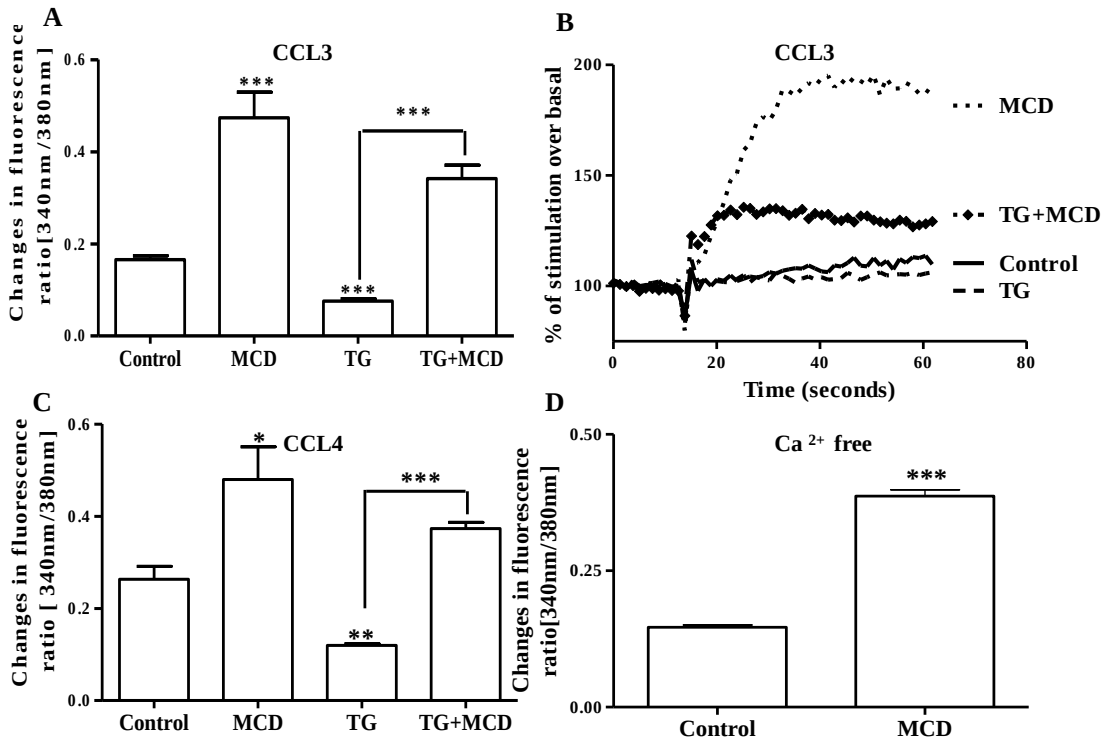

Figure 5.5 Increases in membrane fluidity abrogate the effect of thapsigargin. THP-1 cells were treated with 10 mM MCD, 1 µM thapsigargin or both, and stimulated with 200 nM CCL3 (A, B and D) or CCL4 (C). B shows calcium single traces and D shows cells treated in calcium free medium. Data are expressed 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%. Significant changes towards control cells are indicated by asterisks (**p < 0.01, ***p < 0.001). Data represent mean ± SEM from at least three independent experiments for the bar charts and a representative tracer for the calcium flux data. (Cardaba and Mueller, 2009).

5.3.4.2 Blockage of the lysosomes H+ ATP-ase and stimulation of the RyR drastically enhances the previously amplified calcium response in MCD-treated THP-1 cells.

Due to the results obtained in thapsigargin treated cells, our next aim was to study MCD´s effect on another proton pump situated in vacuoles and other acidic vesicles to see if this protein was also altered by cholesterol depletion. This pump can be blocked by treatment with bafilomycin-A1. As Figure 5.6 shows, bafilomycin treatment of cells produced a huge increase in the already increased response observed in MCD treated cells. Overall, it can be appreciated how simultaneous treatment of MCD and bafilomycin-A1 increases THP-1 cells response to CCL3 stimulation around 8-fold. The mechanism used by bafilomycin-A1 to increase calcium release from intracellular stores

is not understood.

In order to investigate this further, another ER membrane protein was analysed. Cholesterol was next depleted of THP-1 cells and the effect of RyR activators was measured. As it can be observed in Figure 5.7, caffeine and ryanodine treatment of cholesterol depleted THP-1 enhanced even further the amount of intracellular calcium mobilization, pointing once more at the possibility that CCR5 activation stimulates RyR. We suggest that modification of SERCA pump and RyR conformation or function could partly explain the increase in CCR5 signalling observed in THP-1 cholesterol depleted cell.

On the whole these results show that cholesterol depletion does not only change the plasma membrane composition but also alters other intracellular membranes like the ER or the lysosomes membranes having important effects in cell signalling.

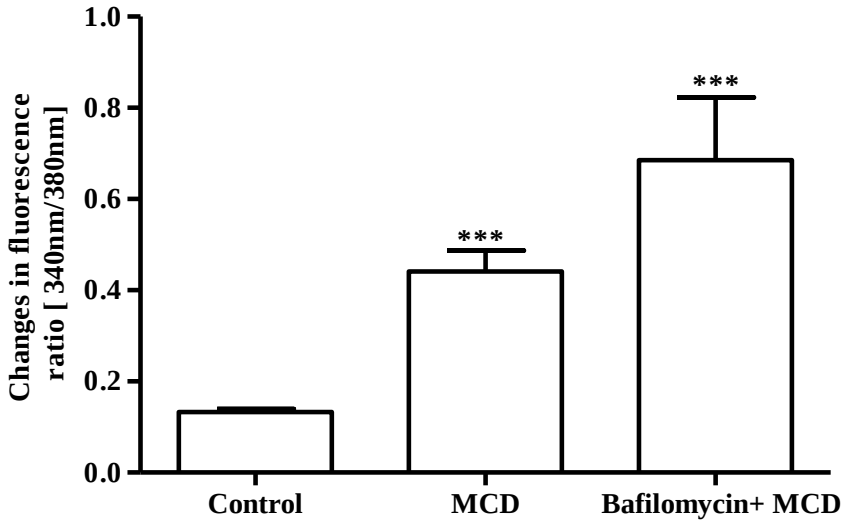

Figure 5.6 Blockage of the acidification process of acidic granules further stimulates calcium release in MCD treated THP-1 cells. THP-1 cells were treated 10 mM MCD or MCD plus 100 µM Bafilomycin for 30 minutes and challenged with 200nM CCL3. Subsequently intracellular calcium concentrations changes were measured. Significant changes towards control cells are indicated by asterisks (*$p < 0.05$, **$p < 0.01$). Data represent mean ± SEM from at least three independent experiments.

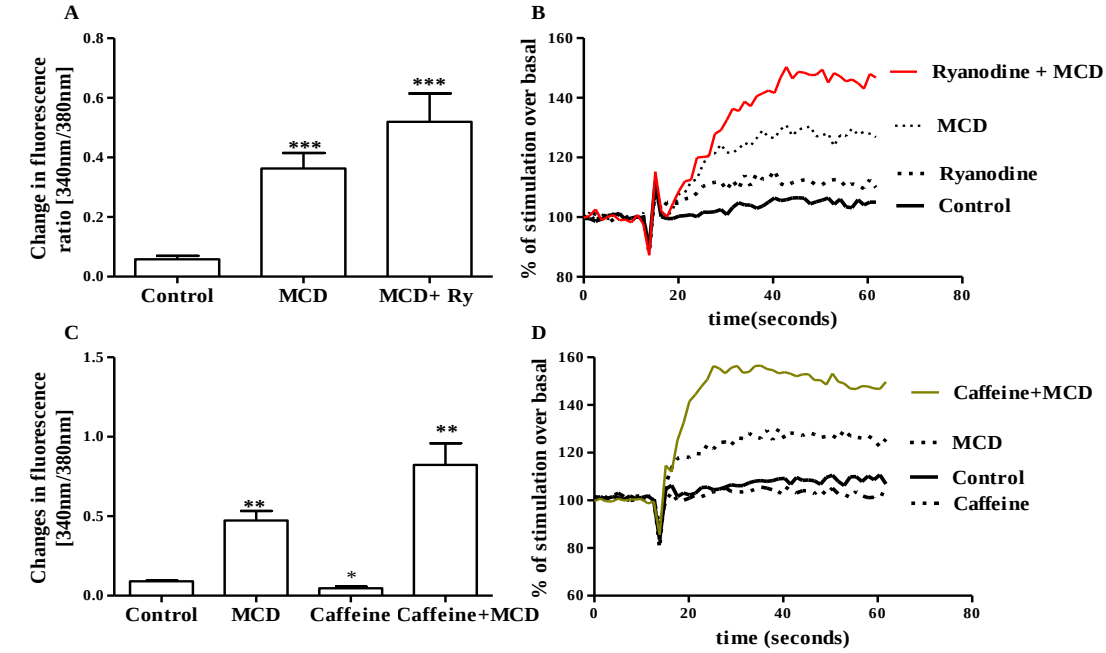

Figure 5.7 Caffeine and ryanodine drastically stimulate calcium signalling in cholesterol depleted cells. THP-1 cells were treated with MCD (10 mM) and then further stimulated with the ryanodine receptor agonist caffeine (10 mM) and ryanodine (10μM) prior to 200 nM CCL3 stimulation. B and D show real traces for a representative experiment of A and D. 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 ± SEM from at least three independent experiments for the bar charts. Significant changes towards control cells are indicated by asterisks (*p < 0.05, **p < 0.01, ***p < 0.001). (Cardaba and Mueller, 2009).

5.4 Discussion

In this chapter the mechanisms through which CCR5 induces calcium release have been investigated focussing on the possible interactions among IP3R and other ER calcium regulatory membrane proteins. Up to date it is still not clear how calcium responses are affected by the modulation of the different ER membrane proteins in chemokine receptors signalling. It is generally accepted that CCR5-dependent activation of the βγ subunits of G proteins activates PLC and causes the production of the second messenger IP3, which binds to the IP3R, producing calcium release from ER stores.

However, the ER membrane has other calcium dependent proteins that may have an effect on the stimulation of IP3R.

Characterising the interactions among ER calcium dependent proteins may provide new perspectives on its role in CCR5 signalling and especially on the possibility that calcium signalling is differently regulated inTHP-1 and CCR5 transfected cells. Hence, the first aim of this chapter was to analyse the relation between the IP3R, the RyR and the SERCA pump, all of them situated in the ER membrane in CCR5 stably transfected and in THP-1 cells. A further aim was to understand if cholesterol depletion with MCD in THP-1 cells modulated the conformation or function any of these proteins which could somehow explain an increase in intracellular calcium mobilization upon CCR5 stimulation only in this cell line.

As explained in the introduction of this chapter, many studies have analysed the possible interaction between signals stimulating calcium release from the IP3R and the RyR but no studies focussing on this area have been performed for CCR5 signalling. Although it is generally accepted that the calcium fluxes produced upon CCR5 stimulation only activate the IP3R, there are reports showing that cADPR pathways activated by the CCR5 ligand CCL5 also take part in inducing calcium responses. Besides, there is the possibility that under physiological conditions stimulation or blockage of IP3R's neighbour receptors or proteins have an important effect on CCR5 signalling.

We initiated the results section of this chapter by analysing the effect of SERCA blockage on CCR5 signalling. To our knowledge, only one study performed in rat microglia has previously shown that CCR5 calcium responses can be blocked by thapsigargin (Boddeke et al., 1999). In accordance with this group, in the experiments here presented, we demonstrated that IP3-evoked flow of calcium into the cytosol has its origin in ER stores since, as expected, thapsigargin treatment of cells has a blocking effect in CCR5 induced calcium fluxes in all cell lines used.

Further evidences that calcium comes from internal stores are highlighted by the fact that the observed signalling was not affected by removal of extracellular calcium.

We could also prove that CCR5-induced calcium release could be potentiated by cells treatment with drugs like caffeine and ryanodine, both able to stimulate RyR dependent calcium release. These data suggest that either CCR5-induced calcium responses through IP3R are dependent on the open state of the RyR or that CCR5-dependent calcium signalling involves direct stimulation of the RyR as has been

reported before (Cancela et al., 2000; Partida-Sanchez et al., 2004; Shideman et al., 2006). Interestingly, we could also show that caffeine treatment of cells, contrary to its effect on CHO.CCR5 and HEK.CCR5 cells, did not activate calcium fluxes in THP-1 cells. On the other hand ryanodine stimulation of the RyR enhanced calcium responses triggered by CCR5 stimulation in all cell lines studies, which could be explained by the property of caffeine to block IP3R. The fact that caffeine treatment of cells increases IP3R dependent calcium release only in CHO.CCR5 and HEK.CCR5 cells highlights the possibility of a difference in RyR conformation or function between CCR5 stably transfected cells and THP-1 cells.

In the present study, the possibility that CCR5 had the ability to release calcium from other organelles different from the ER was considered. We have shown that CCR5-induced calcium release was enhanced in all cell lines when bafilomycin-A1, a blocker of the H+ ATP-ase situated in acidic organelles, was used. A reduction of calcium signalling would have indicated that part of the response originated upon CCR5 stimulation was originated in acidic calcium reserves. However no explanation could be found for a bafilomycin-dependent increase in the amount of intracellular calcium liberation upon CCR5 stimulation.

Remarkably, we have demonstrated that in THP-1 cells, MCD treatment can reverse the blocking effect that thapsigargin has on calcium release upon CCR5 activation (Cardaba and Mueller, 2009). This may be the first time a study has shown that cholesterol depletion might have an effect on proteins located in internal organelles. Considering that membrane cholesterol depletion by MCD has been broadly used as a tool to study the dependence on lipid rafts of numerous signalling molecules, these results might provide new insights into the mechanisms behind it.

Furthermore, the results here presented may be important for studies focussed on thapsigargin as a chemotherapeutic agent. Thapsigargin is currently under investigation for the treatment of slow progression cancers like prostate cancer (Jakobsen et al., 2001; Legrand et al., 2001; O'Neill et al., 2006). Normal chemotherapy is not effective for these type of cancers due to their low proliferative rate and, therefore, new approaches are being investigated with drugs inducing programmed cell death like thapsigargin. The data provided in this study have shown that cholesterol modulation of the membrane can affect the ability of thapsigargin to block SERCA pump in the monocytic cell line THP-

1. In the case that thapsigargin derivatives are finally approved for prostate cancer treatment, the study of how cholesterol fluctuations could affect its function should be further analysed on prostate cells, especially taking into account that cholesterol modulation drugs are commonly prescribed nowadays.

It is worth noting that not only thapsigargin but also MCD is in the spotlight for new therapies. Topical application of MCD has been considered for the prevention of HIV infection and other sexual transmitted diseases (Hughes et al., 2007; Hanna et al., 2002) and also as a co-therapeutic agent in the treatment of certain types of cancers. Of note, numerous tumours have been shown to be sensitive to MCD treatment (Fedida-Metula et al., 2008; Li et al., 2006b). Li's group demonstrated that cancerous cells in breast and prostate cancer patients express more lipid rafts that healthy cells and that MCD-induced raft disruption could be directly linked with apoptosis of cancerous cells. Furthermore, MCD is widely used as an effective tool to deliver drugs into cells. For instance, some recent studies (Yadav et al., 2009; Yallapu et al.) showed that curcumin anticancerous and anti-inflammatory effects were highly increased when it was encapsulated into the MCD cavity. These reports point at the possibility of MCD being used as a drug for human use in the future and highlight the need for a better understanding of its effect on calcium release processes involving ER transmembrane proteins such as SERCA pump.

The work here presented points out some differences between stably transfected cell lines and THP-1 cells. It is possible that the ER membrane of monocytic cell lines is slightly different to that of CHO.CCR5 and HEK.CCR5 cells, which could explain differences in the conformation of ER membrane embedded proteins making them more or less sensitive to certain compounds.

The fact that cholesterol depletion produced such a big increase in intracellular calcium mobilization in thapsigargin-treated THP-1 cells was totally unexpected. It is known that MCD is a cell permeable molecule able to deplete cholesterol from different cellular compartments and affect their function (Ziolkowski et al.). Therefore, the effect cholesterol depletion had on calcium release from ER stores, through RyR and IP3R, and acidic organelles was analysed. Curiously, we found that cholesterol depletion not only abrogated the effect caffeine had on calcium mobilization on untreated THP-1 cells but it also increased calcium release in caffeine treated cells to a further extent than in

MCD-only treated cells. Furthermore, the effect of MCD in THP-1 cells was also increased when cells were treated with ryanodine. Importantly, calcium does not come from the extracellular medium since when the experiment was repeated in a calcium free buffer, the same results were obtained. These data could indicate that MCD, by changing cholesterol concentration from the ER membrane, alters the conformation of some of the proteins resident on it. It could be hypothesised then that a more fluid ER membrane increases the open probability of both, RyR and IP3R while also alters the function of SERCA pump. Cholesterol depletion could be altering IP3R and RyR conformation so that ryanodine potentiates the effect of MCD and caffeine passes from blocking the former in normal conditions to activate the latter in MCD treated THP-1.

However, as it has been demonstrated in chapter 4, the IP3R antagonist 2-APB blocked calcium mobilization from the ER in cholesterol depleted THP-1 cells. These findings might represent the different properties of two IP3R antagonists when binding IP3R. We hypothesise that for unknown reasons caffeine does not block IP3R in an ER fluid membrane whereas 2-APB does.

Similarly to what was observed after ryanodine treatment, the vacuoles and other acidic granules H+ ATP-ase inhibitor bafilomycin-A1which had no effect on THP-1 untreated cells, highly increased calcium release in cholesterol depleted THP-1 cells. It is difficult to understand the mechanism through which Bafilomycin produces such an enhancement in calcium release.

One possibility is that MCD treatment also affects acidic granules membranes so that in the presence of bafilomycin-A1somehow CCR5 signalling gets potentiated.

It is worth mentioning that a study performed in macrophages treated with bafilomycin-A1, showed a reduced cholesterol efflux from lysosomes to other organelles, probably due to the inactivation of the H+ proton pump (Furuchi et al., 1993). This research has some importance since it may mean that cholesterol extraction with MCD would, in bafilomycin-A1 untreated cells, be replenished to some extent with lysosomal cholesterol. However, in this case, due to bafilomycin-A1 inhibition of this process, less cholesterol would be transferred to the membrane after MCD treatment and thus a higher response would be observed in THP-1 cells after CCL3 stimulation.

Essentially, it is known that ER membrane is specially low in cholesterol which makes it more fluid, thinner and more leaky than the plasma membrane (Vangheluwe et al., 2005). Thapsigargin inhibits SERCA by widening the calcium binding site of the enzyme, meaning that only steric factors are involved (Lee, 2002). It has also been shown that cholesterol overload of cells could result in SERCA inhibition (Vangheluwe et al., 2005). Our data could fit in well with these experiments since we could only observe an increase in SERCA activity upon thapsigargin stimulation of THP-1 cells after MCD treatment. It could be speculated that if lipid composition and distribution in the membrane can alter proteins conformation affecting its function (Lee, 2004), it is possible that cholesterol extraction by MCD, by making ER membrane even more fluid results in SERCA conformational changes that makes it thapsigargin insensitive.

An interesting study performed by Huang et al. analysed the effects of cholesterol feeding of rabbits on cardiac function. They reported that Ca2+-ATPase (SERCA)-2 mRNA levels were reduced within few days after cholesterol feeding was started. Considering this interesting result, it could be argued that since an increase in cholesterol caused a reduction in the mRNA levels of the Ca2+-ATPase (SERCA)-2, cholesterol reduction could have opposite effects and increase SERCA levels in THP-1 cells. An increase on the number of SERCA molecules in THP-1 cells could account for the null effect thapsigargin had on these cells upon MCD treatment. However, if this was the case, there is still the need to find out why this effect only occurs in THP-1 cells.

Further research is needed to understand the effect of cholesterol depletion on ER transmembrane proteins in THP-1 cells. We suggest that cholesterol depletion in the monocytic cell line THP-1 alters the ER membrane probably changing proteins conformations and this may increase the affinity of some ligands for their receptor (as can be observed in the case of ryanodine or caffeine) whereas it would reduce the affinity of some antagonists for its receptors (case of thapsigargin on SERCA pumps).

The results analysed in this study could be useful for future research experiments focused on lipid raft disruption. In this thesis I have named several studies focussing on MCD treatment to study the importance of membrane integrity in calcium responses initiated by many receptors. The results that CCR5 stimulation causes calcium fluxes by activation of IP3R and RyR are quite novel. We consider that these data need further

investigations to understand the mechanisms that link CCR5 signalling with the production of second messengers able to activate RyR.

As far as we are concerned, this is the first study showing that that MCD treatment may be altering the conformation and function of RyR, IP3R or SERCA pumps. This new study may be useful for future investigations aiming at understanding calcium response events since the possible effect of cholesterol depletion on internal transmembrane proteins here described, may also be present in other cell systems and should be taken into consideration.

CHAPTER 6 - THE PLEIOTROPIC EFECTS OF STATINS ON CCR5 SIGNALLING, EXPRESSION AND INTERNALISATION.

6.1 Introduction

Atherosclerosis is a major risk factor for many different conditions which on a whole are known as cardiovascular disease (CVD) (Glass and Witztum, 2001). This pathology is considered one of the main problems in developed countries. For instance, in the United States, CVD was the direct cause in 37.3% and a contributing cause in 58% of all US deaths (Hoyert et al., 2005). This disease, considered a chronic inflammatory process, involves the thickening of a wall artery due to the deposition of cholesterol and white blood cells, which can eventually cause thrombosis events, myocardial infarction and stroke (Goldstein, 2007). There are numerous risk factors known to increase the chances of developing this condition (Glass and Witztum, 2001). They can be classified into factors with a significant genetic component and environmental factors. Among the first type, other conditions like diabetes mellitus, obesity, hypertension, metabolic syndrome or an increase in the levels of low density lipoprotein (LDL), typical from people suffering from familial hypercholesterolemia (FH), are known to be of great importance. Environmental risk factors include a high fat diet, smoking, lack of exercise and some infectious agents known to trigger the initial artery damage (Kol and Santini, 2004). Although more than one condition seems to be required to trigger the disease, an excess in the amount of serum cholesterol is believed to be the leading cause that drives atherosclerosis development in humans.

Statins were discovered in 1971 by a group working on the research of microbial metabolites which reduced the endogenous synthesis of cholesterol with the aim to treat diseases characterized by an excess in this lipid (Endo, 2004). The first statin discovered was mevastatin, which was shown to effectively reduce levels of LDL-cholesterol in humans. After this first drug, other statins, with a stronger activity and reduced side effects like lovastatin, pravastatin and simvastatin were approved for use in humans (Endo, 2004). In the next few years these drugs were shown to effectively treat

hypercholesterolemia. Only recently it has been confirmed that treatment with statins is effective in the treatment of atherosclerosis (Nissen et al., 2006).

It is estimated that more than thirty million people are undergoing statin treatment (Kleemann and Kooistra, 2005). These drugs work by inhibiting the rate-limiting enzyme involved in the synthesis of cholesterol, the HMGCOA-reductase. Since only one third of total body's cholesterol reserves come from the diet (Liao and Laufs, 2005), inhibiting *de novo* synthesis of cholesterol results in an effective therapy to reduce hypercholesterolemia and other associated pathologies. The mechanism of action of these cholesterol lowering drugs is very complex and involves several pathways to be added to the widely known pathway leading to cholesterol synthesis. For instance, statins work in a dual way inhibiting the production of cholesterol and promoting its clearance from the bloodstream. The characteristics responsible for this extra effect are the sterol regulatory element binding proteins (SREBP) (see Figure 6.1). These proteins are essential transcription factors which regulate cholesterol synthesis by activating or blocking the enzymes involved in this pathway and also regulates the synthesis of the LDL-R. When cholesterol biosynthesis is blocked by statins, SREBP gets activated and increases the synthesis of LDL-R which in turn accelerates cholesterol uptake in the liver. This cholesterol removal by LDL-R is considered to highly contribute to the cholesterol reducing effects of statins (Kleemann and Kooistra, 2005).

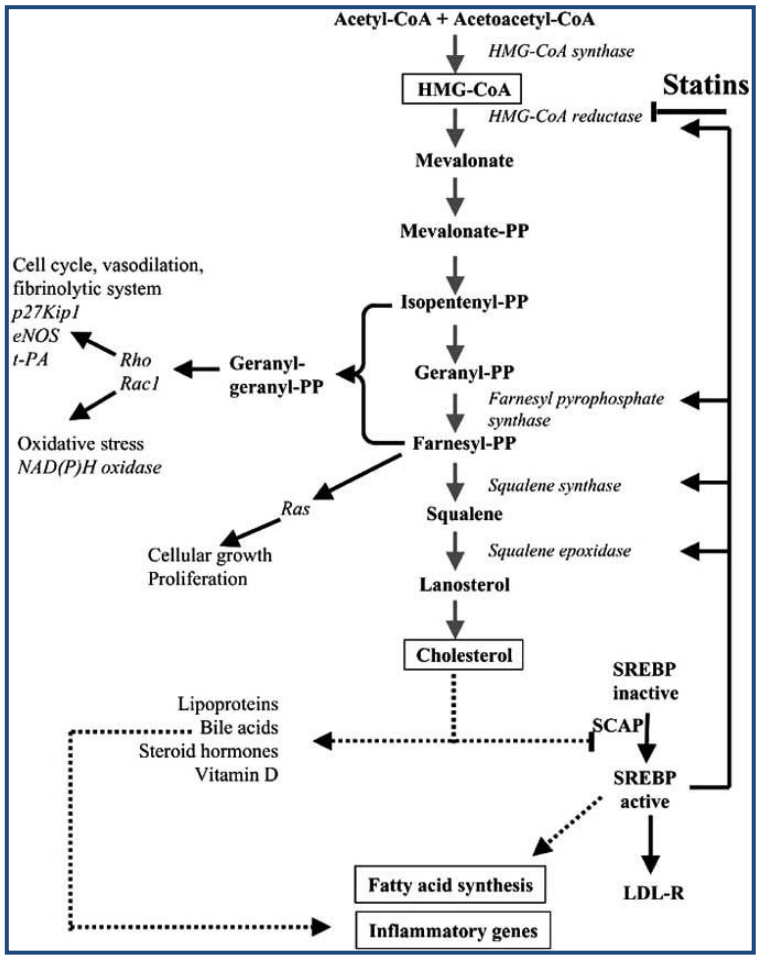

Figure 6.1. Cholesterol biosynthesis pathway and role of isoprenoids on some essential proteins. Sterol regulatory element binding proteins (SREBPs) regulate the activity of HMG-CoA and other enzymes implicated in the synthesis of cholesterol as well as the expression of the low density lipoprotein receptor (LDLR). Diagram shows that blockage of HMG-CoA produces the inhibition of isoprenoid intermediates, impairing posttranslational isoprenylation of key signalling molecules. Diagram from Kleemann et al. (Kleemann and Kooistra, 2005).

In the last few years these cholesterol lowering drugs have been reported to have some pleiotropic effects which could be responsible for a great variety of positive physiological responses upon statins treatment. Some of these cholesterol unrelated effects are believed to occur due to the blockage of the complex mevalonate pathway. HMG-CoA reductase catalyses the second step in the synthesis of cholesterol.

Cholesterol biosynthesis is composed of nine steps, being the middle ones important for the formation of isoprenoid intermediates which are required for the normal functioning of numerous signalling molecules such as Rho, Rac1 or Ras (see Figure 6.1)

Prenylation involves the transfer of prenyl groups (3-methyl-2-buten-1-yl) to proteins to facilitate its attachment to cell membranes. Two types of prenyl groups, farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP), are known to be attached to cytoplasmic proteins at the C-terminal domain (Magee and Seabra, 2003). Rho proteins are small GTP-ases that need to be prenylated to be transported to the plasma membrane where they can get activated and trigger their biological action. Consequently, blocking HMG-CoA reductase impairs key isoprenylation processes leading to the accumulation of inactive small GTP-ases in the cytosol (Liao and Laufs, 2005).

Of note, the γ subunit of G proteins, is also prenylated and needs this posttranslational modification to be able to reach the plasma membrane (Dietrich et al., 1996; Kisselev et al., 1994;Maltese, 1990).

In the past few years, statins have been demonstrated to be important in the treatment of numerous "cholesterol-non-related pathologies". Statins have been confirmed to reduce infection of HIV-1 virus (del Real et al., 2004; Nabatov et al., 2007), to be a possible therapy for multiple sclerosis (MS) (Kuipers et al., 2006) and to be a useful tool to fight the inflammation component of several diseases (Maher et al., 2009). For instance, statins reduce the pro-inflammatory properties of T cells (Blank et al., 2007; Weitz-Schmidt, 2002; Weitz-Schmidt et al., 2001) and inhibit the production of pro-inflammatory cytokines by different cell lines (Grip et al., 2000). Likewise, statins have been shown to reduce the expression of several immunoregulatory molecules such as MHC-II (Kuipers et al., 2005), CCR5 or CCR2 (Fujino et al., 2006; Nabatov et al., 2007; Veillard et al., 2006; Yin et al., 2007) and to block the chemotaxis processes of many cell types (Kuipers et al., 2006; Pozo et al., 2006).

It is believed that all these effects are caused not only by the ability of statins to deplete cholesterol but also by their ability to block the isoprenylation of key signalling proteins. Actually, the antiviral effect of statins has been attributed to the down-regulation of Rho activity through inhibition of geranylgeranylation and not to a reduction in cholesterol levels. Besides, Nabatov et al. confirmed that the ability of statins to block HIV-1 R5 infection was greatly due to the down-regulation of CCR5 receptors in the plasma membrane.

It is of interest for the current study that statins are also known to have the ability to disrupt lipid rafts (Blank et al., 2007; Hillyard et al., 2004) causing the loss of cell surface expression of many signalling molecules (Kuipers et al., 2005). These studies provided some valuable information since many of the processes leading to inflammation start in these membrane microdomains and its disruption could account for some of the anti-inflammatory effects of these drugs. Treatment of U937 cells with fluvastatin caused a substantial reduction in the association of the raft proteins LAT and Lyn with these membrane domains (Hillyard et al., 2004). This group also demonstra that lipid raft disruption was exclusively due to cholesterol inhibition since the use of inhibitors of prenylation was not able to prevent lipid rafts disruption.

Altogether, cholesterol synthesis blockage seems to be involved in inflammatory processes that are highly related to some of the pathways initiated by CCR5. The data provided above gives enough information to speculate that treatment of cells with statins could modulate CCR5 signalling through cholesterol reduction, impairment of the γ subunit of G proteins to reach the plasma membrane and transduce the signal, and to disruption of lipid rafts.

Statins treatment has previously been shown to reduce cell migration towards CCL5 and CCL3 (Kuipers et al., 2006) but no studies have analysed the effect of this cholesterol inhibiting drugs on CCR5-dependent calcium release. Hence, this effect will be investigated. In the current chapter, almost all experiments were performed in CHO.CCR5, HeLa RC49, HEK.CCR5 and THP-1 cells with very similar results. However, for simplicity reasons only the results from HEK.CCR5 and THP-1 cells are shown. Western blot and RT-PCR experiments were only performed in Hela.RCR9 and THP-1 cells.

6.2 Aim

It has been observed in previous chapters that cholesterol depletion can have very different effects in CCR5 signalling depending on the cell line studied. In this chapter cholesterol synthesis inhibition by statins was studied on CCR5-induced calcium release and CCR5 expression levels in stably transfected cells and in the monocytic cell line THP-1. Special emphasis has been put on comparing the findings here presented with the results obtained in cholesterol depleted THP-1 cells.

6.3 Results

6.3.1 Lovastatin reduces intracellular calcium mobilization in all cell lines studied

The first aim of this chapter was to analyse if blockage of cholesterol synthesis with statins had similar effects in the cell lines studied as cholesterol depletion with MCD. Therefore, cells were treated with varying concentrations of lovastatin for 3 days as indicated in materials and methods and stimulated with the chemokine CCL3 prior to the measurement of calcium mobilization. We decided to incubate cells in the presence of statins for 3 days as that is the protocol followed in most of the papers cited in this chapter since it seems to be the most efficient method for this concentration of statins.

These experiments show that treatment of cells with lovastatin blocks CCR5-dependent calcium release in all cell lines studied: HEK.CCR5 (Figure 6.2 A and B), THP-1cells (Figure 6.2 C and D) and CHO.CCR5 (Figure 6.3).

Interestingly, whereas lovastatin treatment has the same effect in both cell lines showed, MCD in combination with lovastatin treatment has different effects in HEK.CCR5 and THP-1 cells. Figure 6.2 A and B show that MCD blocks even further the response of CCR5 upon CCL3 stimulation in HEK.CCR5 cells.

However, when THP-1 cells were treated with MCD and lovastatin, MCD rescued the signalling capacity of CCR5 in lovastatin treated cells (Figure 6.2 C and D).

Lovastatin effects on CCR5 signalling were next verified by the use of simvastatin, another statin with very similar characteristics to lovastatin. Both are pro-drugs, derived from fungi, highly lipophilic and with comparable potencies (Blum, 1994).

Simvastatin treatment of cells had the same effects as lovastatin in the ability to interfere with calcium release triggered by CCR5 stimulation. Figure 6.4 and Figure 6.5 show that in HeLa.RC49 and THP-1 cells, respectively, treated with simvastatin for 3 days, a lower calcium response upon CCR5 stimulation was triggered than in control cells.

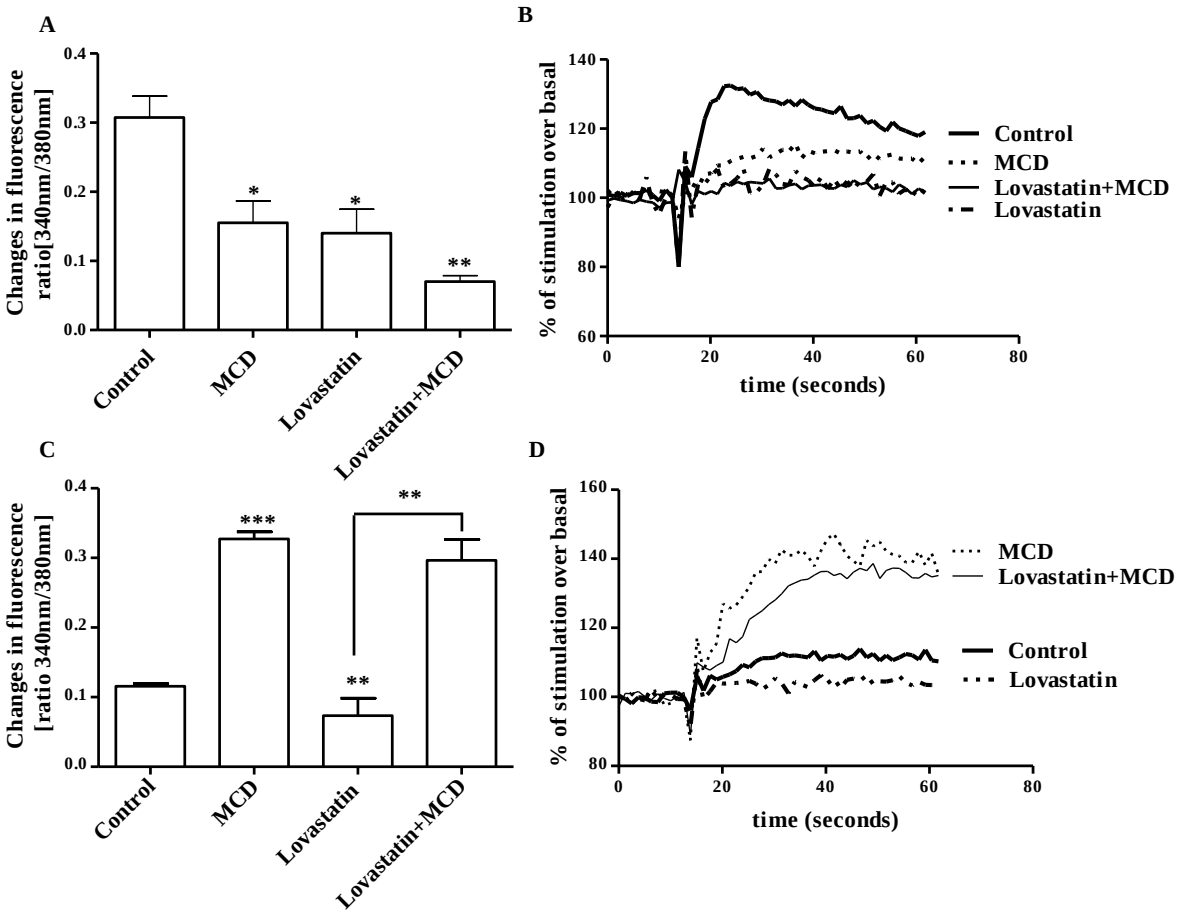

Figure 6.2 Lovastatin blocks calcium mobilization. HEK.CCR5 cells (A and B) and THP-1 cells (C and D).Cell were treated with 10 µM lovastatin for three days, 10 mM MCD for 1 h, both, or left untreated before stimulation with 100 nM CCL3. Data represent mean ± SEM from at least three independent experiments for the bar charts and a representative tracer for the calcium flux. Significant changes towards control cells are indicated by asterisks (*p < 0.05, **p < 0.01). (Cardaba and Mueller, 2009).

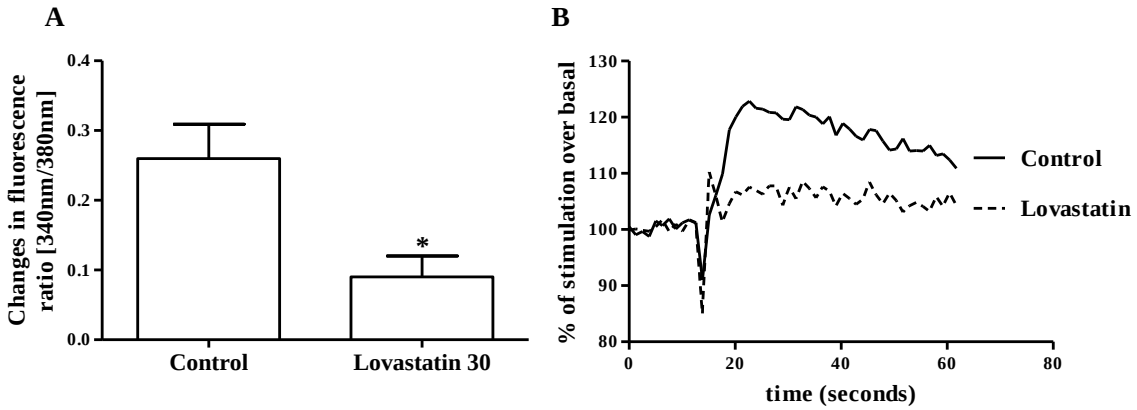

Figure 6.3 Lovastatin effect on CCR5-dependent calcium mobilization in CHO.CCR5 cells. Cells were treated with 30 µM lovastatin for three days before stimulation with 100 nM CCL3. Data represent mean ± SEM from at least three independent experiments for the bar charts and a representative tracer for the calcium flux. Significant changes towards control cells are indicated by asterisks (*p < 0.05). (Cardaba and Mueller, 2009).

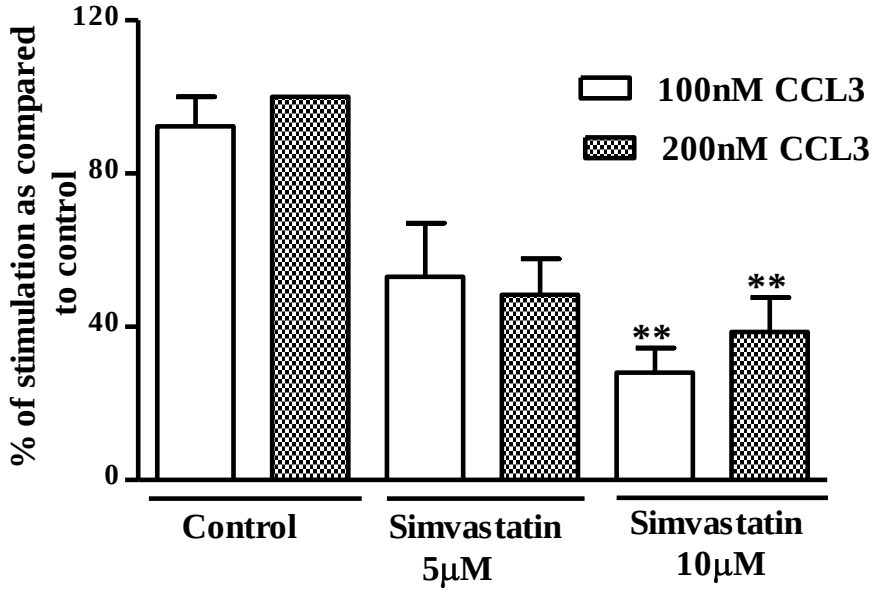

Figure 6.4 Simvastatin reduction of intracellular calcium mobilization in HeLa.RC49 cells. Cells were treated with simvastatin for 3 days and stimulated with CCL3. Data represent mean ± SEM from at least three independent experiments. Significant changes towards control cells are indicated by asterisks (**p < 0.01).

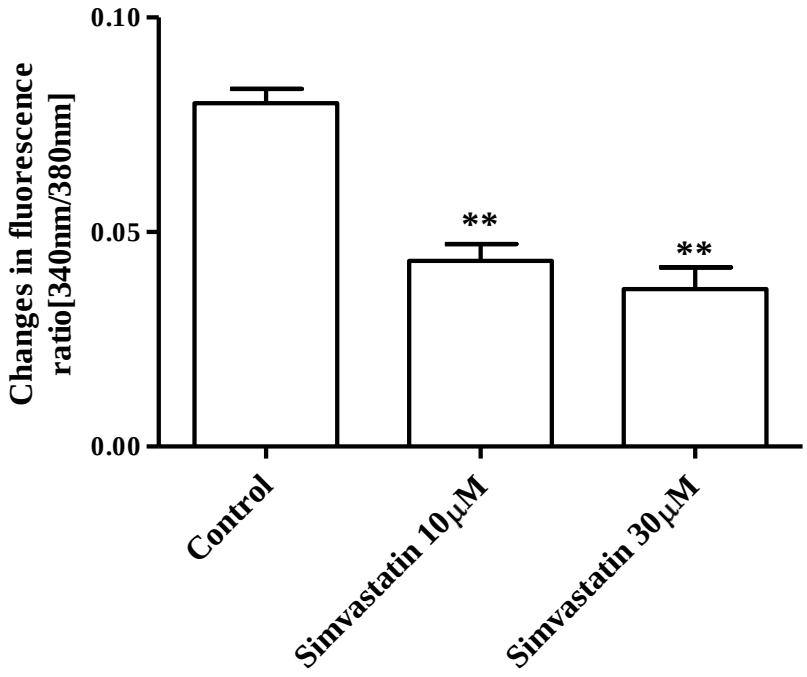

Figure 6.5 Simvastatin reduces calcium responses in THP-1 cells. THP-1 cells were treated with simvastatin for 3 days prior to stimulation with 200 nM CCL3. Data represent mean ± SEM from at least three independent experiments. Significant changes towards control cells are indicated by asterisks (**p < 0.01). (Cardaba and Mueller, 2009).

6.3.2 Lovastatin decreases cellular cholesterol by 30 %

MCD was previously shown to deplete around 70% of total cellular cholesterol. It is now hypothesised that one of the reasons why the effects of MCD are so different from those observed after lovastatin treatment may be due to differences in the amount of cellular cholesterol both drugs are able to deplete. Therefore, the next step was to analyse to what extent blockage of HMG-CoA reductase reduced total cholesterol. As shown in Figure 6.6, lovastatin treatment was able to decrease a maximum of 40% of total cellular cholesterol. However, the concentration of lovastatin used in this study, 10 μM, could only deplete cholesterol by 20%. This assay was performed in THP-1 cells (A) and HEK.CCR5 cells (B) and in both cell types similar results were observed.

The effects of simvastatin on cellular cholesterol were also studied. It can be seen (Figure 6.7) that 10 μM simvastatin treatment of THP-1 cells, which is the concentration used in calcium flux assays, depleted total cellular cholesterol by 40% (*$p < 0.05$).

These results are not very clarifying and do not help understanding the differences in THP-1 signalling upon treatment with statins or MCD. As expected, lovastatin treatment of THP-1 cells reduces cellular cholesterol in a concentration dependent manner (Figure 6.6 A) but it also reduces CCR5-dependent calcium response. However, since MCD produced a massive loss of cholesterol and caused an immense increase of calcium mobilization in a cholesterol dependent way, we suggest that lovastatin must have other effects on the cell independent of cholesterol blockage responsible for the reduction in CCR5 signalling.

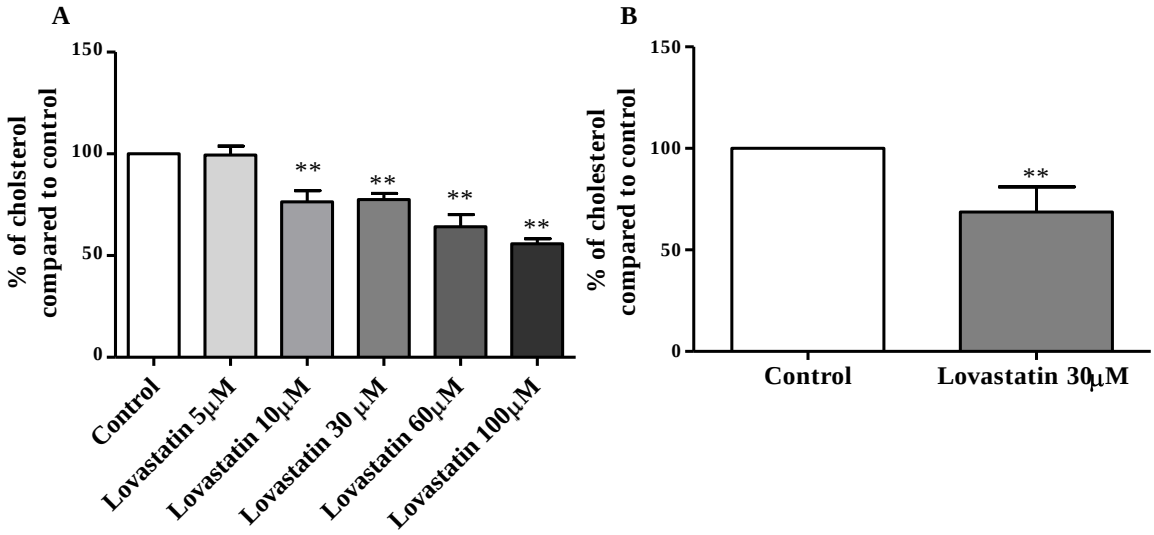

Figure 6.6 Quantification of total cellular cholesterol levels after lovastatin treatment. THP-1 cells (A) or HEK.CCR5 cells (B) were treated with different concentrations of lovastatin and total cholesterol was measured using the Amplex Red cholesterol assay as described in materials and methods. Data represent mean ± SEM from at least three independent experiments. Significant changes towards control cells are indicated by asterisks (**p < 0.01). (Cardaba and Mueller, 2009).

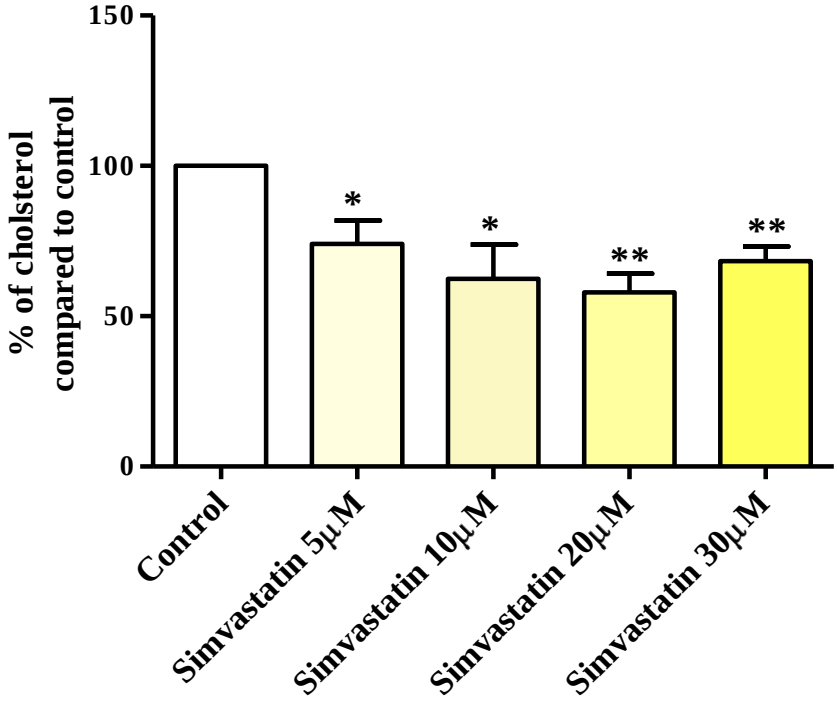

Figure 6.7 Quantification of total cellular cholesterol levels after simvastatin treatment. THP-1 cells were treated with different concentrations of simvastatin and total cholesterol was measured using the Amplex Red cholesterol assay as described in materials and methods. Data represent mean ± SEM from at least three independent experiments. Significant changes towards control cells are indicated by asterisks (*p < 0.05, **p < 0.01). (Cardaba and Mueller, 2009).

6.3.3 Lovastatin treatment of cells reduces CCR5 surface expression.

The results observed in HEK.CCR5 cells (Figure 6.2 A and B) show that cholesterol synthesis inhibition reduces CCR5 signalling in these cells, which was in congruence with previous data obtained from MCD-treated CHO.CCR5 and HEK.CCR5 cells. The main purpose of the next set of experiments was to try to understand why lovastatin did not cause an increase in calcium mobilization in THP-1 cells similar to the one observed upon MCD treatment. MCD appears to cause blockage of CCR5 signalling in CCR5 stably transfected cells without altering its membrane expression [chapter 3 (Cardaba et al., 2008)]. Here, the effect of lovastatin on CCR5 membrane expression in HEK.CCR5 cells and THP-1 cells was analysed by flow cytometry. Figure 6.8 illustrates that lovastatin causes a significant reduction in the membrane expression levels of CCR5 both, in HEK.CCR5 and THP-1 cells whereas MCD does not alter it (Figure 6.8 B). The effects of lovastatin were stronger in HEK.CCR5 cells where the loss of CCR5 molecules reached 70% versus the 50% achieved by lovastatin treatment in THP-1 cells. These findings might indicate the different capacity of these two cell lines to deal with cholesterol synthesis and transport to the membrane.

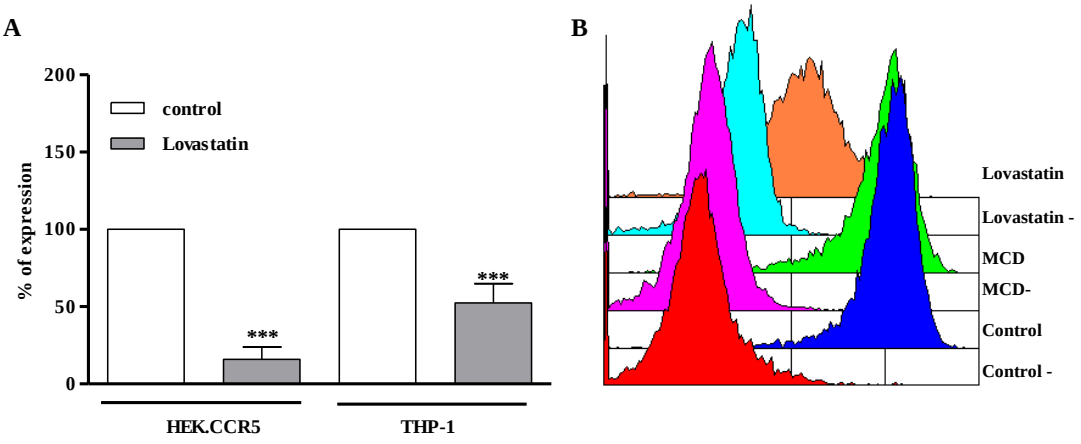

Figure 6.8 Lovastatin reduces CCR5 expression in HEK.CCR5 and THP-1 cells. Cells were treated with 10 µM lovastatin for 3 days or 10 mM MCD for 30 minutes. Next, cells were incubated for 1h with anti-CCR5 antibody followed by FITC-conjugated secondary antibody. A) Shows CCR5 expression levels as measured by flow cytometry represented by mean ± SEM from at least three independent experiments. B) Histogram overlay showing flow cytometry analysis from HEK.CCR5 cells treated with 10 mM MCD, 10 µM lovastatin or left untreated. Significant changes from control cells are indicated by asterisks (***p < 0.01). (Cardaba et al., 2008).

6.3.4 Effects of lovastatin on CCR5 mRNA and protein expression levels

In the previous section we have reported that treatment of stably transfected cells and THP-1 cells with lovastatin causes a reduction in the number of CCR5 receptors in the cell membrane.

This result is in accordance with many other studies showing the effects of statins on chemokine receptors (Fujino et al., 2006; Han et al., 2005; Veillard et al., 2006;Yin et al., 2007). Most of these studies confirmed a reduction in the mRNA levels of CCR5 and CCR2 receptors. However, the fact that, in this study, less CCR5 molecules are found in the plasma membrane does not necessarily mean that there is a reduction in CCR5 mRNA. There is the possibility that upon statin treatment CCR5 is abnormally held in the cytosol but its mRNA and protein levels are kept at normal levels. Another possibility would be that mRNA levels are unchanged but there is a defect at protein levels. In order to find out the source of the reduction of CCR5 molecules in the membrane as measured by flow cytometry, RT-PCR experiments were next performed.

Normalization of RT-PCR experiments requires an endogenous control to account for differences in the amount of total RNA in each sample. The expression of the reference gene should not vary in the tissue or cell line used and should be stable in response to an experimental treatment. GAPDH and β-actin were shown to be unsuccessful for these experiments due to the ability of lovastatin to significantly alter their expression. 18S has been found to be one of the most stable genes by the geNorm method before (Radreau et al., 2009). Accordingly, it could be shown that 18S expression levels were not altered by statins treatment and was, therefore, a good reference gene.

Lovastatin 10 μM was used to treat the cells during 3 days and then CCR5 mRNA levels were analysed. Figure 6.9 shows that CCR5 mRNA levels are increased in the two cell lines used. This increase was 2.5-fold in THP-1 cells (A) and around 9-fold (*$p < 0.05$) in HeLa RC49 cells (B).

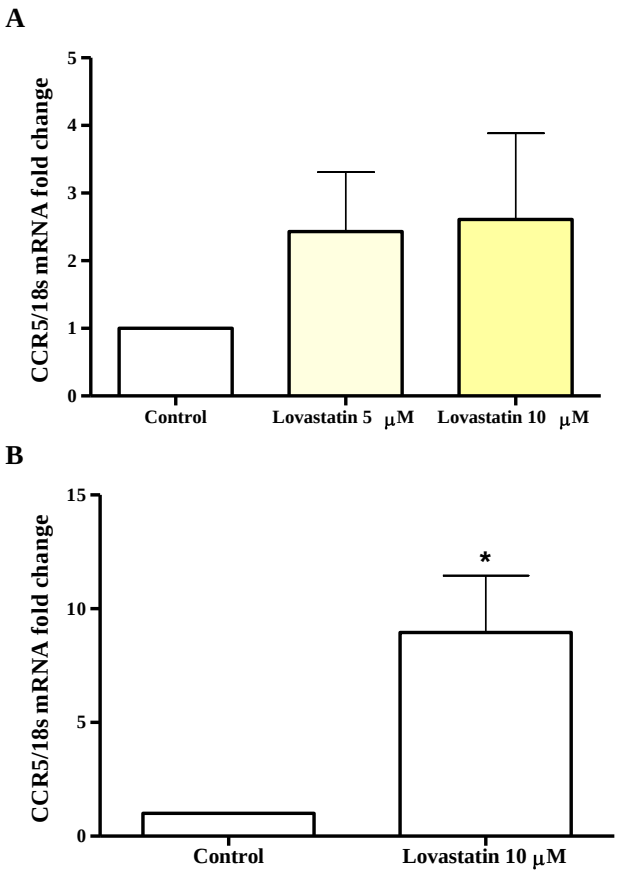

Figure 6.9 Shows lovastatin-induced fold-changes in the target gene when compared to 18S reference gene. CCR5 mRNA folds change from THP-1 cells (A) and HeLa.RCR9 cells (B) treated with different concentrations of lovastatin or left untreated. Data represent mean ± SEM from at least three independent experiments. Significant changes towards control cells are indicated by asterisks (*$p < 0.05$).

Since the results obtained here did not explain a reduction on CCR5 membrane expression levels it was next sought to examine the protein levels of the receptor to see if inhibition of the mevalonate pathway had any effect on them. Two cell lines, HeLa.RC49 and THP-1 cells were used to perform western blots to determine CCR5 protein levels upon lovastatin treatment. Interestingly, it was found that CCR5 levels were reduced in both cell lines as can be appreciated in figure 6.10 when compared to the normalising protein β-actin.

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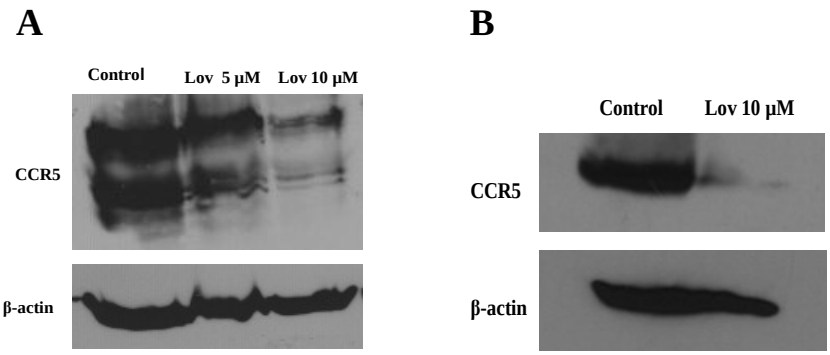


Figure 6.10. CCR5 is down-regulated in THP-1 cells (A) and HeLa.RC49 cells (B) in response to lovastatin treatment. Protein levels were examined by western blot and compared to the normalising protein β-actin. Experiment shows a film representative of two independent experiments the case of THP-1 and a single experiment in the case of HeLa.RC49 cells.

6.4 Discussion

In this chapter the effect of statins on different cell lines expressing CCR5 has been analysed. It had previously been shown that MCD-dependent cholesterol depletion had opposite effects in THP-1 cells and in CCR5 stably transfected cells. MCD-induced cholesterol depletion but not filipin or nystatin treatment caused a huge increase in the amount of calcium being released from ER stores in a CCR5-activation dependent manner in THP-1 cells. On the contrary, MCD completely blocked calcium responses in HEK.CCR5, CHO.CCR5 and HeLa.RC49 cells. It was hypothesised that the differences in signalling among these cell lines upon cholesterol depletion may be due to different cholesterol levels requirement for optimal signalling. Therefore, blockage of cholesterol synthesis with statins, which represents another approach to study how cholesterol modulation alters CCR5 signalling in the different cell lines studied, was next used. Since both treatments are widely known to reduce the amount of total cholesterol in the cell it was expected to observe a decrease in CCR5-induced calcium release in stably transfected cells and a massive increase in calcium mobilization upon CCL3 treatment in the monocytic cell line. Accordingly, when CCR5 stably transfected cells were treated with 10µM of lovastatin, an almost complete inhibition on calcium release was observed. Nevertheless, a similar reduction on CCR5 signalling was obtained after statins treatment of THP-1 cells.

These findings were completely unexpected and led to investigate possible reasons why lovastatin could block CCR5 signalling in the monocytic cell line.

Results from this chapter have shown that MCD treatment in combination with lovastatin treatment in THP-1 cells was able to recover CCR5-dependent calcium signalling. These different effects of MCD and statins in THP-1 cells clearly highlight the cholesterol independent effect of statins in some biological responses. It could be argued that, as shown above, MCD has a much stronger capacity of depleting membrane cholesterol than lovastatin, which could account for the distinct responses observed. The concentration of lovastatin used in these experiments, 10 μM, is only able to deplete about 20% of cellular cholesterol whereas MCD treatment caused 70% of cholesterol loss. However, this does not explain the opposite effects exerted by both drugs.

These findings were completely unpredicted and led to investigate a possible cause why statins could inhibit CCR5-dependent calcium release. Chemokine receptors have been previously shown to be down-regulated from the plasma membrane upon statins treatment. Thus, possible alterations in CCR5 membrane expression upon lovastatin treatment were studied next. Accordingly, flow cytometry experiments showed a reduction in the number of CCR5 receptors in the plasma membrane. This finding clearly indicates that statins effects on CCR5-dependent calcium response could be due to a reduction on the number of CCR5 molecules in the plasma membrane able to interact with the ligand.

The decrease on CCR5 membrane expression was further analysed by studying CCR5 protein levels and CCR5 mRNA levels in HeLa.RC49 and THP-1 cells. The fact that the number of CCR5 molecules in the plasma membrane is diminished could indicate a lack of receptors reaching the plasma membrane or a reduction on the synthesis of the receptor. In order to understand this, CCR5 mRNA levels and CCR5 protein levels were analysed by RT-PCR and western blot respectively. It could be observed that while CCR5 protein levels were considerably reduced, CCR5 mRNA levels were increased, especially in lovastatin treated HeLa.RC49 cells. These findings might represent a sort of compensation mechanism through which cells, in an attempt to recuperate CCR5 receptors in the plasma membrane increase CCR5 synthesis mechanisms. The reason why CCR5 protein levels are reduced upon statins treatment remains unknown. Although this is not the first time that statins have been shown to

reduce the expression of chemokine receptors, in this chapter the reduction of CCR5 expression in the membrane has been linked to a reduction in CCR5 protein levels but not to a reduction in mRNA levels as in the other studies. For instance, statins showed to reduce CCR2, CCR5 and its respective chemokines CCL2 and CCL5 mRNA levels on monocytes (Fujino et al., 2006; Han et al., 2005). Similarly, patients with hyperlipidemia treated with statins were shown to experience a reduction in CCR5 and CCL5 mRNA levels after only 1.5 months of statins treatment (Li et al., 2006a). Statins were also shown to reduce mRNA levels of CCL2, CCL3,CCL4 and the chemokine receptors CCR1, CCR2, CCR4 and CCR5 in endothelial cells and macrophages (Veillard et al., 2006). Veillard's group tried to find a link between the pleiotropic effects of statins and the reduction in the CCR2 and CCR5 mRNA levels. They associated the blockage of the prenylation processes with an increase in the transcription levels of Oct-1, a transcriptional repressor able to down-regulate CCR2 and CCR5 expression levels. Nevertheless, considering that CCR5 mRNA levels were significantly increased in the examples here provided, it seems that statins effects on these cells are not related to the transcriptional repressor Oct-1. Another possibility is that Rho-activation impairment by statins alters lipid rafts formation and, therefore, affects the number of CCR5 receptors in these microdomains. However, once more, this does not explain the reduction in CCR5 protein levels or why lipid rafts disruption with MCD has opposite effects in THP-1 cells. Thus, the reasons behind this reduction in CCR5 expression by statins remains unknown and further investigations should be done in order to understand the exact mechanism behind it.

CCR2 and CCR5 receptors play an important role in atherosclerosis (Boring et al., 1998; Potteaux et al., 2006; Veillard et al., 2004; Zernecke et al., 2006). These receptors are known to induce the recruitment of monocytes to the damaged endothelium upon endothelial cells secretion of chemokines such as CCL2 or CCL5. A good example of this is represented by mice deficient in CCL2 or CCR5, who showed a better prognosis in atherosclerosis development clearly influenced by a reduction in monocytes chemotaxis and adhesion (Boring et al., 1998).

Overall, it seems that statins-dependent reduction of chemokine receptors and chemokines levels might represent one of the bases of the anti-inflammatory effects of this popular drug.

Altogether, this study provides further evidence that statins treatment, by reducing CCR5 signalling can contribute to the recovery and prevention of atherosclerosis and other inflammatory disease. The differences between MCD and statins effects in CCR5 signalling indicate that the inhibitory effects of statins on CCR5 signal transduction cannot be exclusively due to a decrease in cholesterol levels or lipid rafts disruption. Hence this study agrees with previous ones that statins have the ability to greatly alter biological responses independently of cholesterol.

CHAPTER 7- IMPORTANCE OF PKC ON CCR5 SIGNALLING

7.1 Introduction

GPCR expression can be up-regulated or down-regulated according to body's need. CCR5 can undergo two different types of phosphorylation that will lead to receptor internalisation (Pollok-Kopp et al., 2003). The first one is called homologous desensitisation and it is carried out by G protein-coupled receptor kinases (GRKs) when the receptor is occupied by a ligand (Pitcher et al., 1998). The second type of receptor phosphorylation is called heterologous desensitisation and takes place in the absence of ligand or at least is not completely dependent on its binding to the receptor. The enzyme involved in this desensitisation is protein kinase c (PKC) and its activation with PMA or through intracellular second messengers can cause receptor phosphorylation in the absence of ligand binding. It is interesting to note that PKCs can also be involved in homologous desensitisation due to their ability to activate GRKs (Chuang et al., 1996).

CCR5 contains serine and threonine residues which are the target of both GRK and PKC. These enzymes specifically phosphorylate these residues making it possible to differentiate which enzyme is responsible for receptor desensitisation in each case. Ser-337 is exclusively phosphorylated by PKC whereas Ser-349 is phosphorylated by GRK2/3 after ligand stimulation of CCR5. A study using phospho-sitespecific antibodies (Pollok-Kopp et al., 2003) demonstrated that Ser-337 was phosphorylated at lower concentrations of agonist and about 4 times quicker than Ser-349. Accordingly, PKC inhibitors are able to block CCR5 desensitisation coming from heterologous stimulation (Le et al., 2001; Li et al., 2001). Therefore, it seems that PKC is involved in CCR5 desensitisation upon stimulation with other receptors ligands due to cross-talk between CCR5 and this other chemokine receptor.

CCR5 forms heterodimers with the C5aR and, interestingly, it was found that stimulation of C5aR was able to promote CCR5 internalisation not only in a PKC dependent (heterologous) manner but also in a GRKs dependent (homologous) form.

These findings cast doubt on PKCs and GRKs exclusively acting in heterologous and homologous desensitisation respectively. Indeed, we now suggest that CCL3 binding to CCR5, apart from triggering homologous desensitisation of the receptor through GRKs, is also likely to promote PKC dependent desensitisation of the receptor through the stimulation of intracellular pathways. In congruence with this hypothesis, it was shown that CCL5 stimulation of CCR5 was able to promote phosphorylation in Ser-337 (Pollok-Kopp et al., 2003).

Another big difference between these two types of receptor desensitisation can be understood after treatment of cells with pertussis toxin. This molecule only blocks PKC dependent phosphorylation of CCR5 whereas it has no effect on GRKs dependent phosphorylation of the receptor, demonstrating that activation of G proteins is not needed for homologous or agonist-specific CCR5 phosphorylation (Pollok-Kopp et al., 2003). It seems that both, homologous and heterologous desensitisation can be initiated by ligand binding to the receptor but, whereas the first one occurs exclusively due to ligand binding, the latter is initiated by intracellular second messengers.

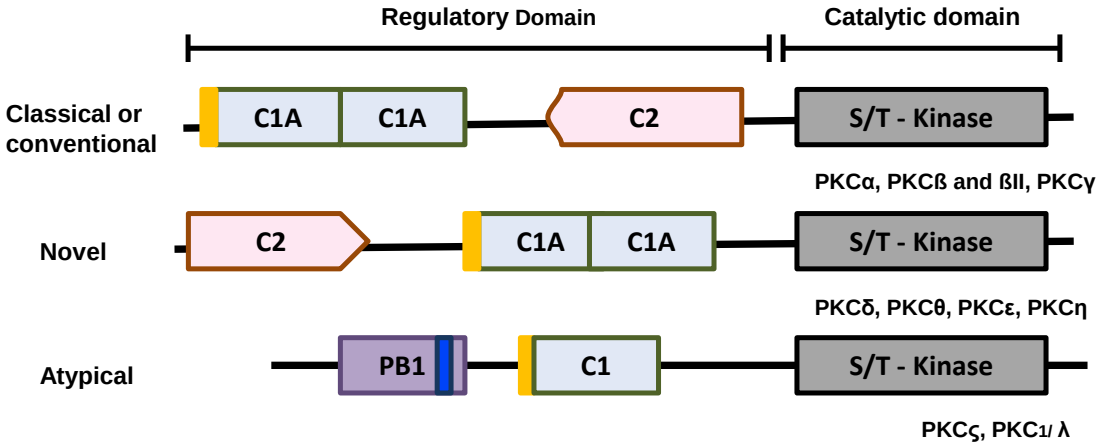


Figure 7.1 Diagram of the primary structures of the different isoforms of PKC enzymes**.** The N-terminus includes a C1 domain which is different among the different members of the family, a C2 domain which is not present in atypical PKCs and a PB1 domain, only present in atypical isoforms. All members share the catalytic domain which contains an ATP binding domain, a substrate binding domain as well as the phosphotransfer sites. Scheme adapted from S. Corbalán-García, J.C. Gómez-Fernández / Biochimica et BiophysicaActa 1761 (2006) 633–654 (Corbalan-Garcia and Gomez-Fernandez, 2006).

There are at least 10 different isoforms of PKC. They are divided in 3 groups: the classical PKCs (cPKCs or calcium and DAG dependent: α, βI, BII, and γ), novel PKCs

(nPKCs or calcium independent: δ, ε ,η , and θ) and the atypical PKCs (aPKCs or calcium and DAG independent: ζ, ι.) (Millar and Newton, 2009). The common structure of PKCs include a N-terminal regulatory domain, activated by the second messengers above described and a C-terminal catalytic domain, which has the ATP binding site and is responsible for the kinase activity of the enzyme (Corbalan-Garcia and Gomez-Fernandez, 2006).To date, the isoforms/isoforms implicated in CCR5 phosphorylation have not been reported.

In the past years PKC modulators have become very important compounds. It is now known that PKC activators can be useful against HIV infection due to their property to reactivate latent virus and down-modulate CCR5 and CXCR4 receptors (Bedoya et al., 2009). Additionally, some studies present PKC inhibitors as effective drugs against HIV infection since they have been shown to stop the virus replication cycle (Kruth et al., 2005). Considering that PKC is a key enzyme in up-regulating HIV transcription through regulating NF-κβ and MAPK signalling pathways (Kagnoff and Roebuck, 1999; Yang and Gabuzda, 1999) it makes sense that blocking this enzyme would inhibit HIV infection. It seems that the use of PKC inhibitors could be beneficial in order to stop viral replication and the use of PKC activators would be helpful to stop viral latency and down-modulate CCR5 and CXCR4. Thus, a combination of both drugs at the correct period of the infection along with conventional HAART therapy might represent a new option for HIV treatment.

On the other side, PKC inhibitors are on the trial to be used as anti-cancer drugs (Faivre et al., 2006; Mackay and Twelves, 2007). PKC is activated by tumour promoting phorbol esters which necessarily connects this enzyme with tumour progression. Additionally, increased PKC levels have been found in several malignancies where this enzyme has also been shown to have a central role in cell growth, differentiation and angiogenesis (Ali et al., 2009). For example the PKC inhibitor enzastaurin (Ly317615) is in phase I study and so far it has been shown to induce apoptosis and suppress cell proliferation in a wide range of tumour cell lines (Mackay and Twelves, 2007). If this drug successfully passes all the clinical trials it would be a key issue to study the way it interacts with some other signalling pathways. Very few studies have analysed how PKC modulators affect CCR5 signalling and due to the role of this enzyme in desensitising CCR5 it would be expected that PKC significantly modulated chemokine receptor dependent intracellular responses.

CCR5 stimulation can activate PKC through DAG production and calcium release and this activation is thought to be important for terminating calcium release stimulated by IP3 production. When IP3 binds the IP3R and raises intracellular calcium, it is known that PKC stimulation can lead to phosphorylation and de-activation of both, IP3R and CCR5 (Van Rossum and Patterson, 2009). Considering these effects it would be assumed that PKC inhibition increases calcium release to the cytosol as a result of CCR5 and IP3R over-stimulation. Accordingly, some reports show how inhibitors for the classical forms of PKC stimulate calcium release in other GPCR (Deshpande et al., 2007; Manes et al., 2003). Furthermore, GRKs are known to be involved in regulating CCR5 signalling. Patients with rheumatoid arthritis (RA), have levels of GRK2 reduced by 50% which might indicate that GRK is involved in cell migration to inflammation sites in RA. A study done in T cells stablished that a reduction of GRK2 highly increased chemotaxis upon CCL4 stimulation of T cells (Vroon et al., 2004). As previously explained, CCR5 signal transduction has been related to inflammatory diseases like RA, MS or cancer. Therefore, the fact that PKC inhibitors could potentially be used for cancer therapy but could at the same time activate CCR5 induced signalling pathways represent a danger associated to the possible beneficial effect they could have as anti-cancer drugs. In addition, it is known that CCR5 stimulation by chemokines can contribute to cancer growth and spread (Azenshtein et al., 2002; Huang et al., 2009a;Manes et al., 2003). However, it has not been established whether the biological response activated through CCR5 stimulation in the tumour microenvironment only help cancer progression or host antitumor response and cancer regression as well. Considering the literature, it is likely that chemokine receptors play a role in both mechanisms depending on other cellular factors (Coussens and Werb, 2002). In any case, a better characterization of how modulating PKC activity can influence CCR5 signalling would be very useful for understanding these chemokine receptors signal transduction networks.

7.2 Aim

PKC-dependent signalling pathways are being intensively investigated due to the role PKC plays in cancer progression and other diseases. The fact that this enzyme has been shown to be involved in CCR5 phosphorylation and desensitisation highlights the possibility that altering PKC activity might have an important effect on CCR5 signalling. Nevertheless no studies have focus on understanding the connection between

these two important kinases. In this chapter, the way specific PKC inhibitors modify CCR5-dependent calcium release and chemotactic pathways have been analysed.

7.3 RESULTS

7.3.1 PKC-dependent regulation of GPCR-mediated calcium release

In this chapter, the effects of modulating PKC activity have been analyzed. Specific inhibitors for each type of PKC isoforms have been used and it has been found that they have different effects on CCR5 signalling. The inhibitor GF109203X can be used as a general PKC inhibitor when added at micromolar concentrations and as a classical PKC inhibitor when added at nanomolar concentration (Toullec et al., 1991). Cells treatment with high concentrations of the drug (5 μM) where it inhibits PKC α, βI, δ, ε and ζ, significantly increases calcium release in Hela.RC49 (Figure 7.2), CHO.CCR5 cells (Figure 7.3 A) and HEK.CCR5 cells (Figure 7.4). The CCL3 dose response curves performed in CHO.CCR5 cells indicates that GF109203X causes a slight decrease in CCL3 potency but augments 2.5-fold the predicted efficacy of the chemokine. These findings confirmed the hypothesis that probably, through a reduction in CCR5 phosphorylation, blocking PKC activity would increase the time and efficiency of CCR5 coupling to G proteins. The specific CCR5 antagonist maraviroc was able to abrogate the effect of GF109203X in HeLa.RC49 cells (Figure 7.2), indicating that this increase in calcium signalling was directly caused by CCR5 stimulation. In order to corroborate this theory, the effect of a PKC activator was analysed in CCR5 induced calcium release. It was suggested that since inhibition of PKC could stimulate intracellular calcium mobilization upon CCL3 treatment, stimulating PKC activity with PMA would further phosphorylate CCR5 and, therefore, reduce its signalling. CCL3 dose response curves were created for control CHO.CCR5 cells and cells treated with PMA. As expected, PMA treatment of cells blocks CCR5-induced calcium release. PMA treatment of CHO.CCR5 cells produces a decrease of the $LogEC_{50}$ from -6.67 to -2.73, having also a marked effect on predicted efficacy (Figure 7.5).

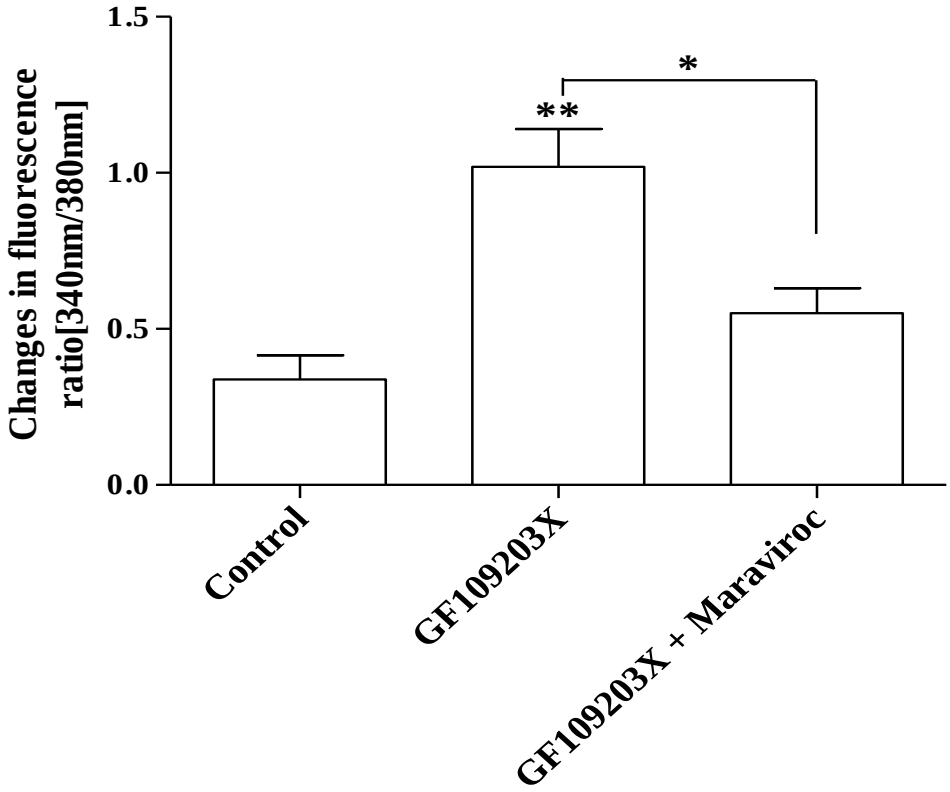


Figure 7.2 General inhibition of PKC causes increases in CCR5 calcium signalling in HeLa RC49 cells. Cells were treated with 5 µM GF109203X, GF109203X and 100 nM maraviroc for 30 minutes or left untreated (control) and stimulated with 100 nM CCL3. Calcium release was measured as previously described. Data represent mean ± SEM from at least three independent experiments. Significant changes towards control cells are indicated by asterisks (* $p < 0.05$, ** $p < 0.01$).

Remarkably, it was found that treatment of cells with GF109203X at 50 nM, where it only blocks the classical PKC isoforms α and βI, slightly reduces the predicted efficacy and increases the $LogEC_{50}$ from -7.18 to -7.45, not having overall a dramatic effect on CCR5-induced calcium release (see figure 7.3 B). These data indicate that novel or atypical isoforms of PKC are most likely responsible for the enhancement in CCR5-dependent calcium release.

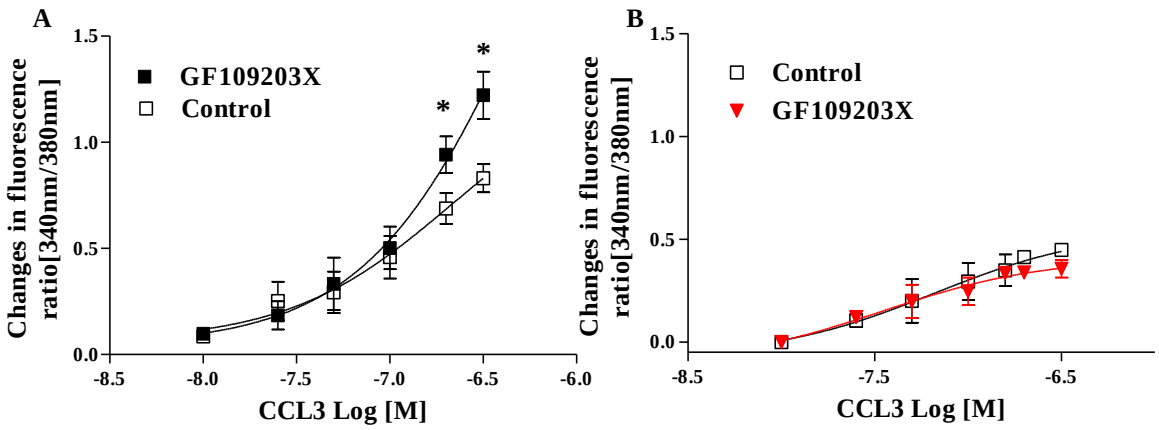


Figure 7.3 General inhibition of PKC increases calcium release whereas blockage of classical PKC isoforms does not. CHO.CCR5 cells were treated with GF109203X (5 µM for A and 50 nM for B) and stimulated with different concentrations of CCL3. Data represent mean ± SEM from at least three independent experiments. Significant changes towards control cells are indicated by asterisks (** $p < 0.01$).

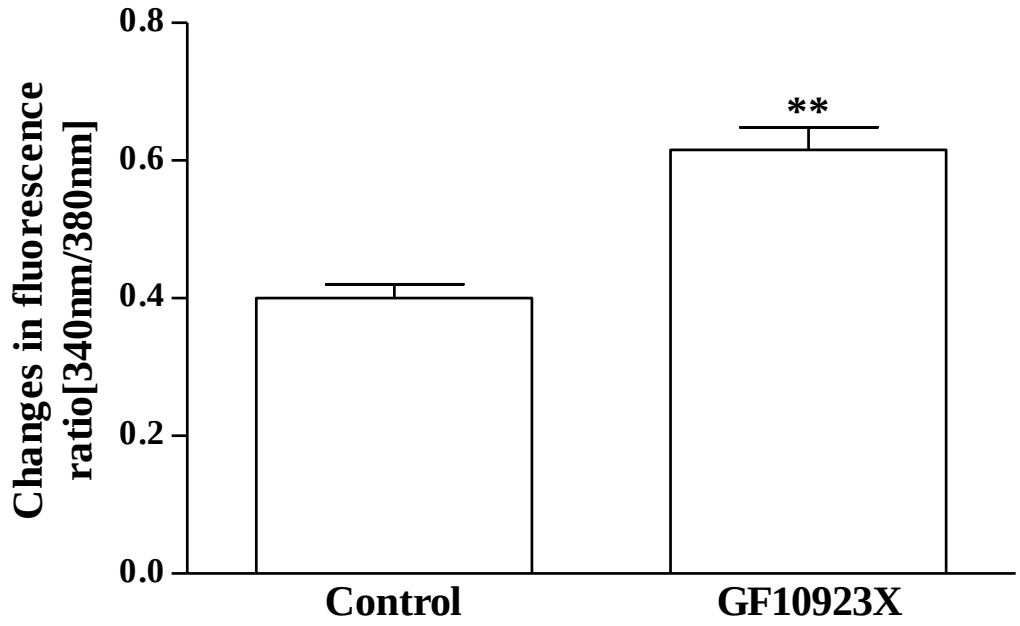


Figure 7.4 General inhibition of PKC causes increases in CCR5 calcium signalling in HEK.CCR5 cells. Cells were treated with 5 µM GF109203X for 30 minutes, stimulated with 100 nM CCL3 and calcium release was measured as previously described. Data represent mean ± SEM from at least three independent experiments. Significant changes towards control cells are indicated by asterisks (** $p < 0.01$).

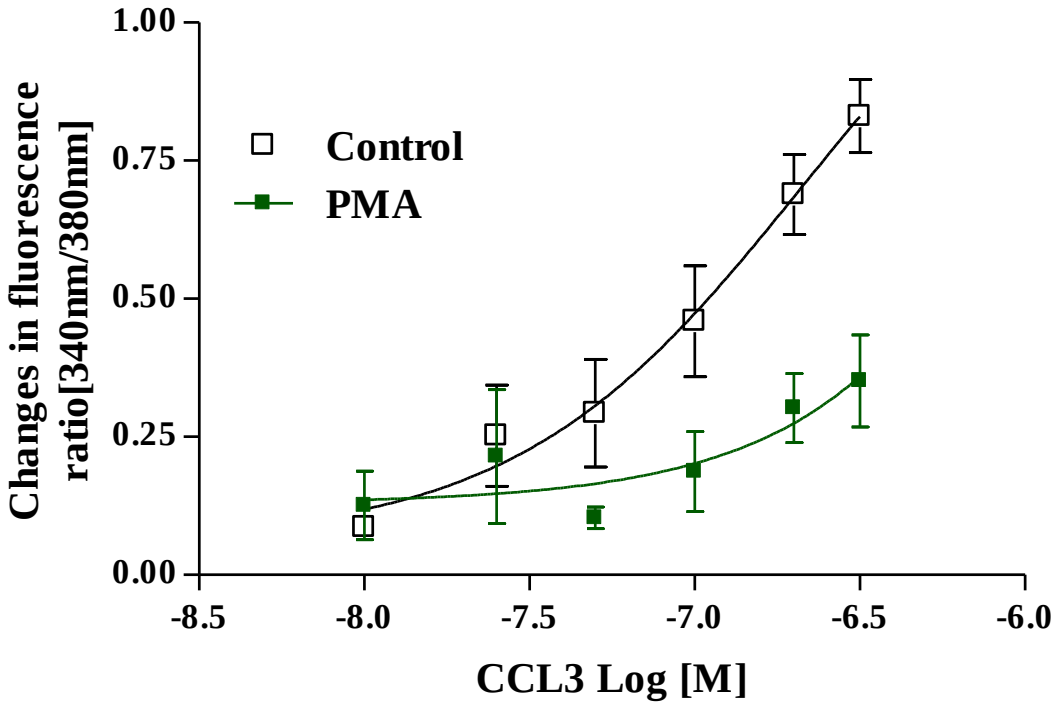


Figure 7.5 Activation of PKC causes a reduction of CCR5 activation upon ligand treatment. CHO.CCR5 cells treated with 100 nM PMA for 30 minutes, stimulated with different concentrations of CCL3 and calcium fluxes were measured. Data represent ± SEM from at least three independent experiments.

7.3.1.1 Effects of classical isoforms of PKC inhibitors on CCR5 calcium release.

In the previous section it has been shown that there is a significant possibility that inhibiting novel and atypical isoforms of PKC could increase the effect chemokines have on CCR5. Thus, it was next sought to focus on which PKC isoforms were involved in CCR5-mediated calcium release. The PKC inhibitor Go6976, specific for the classical isoforms of the PKC enzyme, was next used to verify the results obtained with low concentrations of GF109203X. Surprisingly, this time the results were opposite to those observed after pre-treatment of the cells with 50nM of the general inhibitor GF10923X. Thirty minutes pre-treatment of cells with Go6976 significantly blocked calcium release upon CCL3 stimulation in HeLa.RC49 cells (Figure 7.6). In order to understand why this inhibitor had opposite effects to GF109203X, CCR5 expression after incubation of cells with Go6976 was measured by flow cytometry. Thirty minutes incubation with Go6976 had no effect on CCR5 surface expression (Figure 7.7).

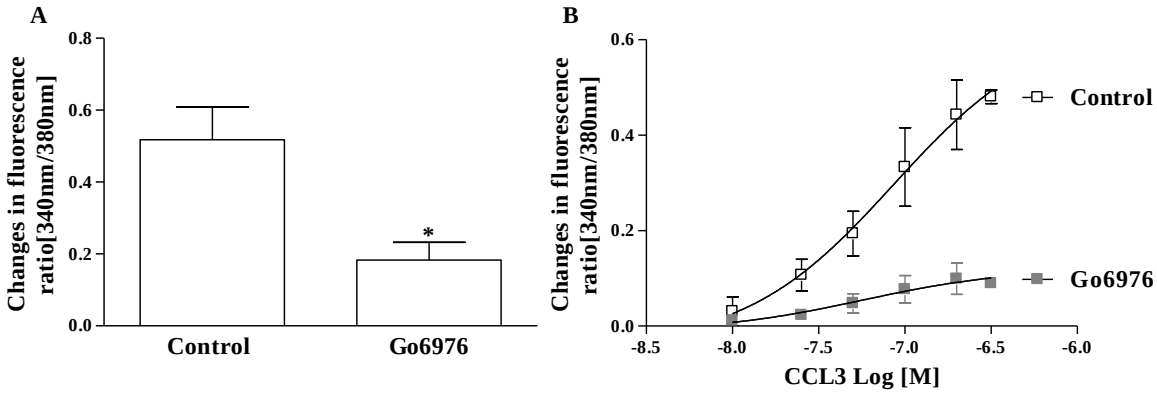


Figure 7.6 Go6976 blocks calcium release induced by CCR5 stimulation. Figure shows HeLa RCR9 cells treated with the 100 nM Go6976 and stimulated with 200 nM CCL3 (A) or with increasing concentrations of the chemokine (B). Data represent mean ± SEM from at least three independent experiments. Significant changes towards control cells are indicated by asterisks (* $p < 0.05$).

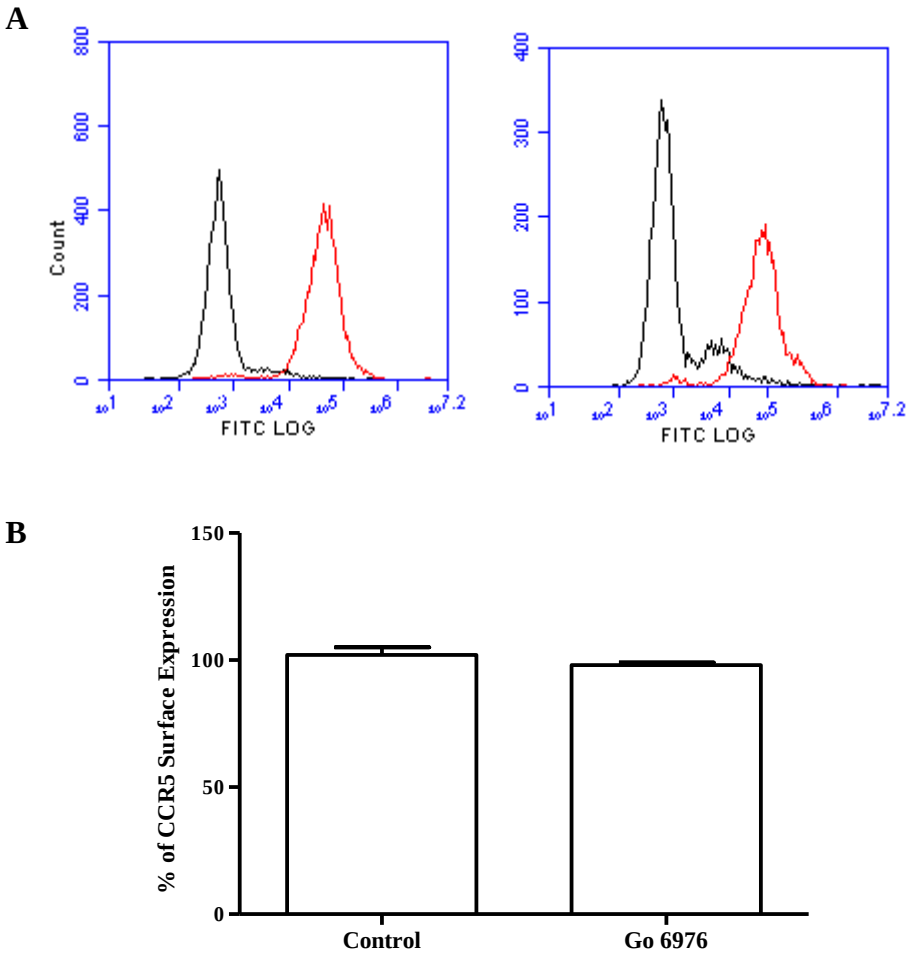


Figure 7.7 Flow cytometry analysis of CCR5 expression. HeLa RC49 cells were treated with the classical PKC inhibitor Go6976 at a concentration of 100 nM for 30 minutes and stained with an anti-CCR5 antibody and a FITC-conjugated secondary antibody. A) Shows a representative histogram B) Shows mean ± SEM from 2 independent experiments.

7.3.1.2 Effects of the novel PKC inhibitor rottlerin on calcium signalling

Rottlerin is a PKC inhibitor with specificity for PKCδ, with an EC_{50} of 3-6 μM (Gschwendt et al., 1994). This inhibitor was then used to analyse the role of PKCδ on CCR5 induced calcium release. As can be observed in Figure 7.8, rottlerin has similar effects on CCR5 signalling than the PKC classical inhibitor Go6976.

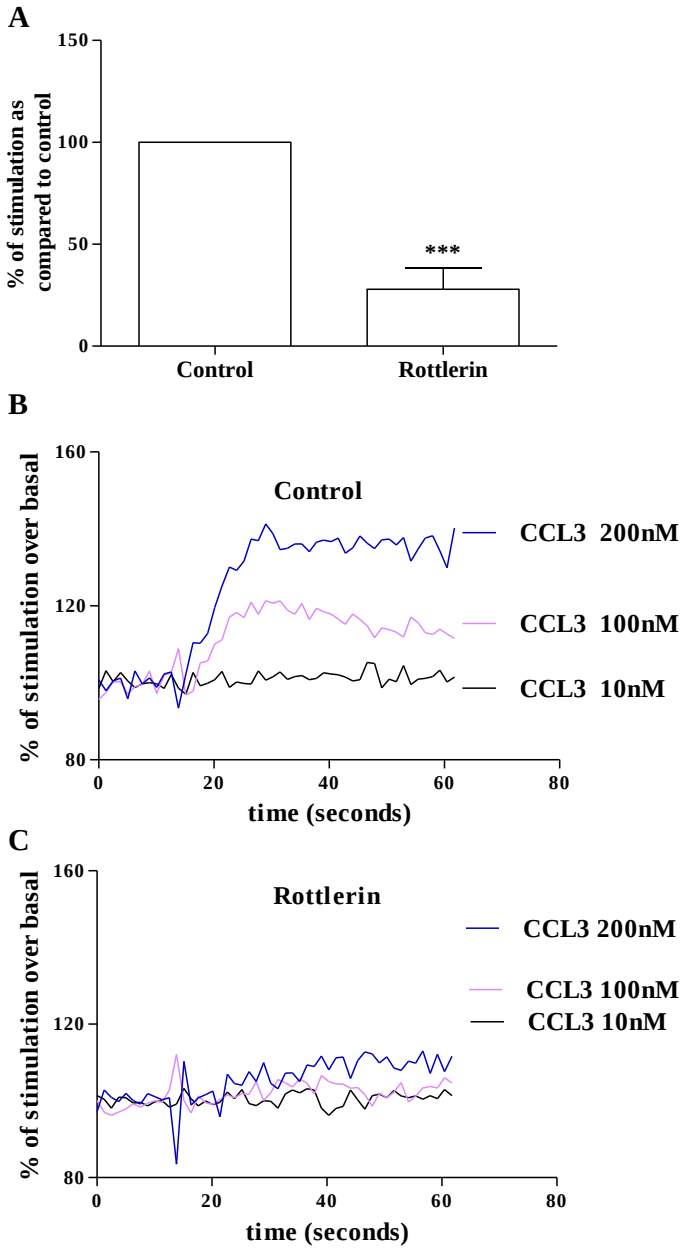


Figure 7.8 Rottlerin has an inhibitory effect in CCR5 dependent calcium release. HeLa cells were treated with rottlerin (4 μM) or left untreated (vehicle) and stimulated with 200 nM CCL3 (A) or with increasing concentrations of chemokine (B and C). Data represent mean ± SEM from at least three independent experiments. Significant changes towards control cells are indicated by asterisks (*** $p < 0.001$).

7.3.1.3 Is there a role for PKCμ in CCR5 induced calcium release?

PKCμ, also named PKD-1 is as an atypical isoform of PKC which was isolated in 1994 and was one of the last PKC isoforms to be discovered (Johannes et al., 1994). Interestingly, the inhibitors Go6976 and Rottlerin can also block PKD-1 (McEneaney et al., 2008). Therefore, we investigated whether the reason why Go6976 and Rottlerin blocked calcium release and GF10923X did not, was due to the ability of the two latter to block PKD-1.

The PKD-1 inhibitor CID755673 is only specific for this isoform at nanomolar concentrations whereas it has been demonstrated to block other PKC isoforms and to have PKD-1 independent effects at micromolar concentrations (Johannes et al., 1994; Torres-Marquez et al.). Consequently, CID755673 was used at nanomolar concentrations to ensure the effects observed were exclusively due to PKD-1 inhibition.

We could demonstrate that blockage of PKD-1 did not affect CCR5 signalling in CHO.CCR5 (Figure 7.9 A) and THP-1 cells (Figure 7.9 B).

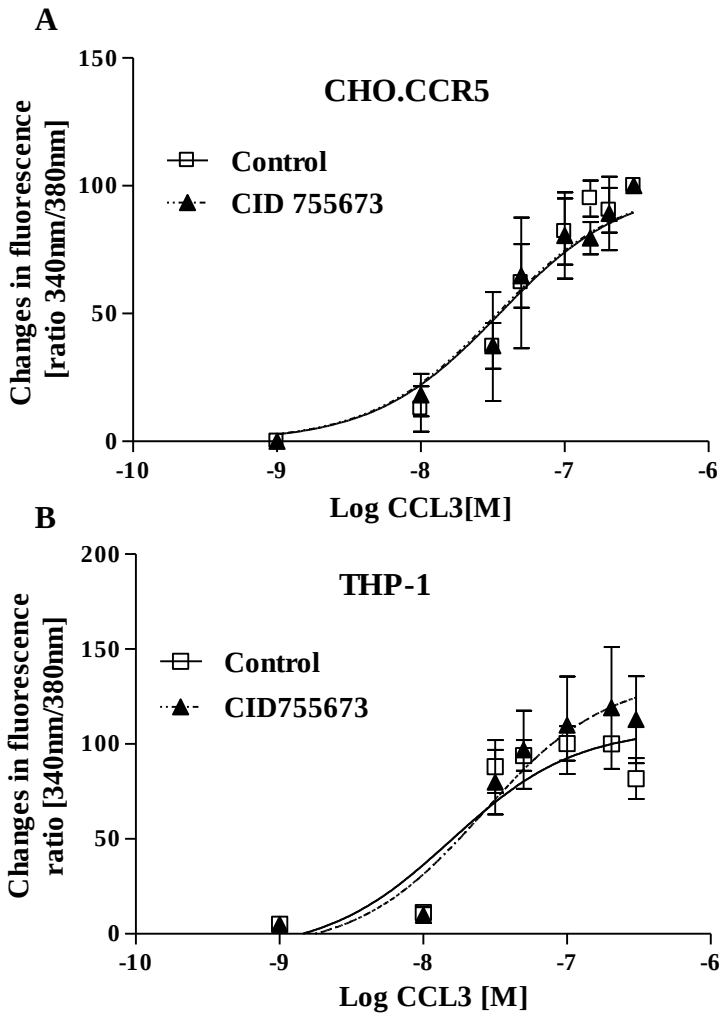


Figure 7.9 Effects of the PKD-1 inhibitor in CCR5-dependent calcium release. CCL3 dose-response curves when cells were treated with the PKD-1 inhibitor CID755673(400 nM) for 30 minutes or left untreated as control in CHO.CCR5 (A) and THP-1 (B) cells respectively. Data represent mean ± SEM from at least three independent experiments.

7.3.2 Are PKC inhibitors affecting ER stores?

The results observed with the classical PKC inhibitor Go6976 and the PKC inhibitor rottlerin had no logical explanation. The fact that a general inhibitor like GF10923X used at a concentration where it blocks all PKC isoforms causes such an increase in CCR5-dependent calcium release meant that some PKC isoforms are involved in desensitizing the receptor or are somehow involved in modulating intracellular pathways leading to calcium release. Since no reports have been found proving the latter and it is known that PKC phosphorylates CCR5, it was hypothesised

that the increase in signalling observed upon GF10923X 5 μM was due to blockage of receptor desensitisation.

It was next intended to understand the reason why treatment of cells with Go6976 and rottlerin could have such a remarkable effect in CCR5 signalling. A possible explanation for this reduction in calcium release had been considered to be a reduction in CCR5 surface expression but it was shown that Go6976 treatment of cells had no effect on it. Another possibility was that these two drugs had an effect on ER stores impairing calcium release in a PKC independent manner. To analyse this last option, cells were treated with Go6976 or rottlerin for 30 minutes and stimulated with thapsigargin, known to empty ER stores through calcium leakage (Thastrup et al., 1990) It was found that Go6976 and rottlerin (see Figure 7.10 and 7.11 respectively) both had the ability to block calcium release induced by the thapsigargin blockage of SERCA. Go6976 was shown to block calcium release induced by TG 1 μM and 1.5 μM in THP-1 cells (Figure 7.10). Likewise, rottlerin inhibited TG-dependent calcium mobilization in HeLa.RC49 cells (Figure 7.11 A and B) and THP-1 cells (Figure 7.11 C and D). This unforeseen effect of Go6976 and rottlerin in ER membrane proteins could easily explain the lack of calcium response triggered by CCL3 in the presence of these drugs. To ascertain that emptying ER stores was responsible for the effects observed, cells were next treated with 5 μM of the general inhibitor GF10923X and stimulated with TG. Interestingly, it could be shown that treatment of cells with this compound did not block TG-induced calcium response, which is in accordance with the increase in calcium release observed in cells treated with this drug and stimulated with CCL3 (Figure 7.12). It is important to note that GF10923X slightly increased calcium release upon chemokine activation which leaves open the possibility that this PKC inhibitor increases calcium release independently of PKC blockage and not due to inhibition of CCR5 desensitisation as has been suggested.

Nevertheless, the increase in the release of calcium observed upon TG injection was not as significant as the increase observed upon CCL3 stimulation which indicates that there must be an additional factor accounting for this increase and this must be CCR5 and PKC dependent.

Taken together the data gathered so far provide some indications that inhibition of certain PKC isoforms could promote a longer activation of heterotrimeric G proteins by

CCR5. However, in this section it has been shown that targeting the PKC isoform γ or PKC classical isoforms with Go6976 did not provide any valuable information due to the PKC independent effects of these inhibitors, which makes it difficult to assess its importance on CCR5-dependent signalling. Consequently, the next set of experiments will focus on analysing the role of the different PKC isoforms on CCR5-dependent cell migration of THP-1 cells. It is expected that cell migration experiments will provide a better understanding of the role PKC plays in CCR5 signalling.

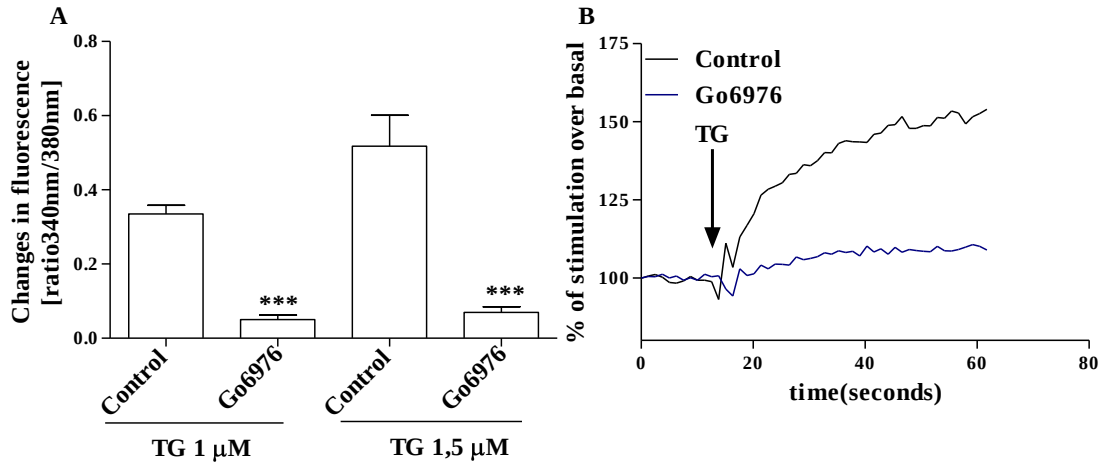


Figure 7.10 Go6976 empties ER stores. THP-1 cells were treated with 100 nM Go6976 for 30 minutes or left untreated (control) and were stimulated with 1 or 1.5 μM of thapsigargin (TG). Figure A illustrates data representative for 3-5 independent experiments and B shows single traces of calcium mobilization. Data represent mean ± SEM from at least three independent experiments. Significant changes towards control cells are indicated by asterisks (*** $p < 0.001$).

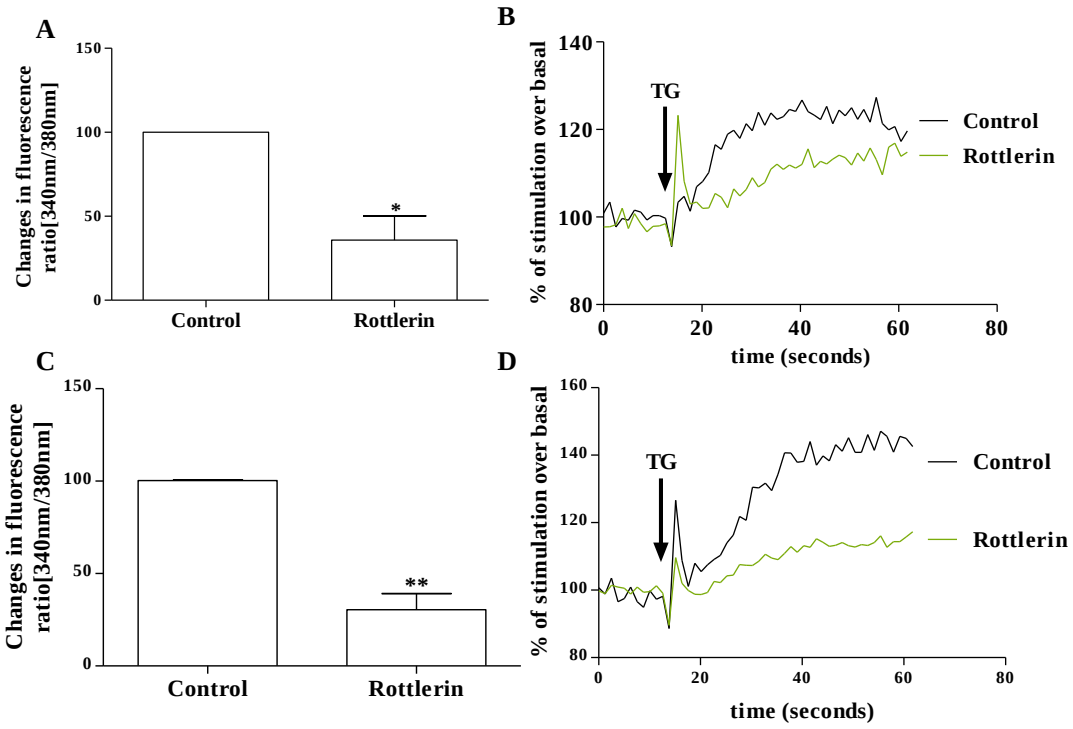


Figure 7.11 Effect of rottlerin on thapsigargin (TG) stimulation of Hela (A and B) and THP-1 cells (C and D). Cells were treated with 4 μM rottlerin for 30 minutes and stimulated with 1μM TG as indicated, and calcium release in rottlerin and control-treated cells was measured. Data represent mean ± SEM. from at least three independent experiments in A and C and single traces for B and D. Significant changes towards control cells are indicated by asterisks (* $p < 0.05$, ** $p < 0.01$).

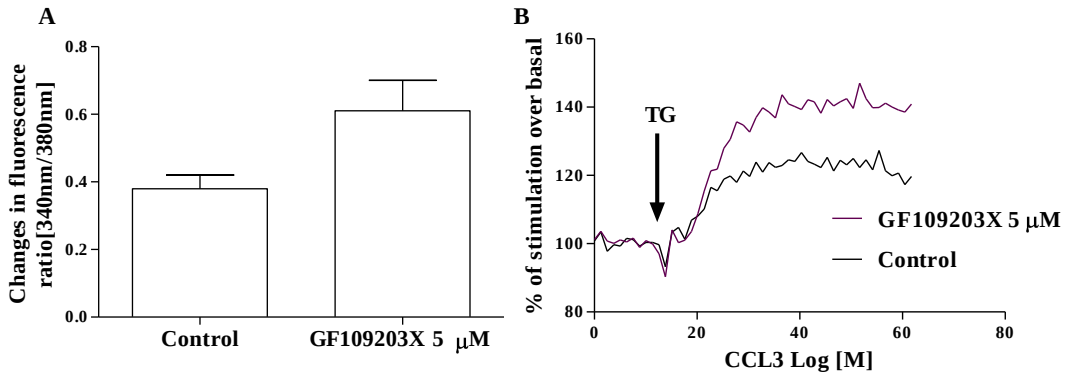


Figure 7.12 The general inhibitor GF109203X does not empty ER stores. A) Shows the effects of thapsigargin on calcium release in HeLa cells pre-treated with GF109203X (5 μM) or vehicle (control) for half an hour. Data represent mean ± SEM from at least three independent experiments in A and single traces in real time for B.

The experiments performed so far indicate that PKC ε, γ or ζ, are the isoforms involved in increasing CCR5 activity as measured by intracellular calcium mobilization. It was next investigated which of these isoforms were expressed in the cell lines used in these experiments to rule out the involvement in CCR5 desensitisation of any PKC isoforms not found. Western blot experiments indicated that PKCα and PKCζ were the most abundant isoforms in HeLa.RC49 and in THP-1 cells (see Figure 7.13). The band for PKCε in THP-1 cells comes up at a lower level than the other isoforms found. There is a possibility that this protein, contrary to other PKC isoforms, gets cleaved or degraded while performing the experiment. HeLa cells seem to express less concentrations of this isoform and in this case, PKC ε seemed to appear at the right level (80 KDa) (Figure 7.13).

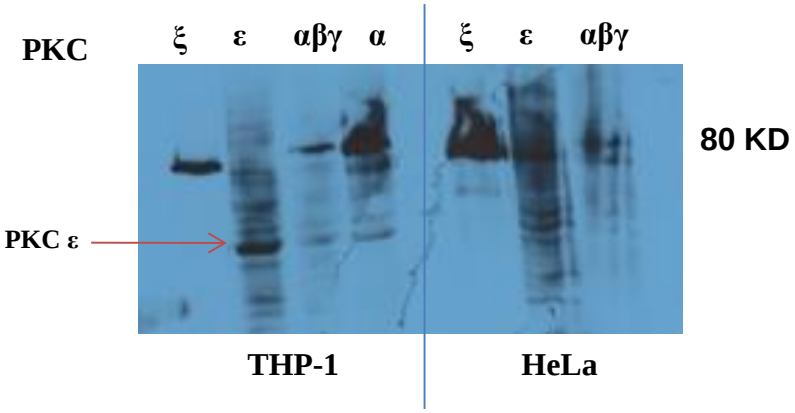
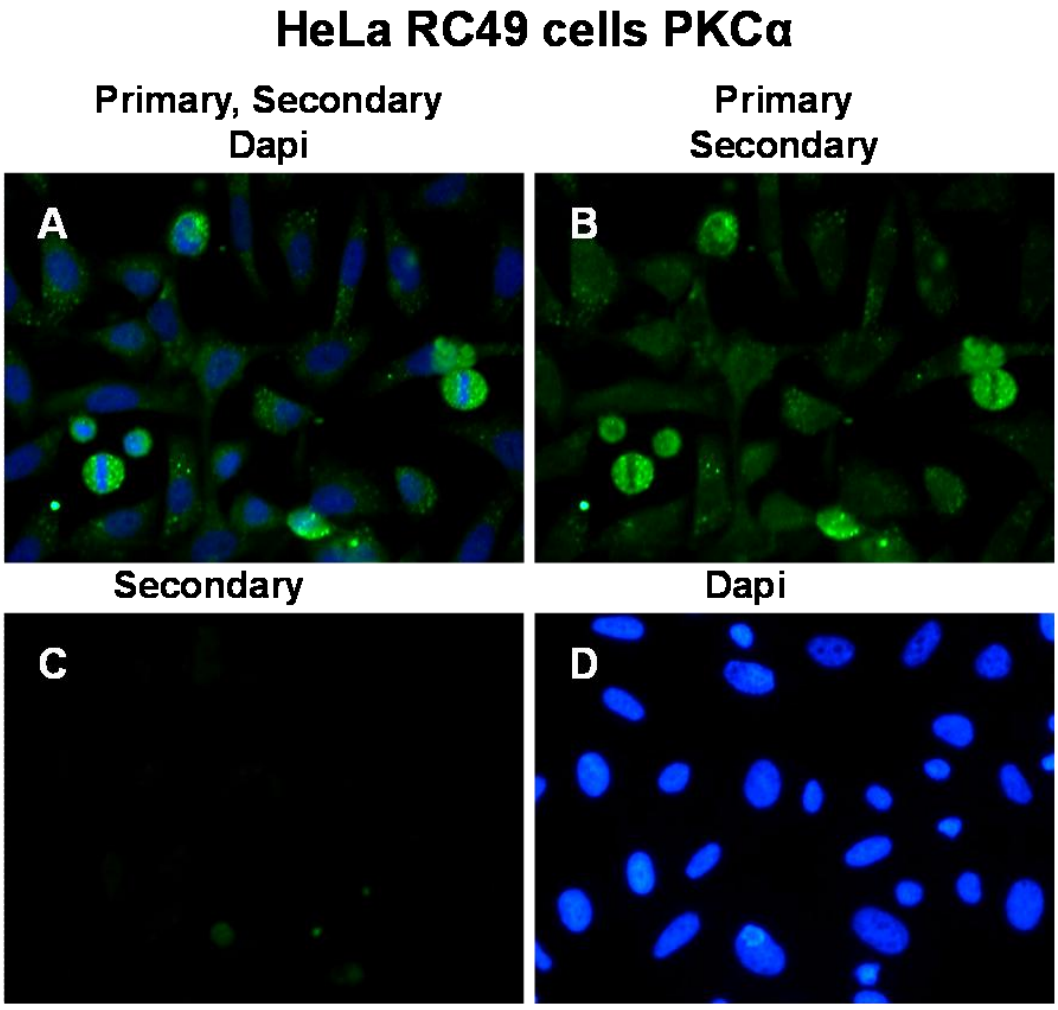
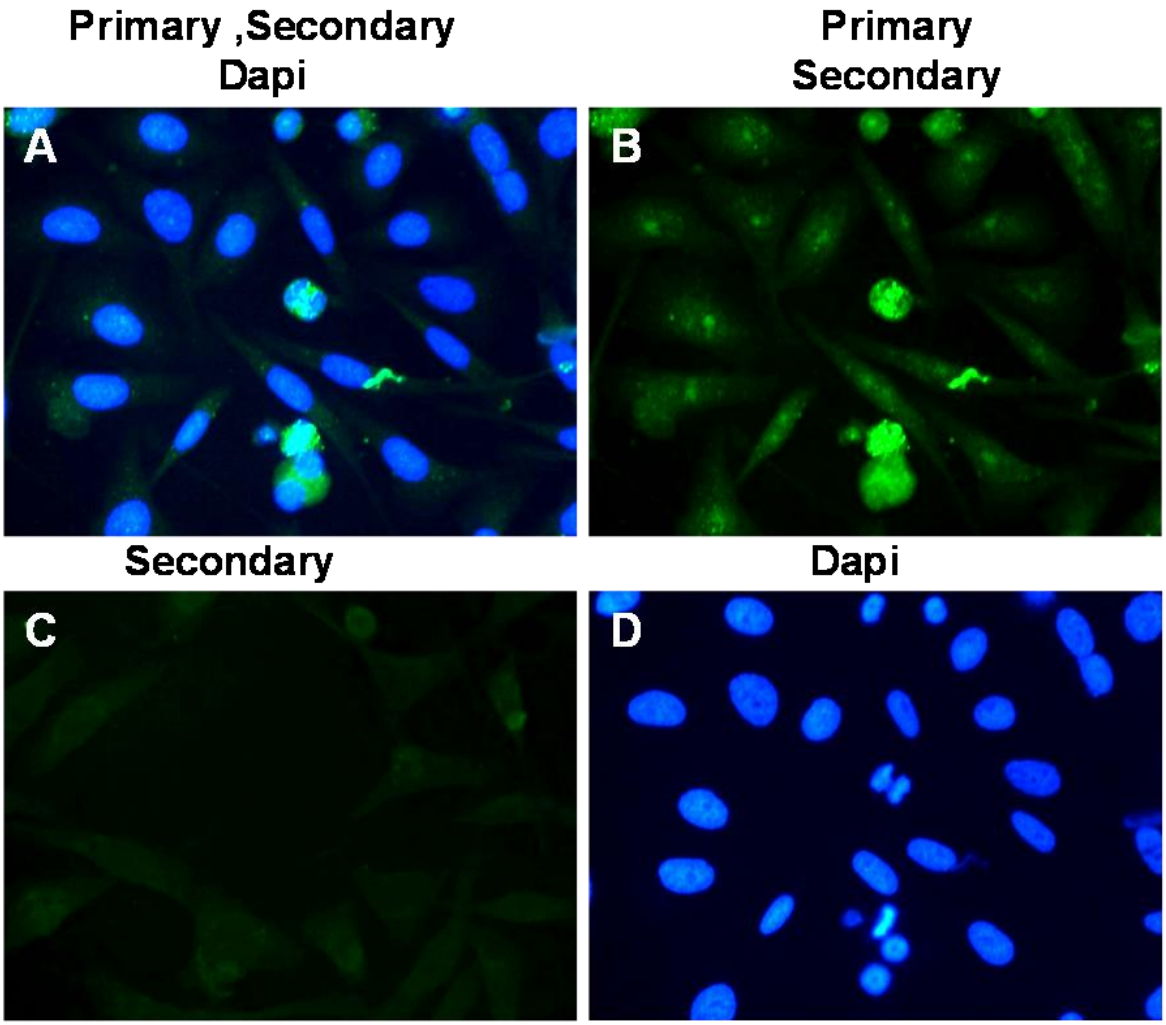


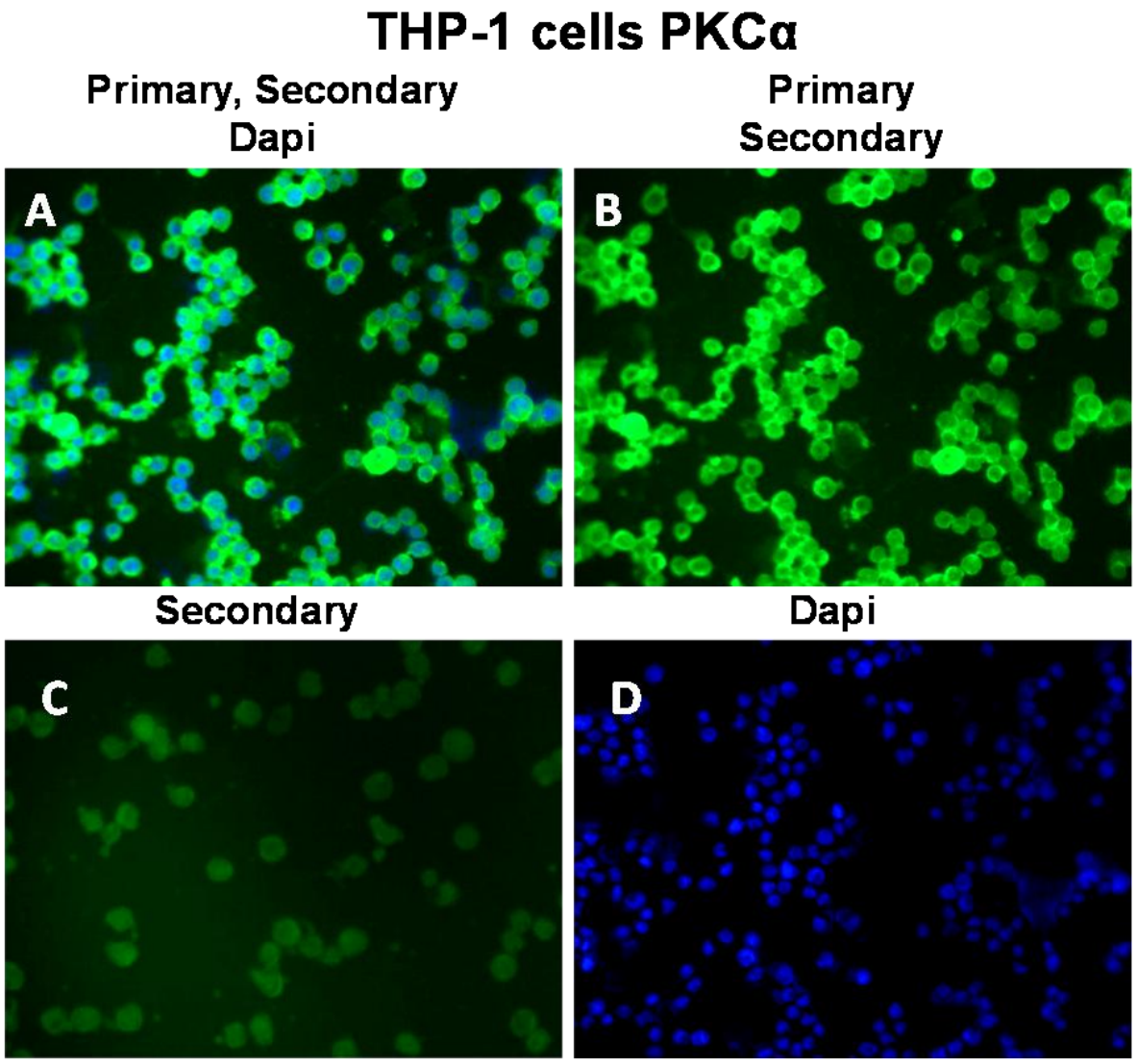
Figure 7.13 PKC isoforms expression in THP-1and HeLa RC49 cells. PKC isoforms were detected by the use of specific antibodies able to specifically recognise PKCζ, PKCε, the PKC isoforms αβγ or PKCα, followed by treatment with a secondary anti-mouse HRP-conjugated antibody. Picture is representative of at least 3 independent experiments.

Immunofluorescence experiments shown in Figure 7.14 illustrate the expression of different PKC isoforms in HeLa.RC49 and THP-1 cells respectively. It can be appreciated that these cell lines express the classical PKC isoform α and the novel PKC isoform ε in the case of HeLa RC49 cells and the classical PKC isoform α, the novel PKC isoform ε and the atypical PKC isoform ζ in the case of THP-1 cells. For unknown reasons PKCζ isoform could not be detected in HeLa.RC49 through immunofluorescence experiments but it was perfectly isolated by western blot experiments.


HeLa RC49 cells PKCα
Primary, Secondary
Dapi
Primary
Secondary
A
B
Secondary
Dapi
C
D

HeLa RC49 cells PKC ε




THP-1 cells PKCα
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Primary
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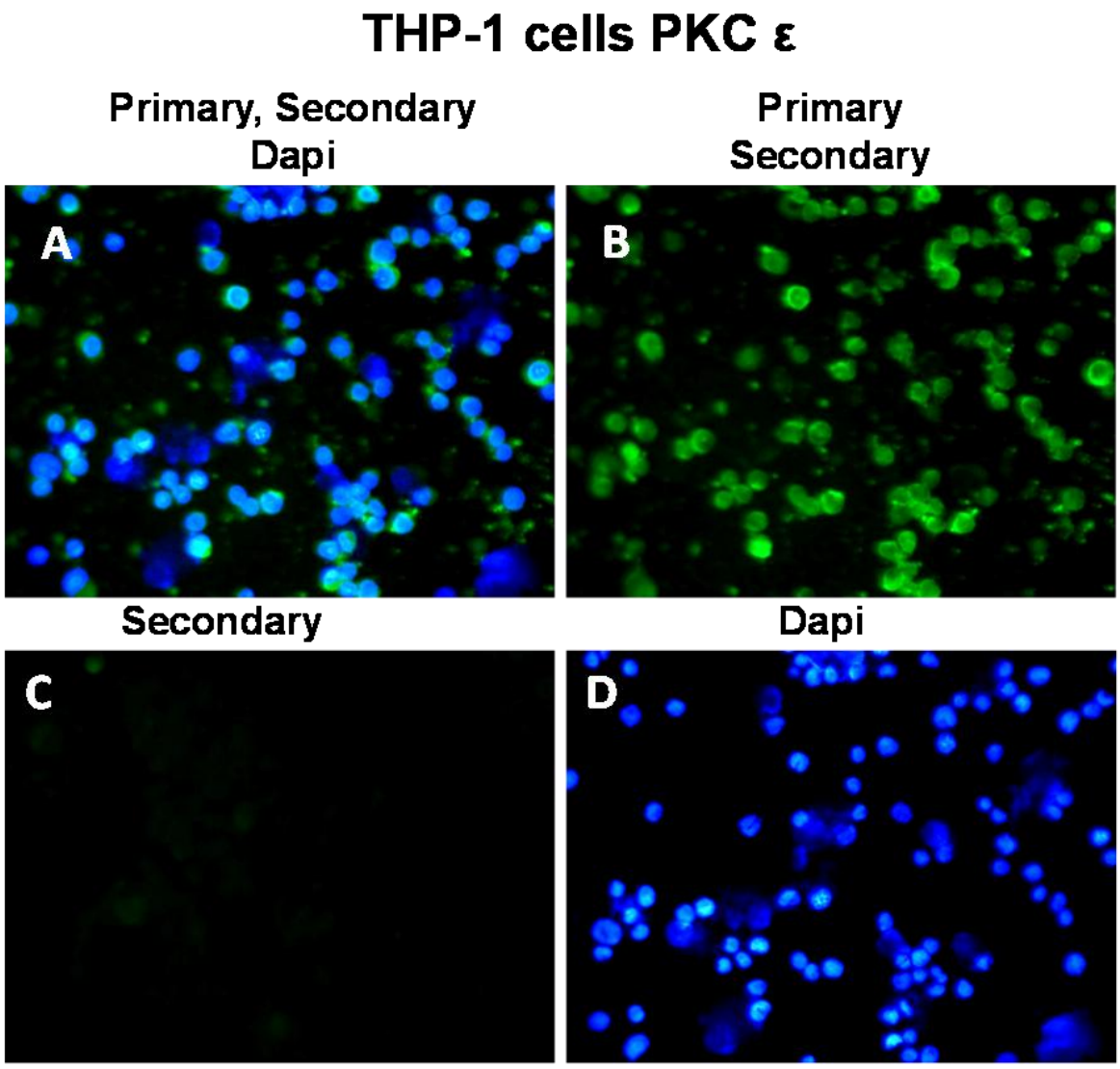
THP-1 cells PKC ε
Primary, Secondary
Dapi
A
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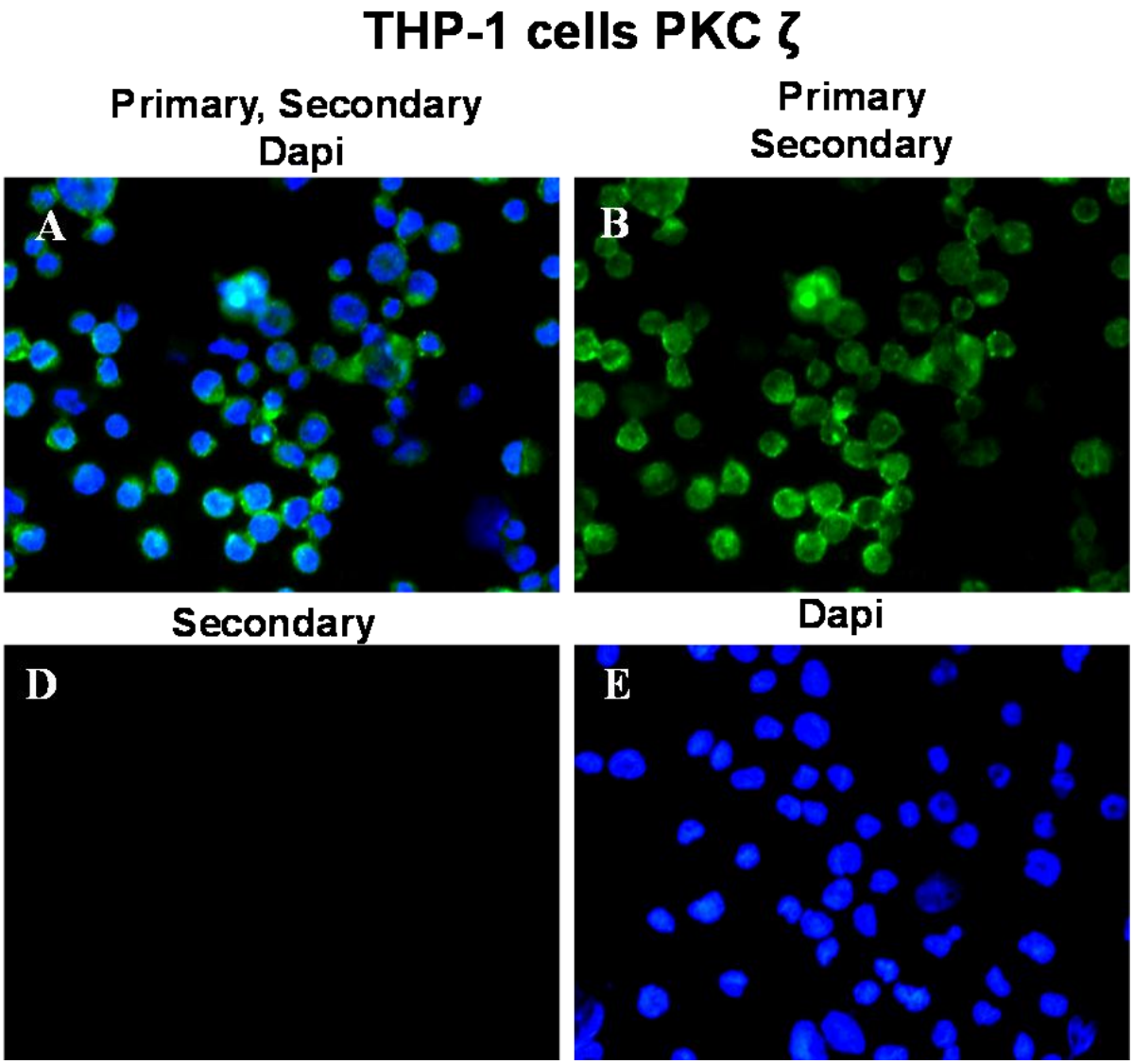

Figure 7.14.PKC isoforms expression in HeLa and THP-1 cells. Pictures illustrate cells treated with primary, secondary antibodies and Dapi, primary and secondary antibodies, only secondary antibody or only Dapi as indicated. Cells were let to dry on coverslips for a few minutes, lysed with acetone, washed and stained for PKCα and PKCε in the case of HeLa.RC49 cells and PKCα, PKCε and PKCζ in the case of THP-1 with specific antibodies for these isoforms followed by treatment with anti-mouse-FITC secondary antibody. Dapi stain was included in the mounting solution.

These data verify that PKC ε, γ or ζ, could potentially be involved in desensitising CCR5 since the three isoforms could be detected in HeLa.RC49 and THP-1 cells.

7.3.3 PKC involvement in chemotaxis: Independence between calcium flux and cell migration events

It has been shown above that Go6976 and rottlerin inhibitors are not a good choice for analysing the effect of PKC blockage in calcium signalling due to their property to deplete intracellular stores. The unexpected effects of these two inhibitors hinder the understanding of the role these PKC isoforms have on calcium signalling. However, the effects observed upon 50nM GF109203X treatment provide enough information about the role of classical isoforms of PKC. Consequently, it seemed clear that another method should be used to know PKC δ involvement in CCR5 signalling. Thereby, chemotaxis assays were next used to test whether any PKC isoforms are capable of regulating THP-1 cells migration. Figure 7.15 and 7.16 show that THP-1 cells migrate towards CCL3 in a concentration dependent manner.

Here it can be observed that pre-incubation of the cells with GF109203X at 50nM does not affect the migration of THP-1 cells (Figure 7.15 A) whereas pre-incubation of cells with GF109203X at 5 μM (Figure 7.15 B) produced a significant increase in the number of cells migrating towards CCL3. These findings are in accordance with the data obtained when analysing calcium mobilization and indicate once more that some non-classical PKC isoforms are involved in desensitising CCR5.

When the effects of Go6976 and rottlerin were studied it was appreciated that rottlerin has no effect on cell migration whereas Go6976 slightly increases chemotaxis (see figure 7.16 A and B respectively). These results are very interesting since they provide good evidence of independence between calcium release and chemotaxis. Go6976 and rottlerin completely empty calcium stores but they do not block cell migration which clearly indicates that chemotaxis processes induced by CCR5 do not require previous calcium fluxes. In order to study this further, the effects of the U73122 inhibitor on THP-1 cells migration was studied. PLC is the enzyme responsible for the formation of IP3 and its blockage is widely known to block calcium release. Here it could be shown that PLC inhibition abrogated cell migration (Figure 7.16 B). The effect of PLC inhibitor would have been attributed to its property to block calcium fluxes.

However, given that it is now known that calcium release and chemotaxis are independent from each other in CCR5 signalling in THP-1 cells, the mechanism through which PLC blockage inhibits cell migration remains completely unknown.

The last experiment performed in this chapter, once more indicates that CCR5 is likely to be dependent on PKC to interrupt its coupling to heterotrimeric G proteins.

Figure 7.17 shows that PKC activation with PMA significantly blocks cell migration which is in accordance with the theory that over stimulation of this enzyme would promote CCR5 internalisation and, therefore, decrease its intracellular signalling responses.

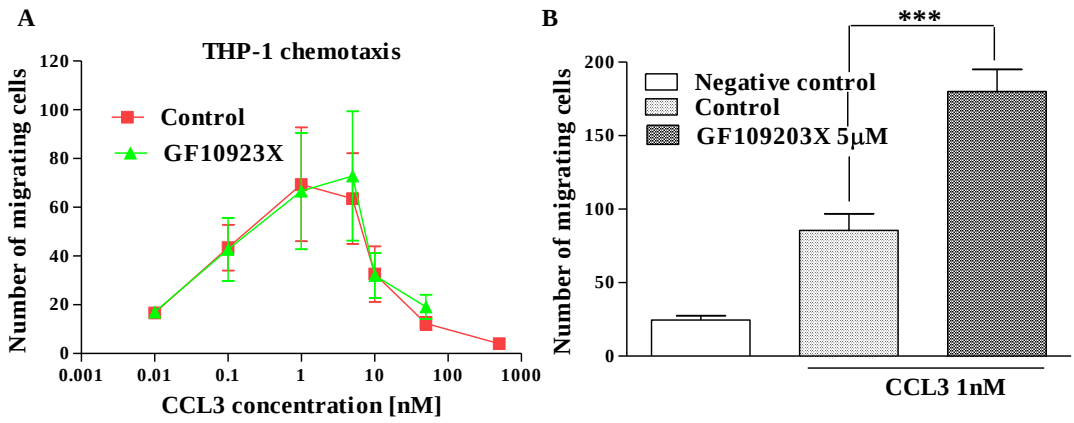

Figure 7.15 Chemokine-induced chemotaxis of THP-1 cells is increased by inhibition of all PKC isoforms but not affected by inhibition of classical PKC isoforms. A) THP-1 cells were assayed for chemotaxis in the absence (basal) or presence of CCL3 at different concentrations and with or without pretreatment with GF109203X (50 nM). B) Shows bar charts were cells were treated with 5µM GF109203X or left untreated, stimulated with 1 nM CCL3 or left un-stimulated. Data represent mean ± S.E.M. of duplicate determinations from three independent experiments. Significant changes towards control cells are indicated by asterisks (***$p < 0.001$).

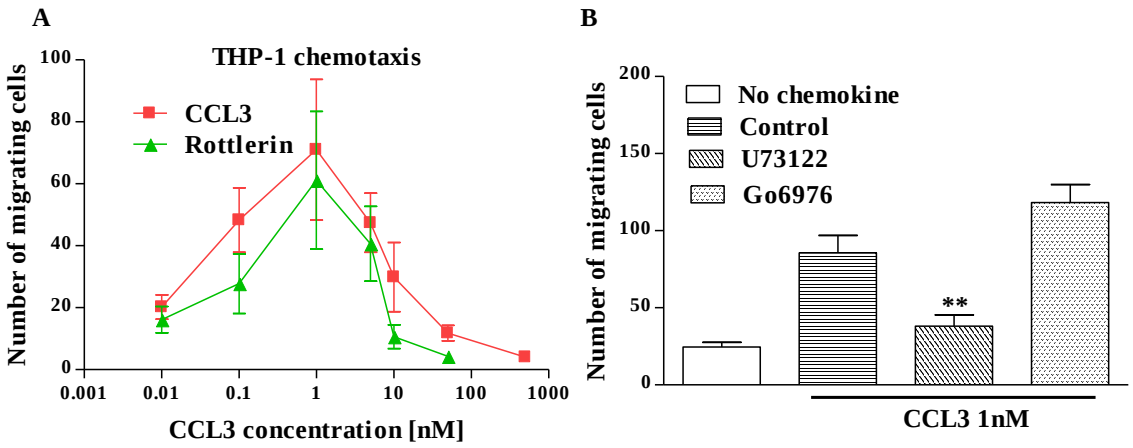

Figure 7.16 Chemokine-induced chemotaxis of THP-1 cells is not affected by treatment with rottlerin. A) THP-1 cells were assayed for chemotaxis in the absence (basal) or presence of CCL3 at different concentrations and with or without pre-treatment with rottlerin. B) THP-1 cells migration towards 1 nM CCL3 in the presence of the inhibitors Go6967 (100 nM) and U73122 (10 µM) for 30 minutes. Data represent mean ± S.E.M. of duplicate determinations from three independent experiments. Significant changes towards control cells are indicated by asterisks (** $p < 0.01$).

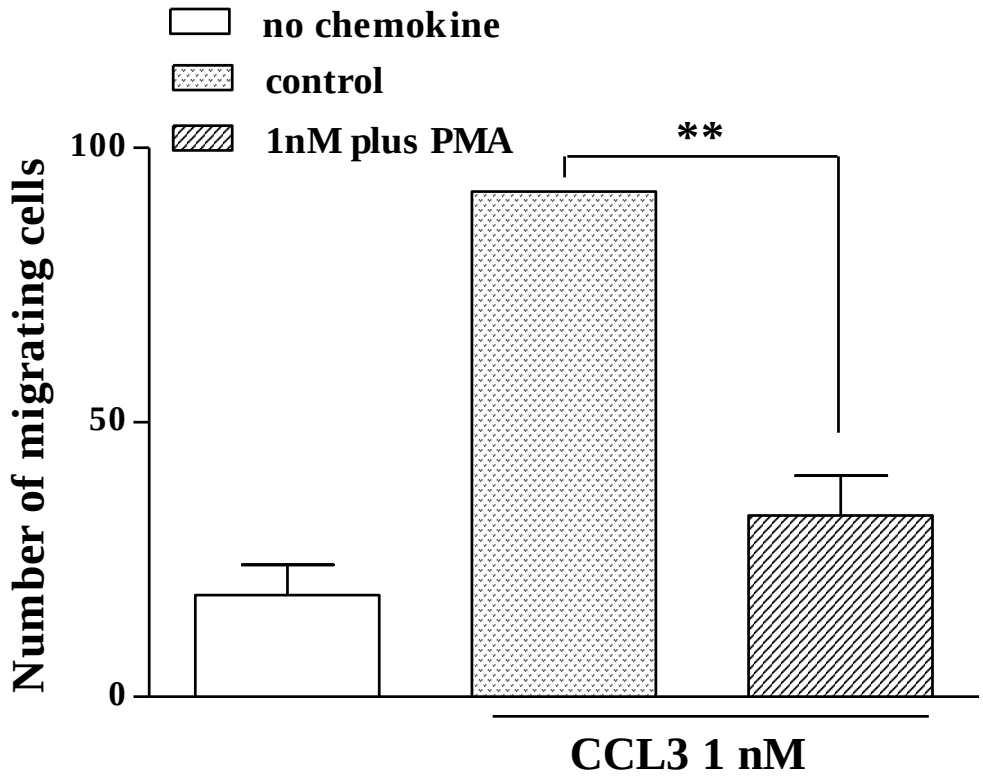

Figure 7.17 Chemokine-induced chemotaxis of THP-1 cells is blocked by a PKC activator. A) THP-1 cells were assayed for chemotaxis in the absence (basal) or presence of 1 nM CCL3 and with or without pre-treatment with PMA (100 Nm). Data represent mean ± S.E.M. of duplicate determinations from three independent experiments. Significant changes towards control cells are indicated by asterisks (**$p < 0.01$).

7.4 Discussion

After stimulation by a ligand, CCR5 gets desensitized thorough a process that starts with phosphorylation of its C terminus by PKCs and GRKs. PKC has been shown to play an essential role in cancer cells and in the last few years different PKC inhibitors have passed the first tests to become anticancer drugs. We have hypothesized that since PKC is involved in promoting CCR5 internalisation, PKC inhibitors might increase the time CCR5 is in contact with G proteins, therefore, increasing receptor signalling, which could have negative effects in pathologies where CCR5 signalling is considered to have negative effects.

Therefore, the main purpose of this chapter was to identify whether some of the PKC inhibitors currently under clinical investigation could have any stimulatory effects on CCR5 signalling.

We have shown that cells treatment with the general PKC inhibitor GF10923X at high concentrations causes a significant increase in calcium mobilization from ER stores. Also, we have shown that this increase could be reduced with the specific CCR5 inhibitor maraviroc, indicating that GF10923X's effect is directly related to an excess of CCR5 signalling. Interestingly, the same inhibitor used at concentrations where it blocked PKC α and β had no effect on the release of calcium induced by CCR5 stimulation.

When other PKC isoforms were investigated, it was found that the PKC inhibitors Go6976 and rottlerin, inhibitors of cPKC and PKCγ respectively, highly reduced calcium release upon CCL3 stimulation without altering CCR5 expression levels. However, we could show that these two inhibitors had the extra effect of depleting ER stores. Unfortunately, this meant that this approach was not adequate to measure if calcium release was affected by CCR5 desensitisation via these PKC isoforms.

The effect of PKD-1 on CCR5 signalling was also investigated in case this enzyme was responsible for CCR5 desensitisation.

Nevertheless, treatment of cells with the specific PKD-1 inhibitor CID755673 showed no alteration of calcium fluxes in any of the cell lines studied.
Altogether these results point at PKC ε, γ or ζ, as the isoforms involved in increasing

CCR5 activity as measured by intracellular calcium mobilization.

The use of PKC inhibitors to measure CCR5 activation through the release of calcium was shown to be inadequate due to the side-effects of some of the PKC inhibitors. Nevertheless, it seemed clear that inhibition of atypical or novel PKC isoforms could be responsible for enhancement of CCR5 activation. Hence, a different way of measuring receptor activity upon PKC inhibition was next employed.

When chemotaxis assays were used to complement the data obtained with calcium signalling, it was shown that similarly to them, general blockage of all PKC isoforms with GF10923X caused an enhancement of cell migration. On the other hand, blockage of cPKC isoforms with low concentrations of GF10923X or of PKC γ with rottlerin had no effect on chemotaxis which suggests that CCR5 desensitisation is triggered by PKC ε or ζ.

It is not the first time the theory that inhibiting PKC can have stimulatory effects in receptors signalling has been studied. In 2007 Deshpande et al. demonstrated that PKC inhibition enhanced signalling pathways such as calcium flux, contraction of airway smooth muscle or cell migration and chemokine production in monocytic cells in response to Leucotriene D4 stimulation of cysteinyl leukotriene type 1 receptor (CysLT1R) (Deshpande et al., 2007). In 2004 Vroom et al. (Vroon et al., 2004) found that in T cells from mice that were heterozygous for deletion of the GRK2 gene (GRK2+/-mice), CCR5 signalling was highly increased. This group had previously shown that in patients with RA the levels of GRK2 were reduced by 50% and thereby they suggest that a reduction of GRK2 might be one of the main causes why CCR5 is a key element in the inflammation that accompanies or causes this inflammatory disease. Additionally, it has been proposed that in RA patients the levels of PKC ζ and ε were reduced as compared to controls (Zini et al., 2008). Interestingly, it was also shown that PKC ζ isoform was down-regulated when cells were treated with inflammatory cytokines like TNF-α or IL-1β, which provides more evidence that in inflammatory conditions, the fact that some PKC isoforms get down-regulated worsens the circumstances. Consequently, taking into account that GRK and PKC have a similar role in desensitizing CCR5, it could be expected that a reduction in PKC activity affects CCR5 signalling in a similar way to down regulation of GRK2. In the experiments here performed it has been demonstrated that inhibition of PKC ζ and/or ε is responsible for

an increase in calcium flux and chemotaxis upon receptor stimulation being thus hypothesised that CCR5 over-activity due to a reduction in PKC ζ and ε could have a key role in RA and be of vital relevance for numerous autoimmune and inflammatory diseases.

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. Numerous studies have shown that ligand binding to CCR5 results in cell movement in a mechanism dependent on actin mobilization and PI3K recruitment to the leading edge of cells (Cheung et al., 2009; Gomez-Mouton et al., 2004). However, the 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 (MM-9). This group strongly prove that the axis CCL5/CCR5 is responsible for cell migration since the use of siRNA against CCR5 highly reduced chemotaxis. Additionally, they could prove that cell migration increased in a CCL5 concentration dependent manner which once more highlights the key role of CCR5 in cell migration of oral cancer cells. In their experiments, treatment of cells with general PKC inhibitors such as GF109203X as well as an inhibitor specific for PKCγ, resulted in a decrease of cell migration, giving this isoform a special role in CCR5- chemotaxis pathway (Chuang et al., 2009). Also, a study performed on HIV-infected macrophages demonstrated that viral-induced cell migration through CCR5 binding could be blocked by PKC inhibition (Kanmogne et al., 2007). 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 (Haughian et al., 2009). Likewise, two nteresting papers reported that in T lymphoblastoid leukaemia cells and eosinophils, (Alfano and Poli, 2001; Cronshaw et al., 2006), chemotaxis was blocked with rottlerin, indicating that PKC γ was needed for cell migration.

Contrary to these reports, in this chapter we have shown that inhibition of PKC ε and ζ causes increases in cell migration whereas blockage of the other isoforms of the enzyme has no effects in CCR5 induced chemotaxis.

Thus, it is reasoned that enhancement of cell migration is due to blockage of receptor desensitisation highlighting that in this signalling system, PKC is not likely to be one of the G-proteins downstream effectors required for cell migration.

The importance of these findings depends on whether CCR5 requires PKC activity to transduce cell migration or not. If the use of PKC inhibitors could stimulate cell migration through an increase in CCR5 signalling but at the same time this migration process was impaired by the inhibition of PKC-dependent intracellular cascades, the outcome would not signify an enhancement in cell migration and the use of PKC inhibitors would thus represent a possible new therapeutic area. However, it appears that PKC isoforms are not required for CCR5-induced cell migration and, therefore, the blockage of the isoforms responsible for CCR5 desensitisation increases cell migration due to a stronger CCR5 activation.

The content of this chapter is significant as this might be the first study showing that Go6976 and rottlerin have a PKC independent effect responsible for a reduction in calcium signalling due to ER stores depletion. Bearing in mind the high interest that is being put on these molecules due to PKC indisputable role in certain cancers, the fact they can alter signal transduction pathways through calcium modification should be taken into consideration. Besides, researchers have widely used these compounds when studying the role of PKC in numerous signalling pathways without considering the PKC-independent effect described in this work. This could lead to incorrect results where the real cause for the action observed could be ER stores calcium depletion and not PKC inhibition. These unexpected data have also provided evidence of an important matter, the non-relation between calcium mobilization from the ER and the chemotaxis process. To date, there is no information (excluding our results presented in Chapter 4) about the connection between calcium release and chemotaxis for CCR5. There are a few examples in the literature on this subject for other GPCR. For instance, chemotaxis responses of T cells were impaired in PLC knock-out mice when compared with wild-type mice (Bach et al., 2007). Also, when these experiments were repeated and calcium was chelated using a pharmacological approach, chemotaxis was blocked resembling the results obtained in PLC knock-out mice which indicates that calcium release is needed for T cell migration. However, a different study (Cronshaw et al., 2006) demonstrated that in the CEM leukemic T cell line and human Th2 cells, chemotaxis was dependent on PLC but not on calcium release from ER stores. Furthermore, a different group

stablished that PLC was not needed for cell migration of neutrophils (Murphy et al., 2000). Interestingly, we have now demonstrated that the role for PLC in chemotaxis is not linked with a need for calcium release since although PLC was required for cell migration, calcium mobilization was not. Hence, it could be stated that there is not a rule for the link between calcium release and chemotaxis among different cell systems or different GPCR. It is possible that second messengers produced by PLC play a key role in cell migration in certain cell types but not in others.

To conclude, at the beginning of this work it was hypothesised that an increase in CCR5 signalling caused by PKC inhibition could be dangerous especially due to a possible increase in cell migration which could potentially contribute to worsening pathological processes such as RA, MS or cancer. Accordingly, it has been shown that inhibition of certain isoforms of PKC, most likely PKC ε or ζ, stimulates CCR5-mediated calcium release and cell migration of THP-1 cells.

CHAPTER 8- FINAL DISCUSSION

In this thesis we have characterised six key points regarding CCR5 signalling pattern:

I. CCR5 signalling has different dependence on cholesterol in CCR5 stably transfected cells and in the monocytic cell line THP-1. Whereas cholesterol depletion with MCD abrogates CCR5 signalling in the former, it causes a massive enhancement of intracellular calcium mobilization in the latter.
II. Cholesterol depletion promotes the signalling of CCR5 through a PTX-resistant G protein.
III. In CCR5 transfected cells, caveolae integrity is not required for CCR5 signalling.
IV. Calcium signalling and chemotaxis processes stimulated by CCR5 in THP-1 cells are independent from each other.
V. CCR5 might initiate cADPR-dependent calcium signalling pathways
VI. Inhibiting PKC ε and ζ increases CCR5 signalling: role for these PKC isoforms on CCR5 phosphorylation?

This report has focussed on understanding the mechanisms activated by the binding of a chemokine to CCR5. It is accepted that CCR5 is coupled to $G\alpha_i$ proteins and that CCR5 activation induces calcium release responses through $G\beta\gamma$-dependent activation of PLC. The $G\alpha_i$ subunit is known to inhibit the enzyme AC and, therefore, to cause inhibition of cAMP accumulation. All these processes have been demonstrated in the present study. It has also been shown that CCR5's ability to transduce intracellular signals is not exclusively due to its coupling to $G\alpha_i$ proteins since MCD treatment of all cell types studied promoted the coupling of the receptor to a PTX-independent G protein. These results are in accordance with those performed by Mueller et al. where they confirmed association of CCR5 with $G\alpha_q$ (Mueller and Strange, 2004a) and with studies showing that CCR5-CCR2 heterodimers signal through $G\alpha_q$ (Mellado et al., 2001). The use of MCD to disrupt lipid rafts in CCR5 transfected cells demonstrated that these cells require membrane cholesterol to produce calcium signalling through CCR5. However, we could confirm that caveolae integrity was not essential for CCR5 dependent calcium signalling as shown by the fact that Caveolin-1 siRNA did not affect

CCR5´s ability to evoke calcium fluxes. This hypothesis was confirmed by the findings that filipin, which we could show to disrupt Caveolin-1 expression in the membrane, did not affect calcium signalling initiated by CCR5.

This work has also revealed interesting data regarding the different effects that the cholesterol depleting drug, MCD, and cholesterol inhibiting drugs, statins, have on CCR5 signalling. While MCD and statins, both reduce CCR5-induced signalling pathways in stably transfected cells, it has been shown that MCD highly increases calcium release in THP-1 cells whereas statins block this signalling. Furthermore, it has been demonstrated that both treatments lead to a reduction of cellular cholesterol and, therefore, we hypothesise that the differences observed between them is necessarily due to a pleiotropic effect of one or both of these drugs. When cholesterol was loaded back to MCD-treated cells it could be observed that CCR5-induced increase in calcium release was returned back to normal. These data indicate that statins might have an extra effect independent of cholesterol reduction which causes inhibition of CCR5 signalling. Indeed, it was shown that statins could reduce CCR5 membrane expression which would, along with other possible effects such as inhibition of G βγ prenylation, account for the reduction in calcium responses observed. Altogether these data provide evidence that support the idea that the anti-inflammatory properties of statins are not exclusively related to cholesterol depletion.

We have been taken by surprise by the fact that THP-1 cells lacking cellular cholesterol are able to increase calcium signalling upon CCR5 stimulation dramatically. This increase in calcium release has been related to the capacity of MCD to alter the conformation of ER and acidic vesicles calcium regulating proteins. Additionally, the CCR5 enhanced calcium signalling has been demonstrated to be dependent on proteins that act downstream of CCR5 such as PI3K, PLC or IP3R, clearly linking the improved calcium responses observed in a cholesterol depleted system with CCR5 activation. However, although some hypothesis have been made in the course of the thesis, the mechanisms behind THP-1 cells behaviour upon CCR5 activation in the absence of cellular cholesterol are far from being understood. The properties of CCR5 calcium responses were looked at in more detail by analysing the role of RyR on CCR5-induced calcium signal transduction. Interestingly, we found that stimulation of RyR with caffeine and ryanodine highly stimulated CCR5 signalling in CCR5 stably transfected cells and especially in THP-1 cholesterol depleted cells. Although there is a possibility

that changing the open state of RyR could stimulate IP3R probably due to an increase in the amount of cytosolic calcium in the proximities of IP3R, we guess that this enhancement in signalling is more likely to be due to the uncharacterised ability of CCR5 to promote the formation of the RyR agonist, cADPR from the intracellular messenger NAD^{+}. The ability of RANTES to induce calcium signalling in a mechanism involving cADRP has been demonstrated before (Partida-Sanchez et al., 2004; Shideman et al., 2006). We suggest that CCR5 dependent calcium signalling may be dependent not only on the known pathway requiring IP3 formation through the action of PLC but also on the generation of the second messenger cADPR through a pathway still not characterised.

In our opinion, a necessary next step in this area would be to understand what other intracellular pathways are stimulated by cholesterol depletion due to the increase of calcium mobilization to the cytosol. Since CCR5-induced calcium release could be implicated in many immune responses, including secretion (Logan et al., 2003) and gene expression (Crabtree and Olson, 2002), a perfect understanding of these signalling mechanisms in monocytes lacking a big percentage of cholesterol, might represent an interesting and unexplored research area. One of the main concerns upon an excessive signalling of certain chemokine receptors would be an excessive migration towards a chemokine gradient which could worsen chemokine-receptors-associated pathologies. For instance, CCR5 expression has been linked with an excess of cell migration in some cancerous processes like breast cancer, prostate cancer or colon cancer, as we have previously explained. Likewise, CCR5-dependent cell migration has been confirmed to play a role in AD and other neurological pathologies as well as in RA. Therefore, in the present work it was studied whether the increase observed in calcium release upon CCR5 stimulation in cholesterol depleted THP-1 cells also implicated an excess in chemotactic processes. Gomez-Moutons' group showed that, during chemotaxis, CCR5 localised to the leading edge of lipid rafts along with PI3K and that these processes were abrogated by cholesterol depletion with MCD (Gomez-Mouton et al., 2004). In accordance with these results, we have shown that THP-1 cells treatment with MCD impeded cell migration towards different concentrations of CCL3, being to our knowledge the first time that independence of calcium release and chemotactic processes have been discovered for CCR5. It appears that increasing THP-1 cell's membrane fluidity causes a drastic increase in the amount of calcium released to the cytosol and a clear reduction in cell migration upon a CCL3 gradient. Independence between calcium

release and chemotaxis has also been reported as a result of experiments performed with PKC inhibitors. We could demonstrate that even if the PKC inhibitors Go6976 and rottlerin depleted ER stores abrogating calcium fluxes in a PKC-independent manner, when these inhibitors were used to study chemotactic processes, these were significantly enhanced. These findings, along with the ones previously explained, clearly discriminate between calcium mobilization and chemotactic responses initiated by CCR5.

Chemokine receptors stimulation with one single chemokine can trigger numerous signalling pathways. For instance, CCL3 stimulation of CCR5 can produce calcium fluxes (Cardaba et al., 2008; Cardaba and Mueller, 2009), inhibition of cAMP accumulation (Cardaba et al., 2008), MAPK and FAK stimulation (Ganju et al., 1998) as well as cell migration (Desmetz et al., 2007; Gomez-Mouton et al., 2004). Two different studies showed that CCR5 stimulation could lead to $G\alpha_i$-dependent calcium release and $G\alpha_i$–independent Janus kinase 2 (JAK2) stimulation, giving some insights into how a single chemokine can activate two different pathways simultaneously (Mueller and Strange, 2004a; Wong et al., 2001). Likewise it is possible that, as we have observed and has been previously suggested (Maghazachi and Al-Aoukaty, 1998), CCR5 activates calcium release and chemotaxis through two independent pathways.

Another interesting research area that has been approached in this thesis relates to the study of PKC inhibitors as potential future anticancerous agents. The proof that PKC-related pathways were over-stimulated in several cancers made researchers study the possibility of targeting PKC to develop new therapies. In this work we hypothesise that since PKC was involved in CCR5 desensitisation, its blockage may increase some of the signalling pathways activated by CCR5. We have shown that, according to our hypothesis, general blockage of PKC isoforms significantly increases CCR5 calcium responses and that PKC ε and ζ inhibition caused an increase in the amount of cells migrating towards CCL3 which suggests, for the first time, a role in CCR5 phosphorylation for these two PKC isoforms. Further work should be able to confirm whether CCR5 is directly phosphorylated by PKC ε and ζ upon ligand stimulation. We report that blockage of PKC ε and ζ may aggravate conditions where CCR5-induced signalling has a negative role due to an increase in CCR5 calcium responses and cell migration.

One of the main points that has been tried to put forward in this research has been that CCR5 overstimulation or increase in signalling could be detrimental in certain conditions. It is well known that CCR5 stimulation can activate MAPKs and Jak-STAT pathways (Mueller and Strange, 2004b; Paruch et al., 2007; Popik and Pitha, 1998) which can lead to cell proliferation and to the secretion of pro-inflammatory cytokines like TNF-α and IL-1β (Sun et al., 2009). We conclude that an excessive signalling through CCR5 could have negative repercussions due to its pro-inflammatory actions. Furthermore, CCR5 interaction with CCL5 has shown antiapoptotic properties in mouse Macrophages (Tyner et al., 2005) and CCR5 signalling is clearly linked to an increase of chemotaxis through PI3K (Gomez-Mouton et al., 2004), what can lead to pathological cell migration as previously mentioned.

On the other hand it is also essential to highlight that CCR5 signalling reduction through statins treatment or through cholesterol depletion in CCR5 stably transfected cells, in the case of calcium release, or THP-1 cells, in the case of chemotaxis, might have positive effects in diseases where an excess on CCR5 signalling may be detrimental. There is a possibility that statins anti-inflammatory effects, which are currently under investigation for the treatment of patients with RA (Full and Monaco, 2010; Steffens and Mach, 2004), are partly due to a reduction in CCR5 signalling.

To sum up, this study has characterised in depth some of the intracellular cascades initiated by CCR5. It has also provided new data regarding how the ability of CCR5 to stimulate important cellular responses can be modulated by different treatments. Interestingly, we have shown that some of the current or future therapies may strongly influence the properties of this chemokine receptor and have recommended further investigations in the area in order to ascertain their save use in some pathological conditions.

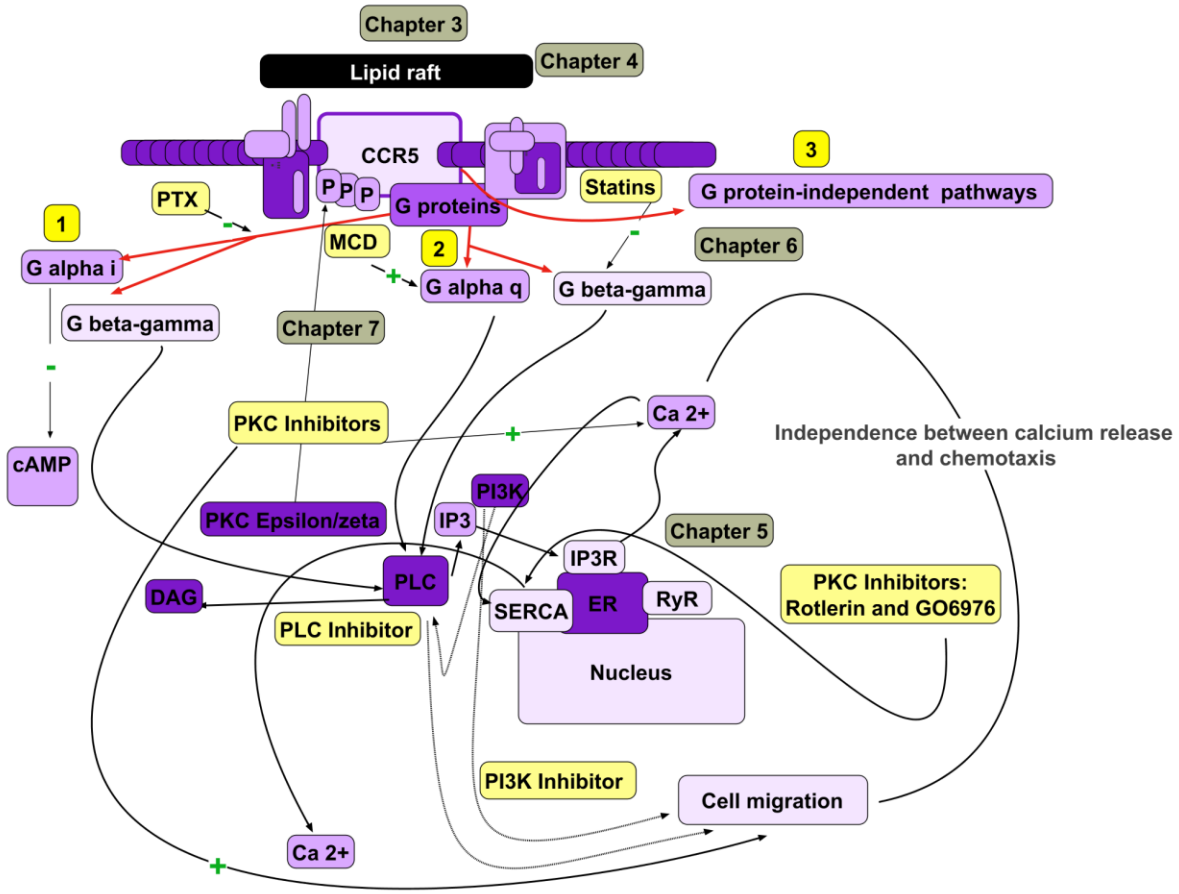

Figure 8.1 Diagram showing some the main pathways suggested to be activated upon CCR5 stimulation in this thesis. Thick lines point towards signalling mechanisms that are widely known to occur whereas thinner lines designate important processes that have been demonstrated to take place in this work. Numbers in yellow circles and red arrows refer to the 3 possible pathways that CCR5 stimulation could activate to generate the intracellular responses observed in this work. Further research should be done to better understand these signalling mechanisms.

8.1 FUTURE DIRECTIONS

This thesis has focussed on analysing the signalling mechanisms of CCR5. Different hypothesis have been made but many of these theories could be confirmed if further experiments were performed. The next few lines reefer to the work that could be done to better understand the mechanisms here proposed.

1. It has been proposed that MCD induces the coupling of CCR5 to PTX-resistant G proteins. However, this idea should be further analysed in order to exclude that other G protein-independent mechanisms are involved. Experiments where G αq/12-13 isoforms are blocked using siRNA technology, dominant negative forms of this protein, or targeting the RGS domain of G $\alpha_{q/12-13}$ with the RGS domain of GRK2, which specifically interacts with Gαq family members, will allow elucidation of whether a PTX-resistant G protein is responsible for the signalling observed in MCD-treated cells.

2. The effect of statins and Caveolin-1 siRNA on the association of CCR5 with G proteins should also be studied in more detail. Statins and Caveolin-1 knockdown are known to affect the composition of the plasma membrane and more specifically, that of lipid rafts. Therefore, understanding if, similarly to MCD treatment, they affect CCR5-Gαi coupling, is a pending task. The use of PTX in statins-treated and Caveolin-1 siRNA transfected cells will allow the characterization of these processes. In this case also, studying CCR5 activation of other signalling pathways which do not require G proteins signalling is highly appealing.

3. It seems clear that the role of the different PKC isoforms on CCR5 signalling should be investigated further. Experiments using phosphosite-specific antibodies in PKC ε and ζ knockdown cells would provide valuable information about the role of these isoforms on CCR5 phosphorylation. Furthermore, the use of siRNA to knock down PKC ε and ζ should be used to measure the effect on calcium release and cell migration upon chemokine stimulation. These two experiments, in combination with the data exposed in this work should give a clear indication of the importance of PKC ε and ζ on CCR5 desensitization and signalling.

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