Calcium Signalling in Mast Cells

Abdullah Alswied

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Dedication

This thesis is lovingly dedicated to my wife, Arwa Alhazmi, for her support, encouragement, and constant love.

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Abstract

Mast cells play a central role in many allergic and inflammatory conditions. These cells are activated following an intracellular rise in calcium, such as that which occurs after the activation of cell-surface receptors. One such important receptor is cysteinyl leukotriene (CysLT) receptor type 1 (CysLT1), which is activated by lipid mediators such as CysLTs LTC_4, LTD_4, and LTE_4. CysLT1 stimulation leads to the hydrolysis of membrane phospholipids such as phosphatidylinositol 4 5-bisphosphate (PIP_2) via phospholipase C-β, which results in the generation of diacylglycerol and inositol trisphosphate. Inositol trisphosphate transiently increases cytosolic calcium levels by releasing calcium from its internal stores. This transient phase is followed by an influx of external calcium caused by the opening of store-operated calcium release-activated calcium (CRAC) channels in the plasma membrane. To understand how CRAC channels are involved in receptor-driven calcium responses, I investigated whether the opening of CRAC channels regulates the production of cellular phosphoinositide. Using cytoplasmic calcium ion (Ca^{2+}) imaging in the mast cell line RBL-2H3, I found that LTC_4 induced repetitive calcium oscillations that ran down in the absence of external calcium and were sustained by calcium entry through CRAC channels. The molecular characterisation of CRAC channel components in RBL-2H3 cells revealed that LTC_4 -mediated calcium oscillations were maintained through calcium entry via Orai1 and that the calcium signal could not be maintained by Orai3 or other calcium- permeable channels. Furthermore, STIM1 (but not STIM2) was the only homologue that supported calcium oscillations in RBL-2H3 cells. The inhibition of the cellular phosphoinositide pool by lithium chloride (LiCl) reduces calcium os-

cillations. Adding the substrate inositol rescued these oscillations, but only when external calcium was present. Pharmacologically blocking CRAC channels with a low concentration of CRAC channel blockers prevented the recovery of oscillations in LiCl-treated cells, even when inositol was present. To further understand how calcium entry contributes to the production of PIP_2, I investigated whether PI4P- or PI5P-specific pools support the oscillatory calcium signal induced by LTC_4. Accordingly, by using pharmacological blockers, concluded that PIP_2 used in LTC_4 -mediated calcium signalling is produced via the conversion of PI4P into PIP_2 by PI5K1 kinases and that the cellular PI5P pool does not contribute to the calcium signal. Moreover, the conversion of PI4P into PIP_2 was possible only when there was calcium entry via CRAC channels. Characterisation of the expressed PI5K1 kinases in RBL-2H3 cells revealed expression of only PIP5K1α and PIP5K1γ and that both kinases are needed to maintain the oscillatory calcium signal induced by LTC_4 and to provide an overlapping function. To further expand current understanding of how calcium regulates PI5K1 kinases, I specifically investigated how calcium entry regulates PIP5K1γ. This was accomplished by looking into PIP5K1γ-regulating proteins, of which talin is a focal adhesion protein shown to activate PIP5K1γ. In this thesis, I show that the cleavage and activation of talin depend on calcium entry via CRAC channels, thereby elucidating a possible mechanism in how CRAC channels mediating calcium entry are involved in phosphoinositide production. This thesis identifies a new role for CRAC channels in mast cell activation. The opening of CRAC channels and calcium entry are required for PIP_2 production and thus the maintenance of agonist-mediated calcium signalling.

Publications from this thesis

Ca^{2+} influx through store-operated calcium channels replenishes the functional phosphatidylinositol 4,5-bisphosphate pool used by cysteinyl leukotriene type I receptors

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Nomenclature

- 2APB 2-aminoethoxydiphenyl borate
- ARC arachidonic acid calcium channel
- ARF adenosine diphosphate Ribosylation Factors
- ATP adenosine triphosphate
- CaMKII calmodulin kinase II
- CysLT1 cysteinyl leukotriene receptor 1
- CysLTs cysteinyl leukotrienes
- DAG diacylglycerol
- DAPI 4,6-diamidino-2-phenylindole
- EGTA ethylene glycol tetraacetic acid
- ER endoplasmic reticulum
- FCDI fast calcium-dependent inactivation
- gDNA genomic deoxyribonucleic acid
- GFP green fluorescent protein
- GPCR G-protein coupled receptor
- HEK human embryonic kidney cell
- ICRAC calcium-release activated calcium current
- IP_3 inositol 1,4,5-triphosphate

- IP_3R inositol 1,4,5-trisphosphate receptor
- IR_K inwardly rectifying potassium channels
- ISOC store-operated calcium current
- KD knockdown
- LiCl lithium chloride
- mRNA messenger ribonucleic acid
- NCS-1 neuronal calcium sensor-1
- pA pico ampere
- PBS phosphate-buffered saline
- pF picofarad
- PI phosphatidylinositol
- $PI(3,4,5)P_3$ phosphatidylinositol (3,4,5)-trisphosphate
- PI3K phosphatidylinositol 3-kinase
- PI3P phosphatidylinositol 3-phosphate
- PI4K phosphatidylinositol 4-kinase
- PI4P phosphatidylinositol 4-phosphate
- PI5P phosphatidylinositol 5-phosphate
- PIP_2 phosphatidylinositol 4,5-bisphosphate
- PIP5K1 phosphatidylinositol-4-phosphate 5-kinase 1

- PKA protein kinase A
- PKC protein kinase C
- PLC phospholipase C
- PMCA plasma membrane calcium-ATPase
- RBL rat Basophilic Leukaemia cells
- RNAi ribonucleic acid interference
- SAM sterile-alpha motif
- SCID severe combined immunodeficiency
- SERCA sarcoendoplasmic reticulum ATPase
- siRNA short interfering ribonucleic acid
- SOAR STIM-1 Orai-1 activating region
- SOCE store-operated calcium entry
- STIM stromal interaction molecule
- TRPC canonical transient receptor potential channel
- WT wild type
- YFP yellow fluorescent protein

Chapter 1

Introduction

1.1 Calcium oscillations

In resting cells, intracellular calcium levels are kept within nanomolar ranges with an estimated average of 100 nM [1]. A transient rise in intracellular calcium levels to the micromolar range following the activation of cell receptors is responsible for regulating various cellular functions and processes [2]. The ability of a single messenger to regulate such a vast number of processes is attributed to the diverse spatiotemporal profile of calcium signalling [3]. In non-excitable cells, the stimulation of cell receptors with a physiological agonist results in the development of calcium oscillations that vary in number and amplitude depending on the agonist, concentration and exposure. The decoding of both the frequency and amplitude by calcium-sensitive intercellular proteins, enzymes, transcription factors, and other intracellular molecules and organelles is thought to modulate their function. Moreover, it is believed that cells adopt calcium oscillation to avoid the toxic effect of

sustained calcium elevations. Moreover, receptor desensitisation is less likely to occur in response to an oscillatory calcium event [4].

Calcium oscillations in excitable cells are generated following changes in the membrane potential, leading to the periodic opening and closing of the voltage-gated calcium channels. In non-excitable cells, calcium oscillations are generated following the binding of an agonist to its receptors on the cell surface, thereby causing the generation of inositol 1,4,5-trisphosphate (IP_3). The activation of phospholipase C (PLC) that follows the binding of external ligands to their receptors on the cell surface precipitates the hydrolysis of phosphatidylyinositol-4, 5-bisphosphate (PIP_2). The hydrolysis of minor phospholipid PIP_2 induces the generation of two second messengers: IP_3 and diacylglycerol (DAG) [4]. IP_3 binds its receptors primarily to the endoplasmic reticulum (ER), thus causing the release of calcium into the cytosol [5].

Research has reported that multiple subtypes of IP_3 receptors (IP_3R1-IP_3R3), with various splice variants for each subtype, exist. The expression of IP_3 receptors among different cells is thought to vary, although most cells express all of them [6]. The modulation of IP_3 levels or IP_3 receptors was suggested to contribute to the development of calcium oscillations [6]. Two hypotheses for the generation of calcium oscillations have been suggested in which either a constant or periodic fluctuation of IP_3 levels can be observed. The first hypothesis posits that there is a constant amount of IP_3, and that calcium oscillations are due to the positive and negative feedback of calcium on IP_3 receptors. High levels of calcium inhibit IP_3 receptors, whereas low levels can potentiate the activity of the receptors [7, 8]. Calcium regulates IP_3 receptors via two binding sites: an inhibitory and an

activating calcium-binding site [5]. It was shown that the binding affinity and dissociation constant differs between the sites, which results in the inhibitory sites being occupied only after a prolonged elevation of cytoplasmic calcium [1]. By contrast, the second hypothesis for how calcium oscillations are generated holds that they stem from the fluctuation of IP_3 levels irrespective of cytoplasmic calcium levels [9, 10].

The sensitivity of IP_3 receptor subtypes to IP_3 levels is believed to vary, and accordingly, oscillations were also attributed to the expression levels of IP_3 receptor subtypes within cells [11]. Regular, continuous, and long-running oscillations are observed when IP_3R2s are activated. Conversely, IP_3 R1 activation leads to the generation of momentary episodic oscillation [12]. Since the knockdown of IP_3R3 prompts an increased number of oscillations, it was suggested that IP_3R3 receptors play a negative regulatory role when it comes to calcium oscillations [11, 13].

The phosphorylation of IP_3 receptors was also suggested to contribute to the generation of calcium oscillations [14]. Protein kinase A (PKA) was shown to phosphorylate all subtypes of IP_3 receptors [15], and the phosphorylation of IP_3 R1 by PKA was shown to potentiate the effect of the receptors by increasing their sensitivity to IP_3[16]. CaMKII Kinase was also considered to regulate IP_3 receptors via phosphorylation [17], specifically that CaMKII-mediated phosphorylation of IP_3 R2 is required for histamine-mediated calcium oscillations [18]. The direct binding of multiple partner proteins to IP_3 receptors such as the neuronal calcium sensor 1 (NCS-1), Bcl-2, and cytochrome C was also suggested to contribute to the development of calcium oscillations [19].

Following IP_3-mediated release of calcium from the internal store after activation of

cell surface receptors, oscillations eventually run down because part of the released calcium is pumped out of the cell by the plasma membrane calcium ATPase [4]. This rundown of calcium oscillation was not observed when cells were bathed in a calcium-containing external solution, an event that was attributed to calcium entry via store-operated calcium channels at the plasma membrane [4].

1.2 Store-operated calcium entry

The model of store-operated calcium entry (SOCE) was first introduced by James Putney [20], who hypothesised that a pathway exists between internal stores and plasma membrane channels. Emptying of the calcium stores will activate the calcium channels at the plasma membrane as illustrated in figure 1.1. Consistent with this, intracellular dialysis of cells with IP_3 resulted in the development of a calcium current detected in patch-clamp experiments [21–24]. The introduction of thapsigargin as a non-reversible selective inhibitor of the sarco/ER calcium ATPase (SERCA) pump strongly supports Putney's model [25, 26]. Thapsigargin caused the depletion of ER calcium, thus activating calcium entry via the store-operated plasma membrane channels [27]. A few years later, Hoth and Penner described a highly selective, inwardly rectifying calcium current following the depletion of ER calcium stores called ICRAC [28]. The same current was subsequently described in T cells by Zweifach et al. [29].

For several years, the mechanism through which the message is conveyed from internal stores to the plasma membrane remained elusive. First, the calcium influx factor (CIF) was introduced as a soluble messenger that travelled from the ER to

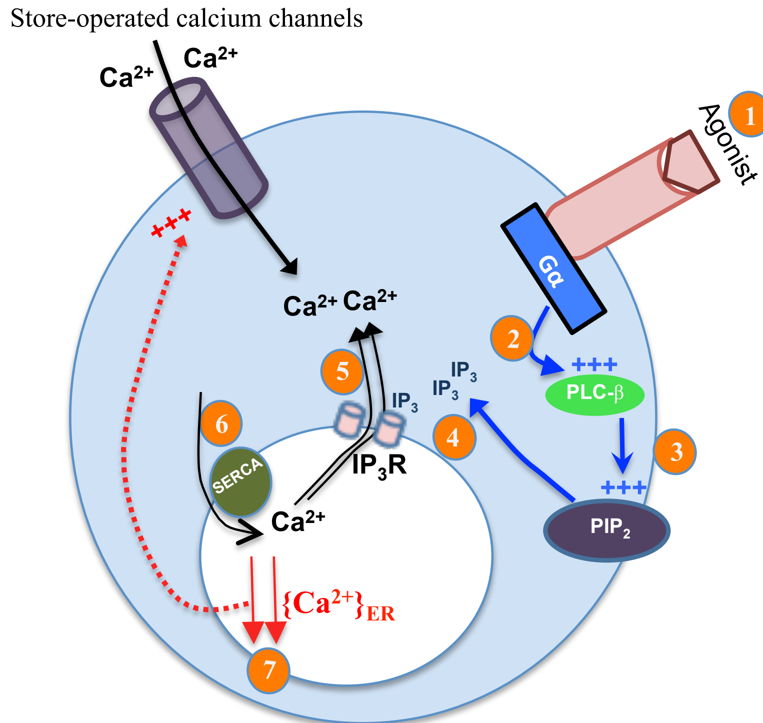

Figure 1.1. Store-operated calcium channels signalling mechanism
(1) Binding of external agonist that links the Gq-signalling pathways to their receptors on the surface of the cell will lead to (2) PLC activation and (3) hydrolysis of PIP_2 . The cleaved PIP_2 will lead to the generation of (4) IP_3 that will bind its receptors on the ER, thus promoting (5) calcium release and accordingly cytosolic calcium rise. (6) Some of the released calcium will be transported back to the ER via the SERCA pump. (7) Eventually the calcium levels within the ER lumn will drop and signal to the calcium channels at the plasma membrane to open. Extracellular calcium entry replenishes stores and maintains the calcium signal within the cell.

the plasma membrane in T cells [30]. Thereafter, it was proposed that cGMP regulated SOCE in pancreatic acinar cells [31]. Tyrosine kinase was also proposed as a diffusible messenger that controls SOCE in platelets [32]. Other hypotheses unrelated to diffusible messengers were also suggested that propose that the luminal domain of IP_3 receptors has a regulatory calcium-binding site and that its cytoplasmic domain interacted with the plasma membrane [33]. Not until 2005, when the first of two components of SOCE were introduced, was the stromal interaction molecule proposed as the ER sensor of SOCE [34, 35]. A year later, RNAi screen studies in drosophila and the gene mapping of severe combined immunodeficiency patients suffering impaired SOCE resulted in the discovery of Orai1 as the second component of SOCE mediating calcium entry at the plasma membrane [36–38]. The STIM and Orai families of proteins are now recognised as constituting store-operated calcium release-activated calcium (CRAC) channels. These channels are the best characterised store-operated calcium channels [4].

1.3 Molecular components of the CRAC channels and their interaction

1.3.1 STIM proteins

STIM1 and STIM2 were discovered as potential candidates in regulating SOCE following the siRNA screening of a library of more than 2,000 proteins [34, 35]. Mammalians have two homologues of the STIM protein; namely, STIM1 and STIM2, and SOCE was shown to reduce following the knockdown of STIM1, but not

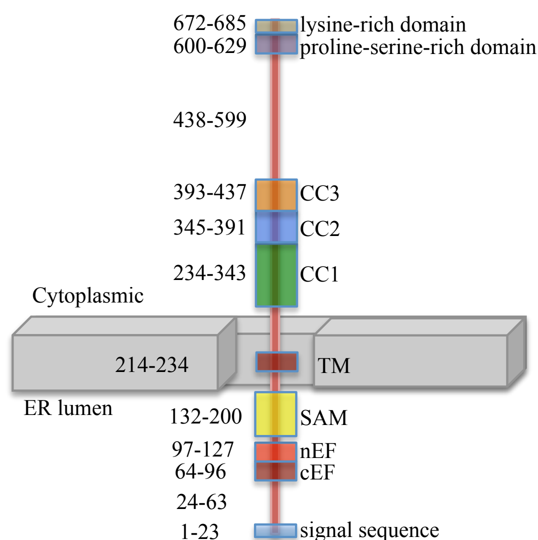

Figure 1.2. Schematic image showing the sequence domains of STIM1. cEF: canonical EF-hand. nEF: noncanonical EF-hand. SAM: sterile-motifs. TM: transmembrane domains. CC: coiled-coil regions.

STIM2 [35]. STIM proteins chiefly localise to ER as a single-pass transmembrane protein, although reports of plasma membrane localisation for STIM1 have been published [39]. Proximal to the N-terminus of STIM proteins and within the ER lumen, STIM has two EF hands and a sterile alpha motif (SAM) domain. Only one of the two EF hands has a calcium-binding domain: the canonical EF-hand domain. The cytoplasmic end of the STIM protein is composed of three coiled-coil domains, a proline-serine-rich domain, and a lysine-rich domain. Fig 1.2 shows a schematic image of the STIM1 structure.

Following a substantial reduction of ER calcium, calcium dissociates from the canonical EF-hand domain, resulting in the unfolding of both EF-SAM domains with the oligomerisation of the protein. The oligomerised protein moves and clusters close to the plasma membrane, specifically at ER-PM junctions where interaction with the plasma membrane channel Orai1 occurs. Once the stores are refilled

following calcium entry via Orai1, STIM1 returns to its resting state [40]. Constitutive oligomerisation and clustering of the STIM protein were observed following the mutation of the canonical EF-hand domain [41]. Unlike STIM1, STIM2 was reported to localise at the plasma membrane with modest ER depletion due to different calcium affinities [42].

The STIM1 Orai-activating region (SOAR) is the domain of STIM1 believed to interact with Orai1 and to facilitate calcium entry via Orai once the stores are depleted [43]. The SOAR region of STIM1 consists of 98 amino acids extending from the 344 - 442 amino acid sequence of STIM1 [43]. It was shown that the SOAR region is inhibited by the coiled-coil domain of STIM1 when ER calcium levels are high. Once the stores are depleted, the acidic residues of the STIM1 coiled-coil domain release the SOAR region, thereby enabling its interaction with acidic residues within the carboxyl terminus of Orai1 [44]. For the activation of CRAC channels, the ratio of STIM1 to Orai1 proteins was suggested to be crucial to the activation of Orai1 [45].

STIM1 was also observed to mediate calcium entry via calcium channels other than Orai1. It was reported that STIM supports calcium entry via certain members of the transient receptor potential cation (TRPC) channels [46], and also that the STIM1 interaction with TRPC channels is an electrostatic interaction [47]. Moreover, STIM1 was found to regulate the arachidonate-regulated calcium (ARC) channel, in which it is believed that following arachidonate release, STIM1 at the plasma membrane interacts directly with ARC channels, thereby causing their activation [48].

STIM2 is highly similar to STIM1, and most of the homology occurs at the amino

terminus of the protein [49]. STIM2 was shown to be more sensitive to reductions in ER calcium contents than STIM1 [42]. Accordingly, it was suggested that STIM2 was more involved than STIM1 in the regulation of the basal levels of cellular calcium. The role of STIM2 in regulating basal calcium is supported by the finding that the overexpression of the protein led to constitutive calcium entry, while its knockdown resulted in reduced levels of basal calcium [42, 50].

A few other differences in the structure of STIM2 compared to STIM1 were reported, including the amino acid sequence of the inhibitory helix C-α-3 [51]. Moreover, it was shown that calmodulin binds to STIM2 at a different site to that of STIM1 [52]. Lastly, the inhibitory domain of STIM2 responsible for calcium-dependent inactivation also differs slightly in its amino acid sequences when compared to STIM1 [53].

1.3.2 Orai1

Mammalian species have three homologues of the Orai protein: Orai1, Orai2, and Orai3. The Orai protein has four transmembrane domains with two extracellular loop regions that connect the first and second transmembrane domains, as well as the third with the fourth transmembrane domain. Additionally, an intracellular loop region connects the second and third transmembrane domains (fig 1.3) and is thought to be conserved among all the homologues of the Orai protein [54, 55]. The intracellular loop was found to play an important role in the fast calcium-dependent inactivation (FCDI) of the channel [56].

Both the Orai amino and carboxyl termini are located intracellularly as a continua-

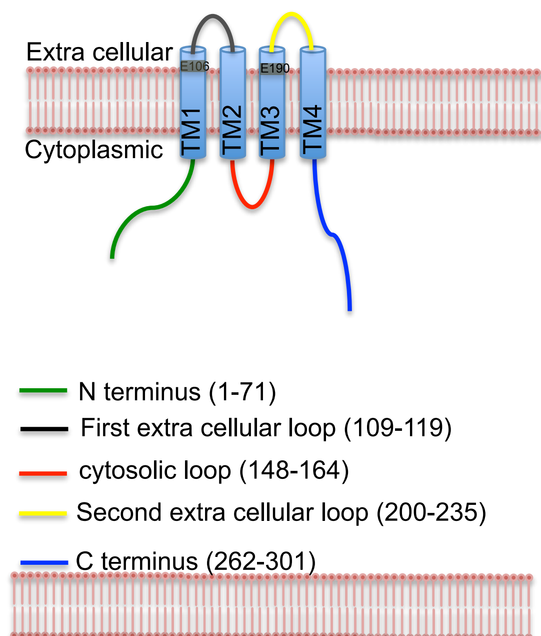

Figure 1.3. Diagram showing the basic structure of Orai. The four transmembrane domains are shown in the image and labelled as TM1-4.

tion of the first and fourth transmembrane domains, respectively. The knockdown of Orai1 was shown to substantially decrease calcium entry [54, 57], and following store depletion, both Orai1 and STIM1 cluster and co-localise at the ER-PM junctions [58, 59]. It was also shown that both the amino and carboxyl termini of Orai1 interact with STIM1 [60].

The charged residues within the C-terminus of Orai were shown to interact with STIM1 [61]. Specifically, amino acids 272-292 were found to interact with STIM1-SOAR, and once deleted, the calcium current is lost [62, 63]. The other homologues were also shown to contribute to calcium entry but only when strongly overexpressed [64].

The amino terminus of Orai1 is thought to be crucial for the channel's activation [62]. It also proposed that the region proximal to the proline- and arginine- rich

regions, that is the membrane-proximal N-terminal domain of Orai1 between amino acid 68 and 91, plays a role in the FCDI of the channel and was found to bind calmodulin in a calcium-dependent manner [65]. The fast inactivation of Orai1 was attributed to the intracellular loop [56], while the acidic residues within the transmembrane domains were linked to FCDI [66]. FCDI was reduced in cells in which the negatively charged residues of the CRAC modulatory domain of STIM1 were mutated, indicating that STIM1 is also involved in FCDI [53].

In aiming to understand the basic structure of Orai1 selectivity towards calcium, it was proposed that acidic residues in transmembrane domains play an essential role in channel selectivity [66]. Two elegant studies that examined the pore structure of Orai1 concluded that the channel's pore was formed by residues of the first transmembrane domain, while the fourth transmembrane domain was the most distal and outermost from the channel's pore [60, 67]. E106 and E190 are acidic residues in the first and third transmembrane domains, respectively, that are believed to mediate most of the channel's calcium selectivity [67]. It was initially reported that the channel was formed by a tetramer of Orai subunits, although this was recently challenged, and it was reported that six subunits of Orai1 are required [68, 69].

1.3.3 Orai2 and Orai3

The role of Orai2 and Orai3 in CRAC channels is unclear; contrary to observations in Orai1, the knockdown of Orai2 and Orai3 shows a modest reduction in CRAC current [70, 71]. However, overexpression of Orai2 and Orai3 can lead to store-operated calcium current [72].

Following the discovery of Orai1, it was found that Orai2 contributed to the development of a calcium current upon store depletion [64]. Based on messenger RNA expression levels, it was found that Orai2 is widely expressed in tissues where mast cells are residing [73]. Orai2 has two splice variants in mice, one of which is believed to negatively regulate calcium entry [74]. Compared to Orai1, Orai2 shows slower FCDI [53], and the overexpression of the dominant negative mutation of Orai1 was found to completely eliminate the calcium signal, implying that Orai1 is necessary for the function of Orai2 [36]. By contrast, mast cells isolated from Orai1 knockout mice were found to have a small CRAC-like current insensitive to 2-APB, indicating possible mediation by Orai2 [75]. The lack of specific antibodies against Orai2 has significantly impacted the progression of research related to its pathophysiological role [76]. One study has concluded that Orai2 changes its expression levels following the modulation of oestrogen receptor activity in parathyroid tumour cells [77].

Like Orai2, Orai3 was shown to be widely expressed in mammalian species with variable tissue distribution [78–80]. Added to the extracellular loop that connects the third and fourth transmembrane domains of Orai1, the cytosolic amino and carboxyl termini of Orai3 are areas where the most differences in amino acid sequencing, compared to Orai1, are observed [48]. The knockdown of Orai3 did not affect the measured SOCE current in human embryonic kidney (HEK) 293 cells [64]. Other research groups have reported that Orai3 is capable of mediating SOCE when Orai1 is knocked down, though at a lesser rate [81, 82]. The inactivation kinetics of Orai3 were shown to be much faster [82], and unlike Orai1, the FCDI of Orai3 was suggested to be mediated by the channel's C-terminus [83].

Orai3 was linked to ARC channel activation via the binding of arachidonate to the N-terminus of Orai3 [84]. Orai3 was found to be highly expressed in breast cancer cells and to mediate SOCE [85]. Furthermore, the knockdown of Orai3 inhibited proliferation of the cells via interference with the cell cycle [85, 86]. In some haematological cells lines, Orai3 was found to induce apoptosis, and it is believed that the active mechanism of tipifarnib, a farnesyltransferase inhibitor, occurs via the activation of Orai3, thereby inducing the apoptosis of white blood cells [87]. While Orai3 plays a protective role in haematological malignancies, it was found to also play a pathological role in vascular disorders. Orai3 was reported to be upregulated in neointimal hyperplasia, in which it can induce vascular occlusive disease [88]. In human lung mast cells, Orai3 was shown to contribute to the inflammatory process and degranulation of mast cells following the activation of the FcεR1receptor [89]. Lastly, Orai3 plays a major role in protecting effector T-helper lymphocytes from reactive oxygen species at the site of inflammation [81].

1.4 CRAC channels and diseases

T-cell activation and proliferation are mediated by CRAC channels. It was shown that blocking CRAC channels resulted in inhibition of T-cell activation and IL-2 production [90]. Moreover, the expression markers of T-cell activation were shown to be reduced when CRAC channels were blocked with the CRAC channel blocker lanthanum [91]. Mutation studies have shown that both STIM1 and Orai1 are involved in T-cell activation and cytokine production [92]. Furthermore, the

release of mediators from cytotoxic T lymphocytes following interaction with non-self cells was found to be mediated by calcium influx via CRAC channels [93]. T cells isolated from patients diagnosed with severe combined immunodeficiency were observed to have an impaired store-mediated calcium entry and consequently impaired T-cell activation and cytokine production. Moreover, both B cells and fibroblasts isolated from the same patients also exhibited impaired store-mediated calcium influx [94, 95]. Isolated STIM1 mutations were observed to induce a syndrome of immune deficiency that results in impaired immune cells, as well as autoimmune haemolytic anaemia and thrombocytopenia [96]; the latter was reported in patients lacking Orai1 [97]. Mice lacking STIM1 or Orai1 were shown to die perinatally, a fact that was attributed to failed skeletal muscle development [98]. Given the observed mortality in the early stages of mice development, several approaches have been used to generate mutated STIM1 or Orai1 mice, such as conditional gene targeting deletion [99]. Orai1 deletion in mice resulted in an impaired allergic response due to defective mast cell activation and the release of mediators following antigen linking to the FcεRI receptor [75].

Myopathies were observed in patients lacking CRAC channel components [98]. An in vitro investigation revealed the vital role of calcium entry via CRAC channels in human myoblast differentiation [100]. Muscle tone was observed to be reduced in patients with Orai1 mutation; it was also observed that affected infants and toddlers showed a delayed onset of motor developmental skills [97]. A histological analysis of muscle fibres showed defective fast-twitch muscle fibres [97], and consistent with the role of CRAC channel components in muscle development, mice lacking STIM1 were reported to show severe fatigability and contractility defects

[101].

Ectodermal tissue development and differentiation were linked to calcium entry via CRAC channels [97]. Patients lacking either STIM1 or Orai1 were reported to have impaired tooth enamel formation, which was attributed to the impaired calcification of the enamel layer [96]. Moreover, consistent with the vital role played by CRAC channel components in ectodermal tissues, patients lacking Orai1 also displayed dry skin, heat intolerance, and hypohidrosis [97].

The role of CRAC channels in the vascular system is well established, and the proliferation and differentiation of vascular tissues were linked to the opening of CRAC channels [6]. In smooth muscle cells isolated from rat aorta, the knockdown of STIM1 and Orai1 significantly affected cell proliferation and migration [71]. The repair process mediated by vascular smooth muscle migration following a physical insult to blood vessels was shown to depend on STIM1 and Orai1 [102]. Animal models of metabolic syndrome showed a greater expression of CRAC channel components in vesicular smooth muscle cells, and the expression of STIM1 and Orai1 diminished when risk factors of the syndrome were modified [103]. A significant expression of CRAC channel components was also observed in the vascular bed of rat models with hypertension, and the application of CRAC channel blockers attenuated the muscles' contractile force [104].

Primary platelets pathology is linked to bleeding and coagulating disorders. Knockout mice models of STIM1 showed impaired platelet activation and aggregation after stimulating platelets with multiple physiological stimuli [105]. Similar to STIM1 knockout mice, Orai1 knockout mice showed a reduced calcium signal, though aggregation was impaired only in agonists that link to the tyrosine ki-

nase pathway, not those that activate platelets via G protein-coupled receptors (GPCRs) [106]. Furthermore, platelets of Orai1-deficient mice were shown to play no role in the formation of pathological thrombi [106].

The expression of CRAC channel components in endothelial cells from both arterial and venous tissues has been confirmed, as has linkage between multiple physiological functions of endothelial cells and CRAC channels [6]. The knockdown of either STIM1 or Orai1 was shown to impair the proliferation of endothelial cells isolated from the human umbilical vein [107].

It has also been proposed that CRAC channels play a role in cardiac hypertrophy, and the knockdown of STIM1 was shown to protect against the hypertrophic response induced by endothelin-1 [108]. Furthermore, the knockdown of STIM1 did not affect the size of muscle tissues when there was no stress and it attenuated only the hypertrophic effect of phenylephrine [109].

It is exceptionally clear that calcium entry via CRAC channel components is linked to multiple pathophysiological pathways and conditions. In this sense, further knowledge of how calcium entry via CRAC channels is involved in activating multiple downstream effectors can expand the current understanding of all of the diseases and pathological conditions associated with CRAC channels.

1.5 Pharmacology of CRAC channels

Given that CRAC channel components were discovered only recently, the availability of channel activators and inhibitors remains limited. To date, only one

direct activator of the channels is known, namely 2-aminoethyl diphenyl borinate (2-APB) [110, 111]. It was shown that using 2-APB in a range of 1-20 M induced CRAC channel activation. The use of 2-APB in cells expressing Orai2 and Orai3 showed that Orai2 is insensitive to the application of 2-APB, unlike Orai3, which showed greater sensitivity [112, 113]. 2-APB-mediated activation of Orai3 occurs independently of store depletion, whereas the 2-APB-mediated activation of Orai1 is suggested to be primarily store dependent [112, 114]. The observed range of the 2-APB-mediated activation of Orai1 and Orai3 was reported to differ, for while 1-20 µM is sufficient to activate Orai1, Orai3 was activated only at greater concentrations (30-75 µM) [115]. Using a concentration of greater than 30 µM of 2-APB inhibited Orai1, which specified it as an Orai3 activator at these concentrations [110, 111]. The 2-APB-mediated activation of Orai3 is thought to occur by increasing the pore size of the channel, whereas enhancing the interaction between STIM1 and Orai1 was proposed as the activation mechanism of Orai1 [115, 116].

Trivalent lanthanides such as La^{3+} and Gd^{3+} are potent inhibitors of CRAC channels [117]. Lanthanides are non-selective calcium blockers that can block both voltage-gated calcium channels and TRPC channels [118]. Lanthanides interact with the negatively charged amino acids of the first extracellular loop of Orai1 [119]. Pyrazole derivatives were shown to be potent blockers of CRAC channels [120, 121], and three particular pyrazole derivatives were suggested to inhibit CRAC channel activity, namely, BTP1, BTP2, and BTPT3; the second of which is the most effective [121]. The half maximal inhibitory concentration of BTP2 was found to be 10 nM, with prolonged pre-treatment providing better blocking [122]. The specificity of BTP2 for CRAC channels is greater than that reported for triva-

lent lanthanides or 2-APB [118]. BTP2 has been suggested to inhibit CRAC channels by inhibiting the actin-reorganising protein, drebrin, which mediates CRAC channel activation [123]. Synta was introduced as a specific potent CRAC channel blocker in multiple cell types [124, 125, 125] and reported to be highly specific towards CRAC channels, but exerting no effect on TRPC-1, TRPC5, TRPC6, or TRPV4 [124]. The half maximal inhibitory concentration of Synta in RBL-1 cells was reported to be 3 µM [125]. Though the mechanism of Synta's inhibitory action is poorly understood, it clearly had no effect on STIM1 puncta formation [124].

1.6 TRPC channels

Prior to the discovery of Orai channels as the chief component of CRAC channels, TRPC channels were thought to mediate SOCE [126]. The deletion of the trp gene was shown to attenuate calcium entry of the drosophila light-sensitive channels. [127]. Further research identified the human homologue of the identified drosophila TRP protein and was named TRPC1 [128]. At present, there are six known TRPC proteins in humans: TRPC1 and TRPC3-TRPC7 [129]. Based on the sequence homology, TRPCs are categorised into one of two subfamilies; TRPC1, TRPC4, and TRPC5 form one subfamily, while TRPC3, TRPC6, and TRPC7 form the other [130]. TRPCs are formed by transmembrane domains spanning segments with a hydrophobic pore domain between the last two transmembrane domains, and four TRP subunits are needed to form a functional channel [131]. The calcium selectivity of TRPCs is low compared to CRAC channels, and the electrophysiological profile of TRPCs differs from that of CRAC channels [132,

133].

1.6.1 TRPC3 channel

Special attention has been paid to TRPC3's role in mediating calcium oscillations [134, 135], which is also one focus of this thesis. TRPC3's expression varies between organs, including the heart, lungs, brain, and pituitary gland [136]. TRPC3 is formed by 848 amino acids with a cytosolic amino terminus containing a distinctive ankyrin-like repeat domain, six transmembrane domains and a cytosolic C-terminus [137]. It has been proposed that a functional channel is formed by tetrameric complexes [138] and that endogenous TRPC3 shows continuous basal activity attributed to the glycosylation of the channels amino terminus [139].

In addition to evidence implicating TRPC3 in SOCE, it is also reportedly activated by the product of PIP_2 hydrolysis: diacylglycerol (DAG) [140]. Unlike the activation of TRPC3 by DAG, channel activation by store depletion remains controversial. The ability of TRPC3 to respond to changes in calcium levels within internal stores or to DAG was shown to depend on TRPC3's interaction with other TRPC channels [141]. Overexpression models of TRPC3 in the HEK 293 cell line and neurons revealed that TRPC3 associates with TRPC6 and TRPC7 but not with the other members of the TRPC family [142, 143]. The interaction of TRPC3 with the members of its subfamily results in a complex of receptor-operated channels [144]. As for the role of TRPC3 in SOCE, an intermolecular electrostatic interaction was shown to occur between STIM1 and the amino terminus of TRPC3 [47]. More recently, it was found that TRPC3 does interact with TRPC1 and that the interaction is necessary to form a complex of store-operated calcium channels [141].

Another hypothesis regarding how TRPC channels contribute to SOCE suggested that Orai-mediated activation of TRPC channels followed store depletion [145]. TRPC3 was also shown to interact with IP_3 receptors, thus possibly enabling the channel's response to calcium level changes in the ER [19].

Similar to Orai channels, TRPC3 channels were shown to be inhibited by 2-APB and the lanthanide gadolinium [146, 147]. Other more specific pharmacological blockers of TRPC3 were also reported, including SKF96365, dye ruthenium red, and Pyr3 [138].

TRPC3 was found to play a physiological role in many non-excitable cells. In store independent pathways, TRPC3 was implicated in the activation of T cells [148] and was shown to help to reduce atrial fibrillation by enhancing the proliferation of fibroblasts [149]. TRPC3 was furthermore shown to partly regulate multiple processes of endothelial cells [150]. Perhaps above all, the TRPC3 channel seems not to be expressed in mast cells, though the heterologous overexpression of TRPC3 in mast cells has identified multiple cellular functions which the channels can control [151–153].

1.7 Mast cells

Belonging to the myeloid linage, which plays a crucial role in both types of immune system, mast cells have been proven to play an essential role in many allergic conditions and in anaphylaxis [154, 155]. Mast cells are long-living cells that originate from CD34 pluripotent stem cells in bone marrow and mature in tissues under the effects of stem cell factors, cytokines, and other factors in their tissue of residence.

Accordingly, mast cells show variable functionality and heterogeneity according to their final organ destination [156–158]. Mast cell number, distribution, and activation change with several physiological conditions such as wound healing and fibrosis, as well as with immunological and non-immunological chronic and acute inflammatory conditions and infections [159].

How mast cells are directed to a particular organ or body area in which they will reside is not fully understood. Immature mast cells migrate via blood and depend on multiple factors, including the expression of certain chemotactic receptors, integrins, and adhesion molecules on the surface of mast cells [160]. For example, in addition to Interleukin-3 (IL-3) and stem cell factor (SCF), alpha4beta7 ($\alpha4\beta7$) integrin was shown to be crucial in attracting mast cells to the small intestine [161]. The expression of the chemokine receptor type 2 on the surface of mast cells was deemed essential to direct mast cells towards lung tissue. Moreover, the interaction of $\alpha4$ integrins with the endothelial vascular cell adhesion molecule 1 was also found to be critical for mast cell migration towards the lungs [162]. At the same time, chemokine receptor type 3 was shown to play a role in localising mast cells at synovial tissues and was consequently involved in the pathogenesis of rheumatoid arthritis [163]. Moreover, it was suggested that resident mast cells release mediators that attract immature circulating mast cells to certain body areas. For instance, leukotriene B_4 secreted from activated mast cells following exposure to an antigen, was shown to attract immature mast cells to the lungs. Such sensitivity to B_4 as a chemotactic factor was shown to decrease as the mast cells aged [164].

Mast cells primarily localise at skin and surface mucosa proximal to body orifices

such as the respiratory mucosa, bladder mucosa, and gastrointestinal mucosa [165]. Also reported is the fact that mast cells abound in connective tissue surrounding muscles, glands, and vascular tissue [166]. In mice, it was shown that the contents of granules and types of expressive receptors differed between mucosal and connective tissue mast cells, and accordingly, mast cells were classified as mucosal or connective tissue mast cells [167]. In humans, mature mast cells are classified differently depending on the contents of the granules, not the histological location of the cells. Chymase- and tryptase-containing cells were classified as one subtype, while cells with only tryptase were classified as another [168]. Though those two subtypes of human mast cells represent most of the mast cell population, a third subtype was identified in asthmatic patients that contains tryptase and carboxypeptidase A3 [169].

Mast cells interact with their surrounding environment via multiple receptors such as NOD-like receptors, Toll-like receptors, Fc receptors, and leukotriene receptors [170, 171]. Upon activation, mast cells release a significant amount of mediators from secretory granules in addition to other substances that immediately become active [172]. The size of mast cell granules is large up to 1,000 nm. Once activated, mast cells may secrete up to one thousand granules. Compared to the well-studied secretory system in neurons, mast cells take minutes to release all of their contents, while the same event takes a fraction of a second in neurons. The recycling of these granules also takes a considerably long time, estimated to last a day, if not more [172, 173]. Mast cells' secretory granules have been shown to contain multiple mediators such as amines, proteoglycans, and proteases. Among the amines, histamine is the most abundant, well-recognised mediator, while heparin and chon-

droitin sulphates are identified stored proteoglycans. Finally, proteases containing granules were shown to contain tryptase and chymase [174]. More specific mediators were also revealed to be released by mast cells, including transforming growth factor-β, tumour necrosis factor-α, and both anti- and pro-inflammatory cytokines [175]. The choice of secreted mediator is thought to depend on the activated receptors on the mast cell surface [174]. The process of mediator release can be classified into two main categories based on the amount of released granule contents: piecemeal degranulation (PMD) and anaphylactic degranulation (AND) [176, 177].

Mast cells are considered to provide an excellent model for studying calcium signalling in non-excitable cells. Many earlier discoveries of molecules, channels, and organelles that regulate calcium levels in cells occurred in mast cells [178]. RBL-2H3 cells constitute a rat cell line isolated from RBL-HR+, a subline of cells isolated from rat tumour growth following the subcutaneous injection of leukemic cells [179]. Although the hematopoietic lineage of mast cells and basophils differs, significant functional similarity between the two is reported, and the RBL-2H3 cell line is often referred to in the literature as a mast cell line [180]. RBL-2H3 cells expressed closer similarity with mucosal mast cells than connective tissue mast cells [180]. The human mast cell line (HMC-1) and murine P-815 mast cells were shown to be comparable to RBL-2H3 in many functional aspects [181]. Accordingly, RBL-2H3 is believed to constitute an excellent in vitro model for studying and understanding mast cell physiology.

1.7.1 Mast cell activation

Mast cells participate in the immune response following their activation by several agonists. Mast cells are activated after challenging their receptors with an agonist that links to the PLC pathway, thereby causing the release of calcium from internal stores with the consequent opening of CRAC channels [155]. Calcium entry was shown to be essential for mast cell activation [28, 132]. The immunoglobulin E (IgE)-mediated activation of mast cells via the high-affinity FcεRI receptor has been investigated for years. Aside from the antigen-mediated activation of mast cells, many other stimulants used the PLC-mediated release of calcium. Challenging receptors forming G protein-receptor complexes with agonists such as the adenosine receptors and cysteinyl leukotriene (CysLT) receptors lead to the release of calcium by linking to PLCβ [3, 182, 183].

Mast cells have been used to establish a connection among changes in the levels of intracellular calcium and the initiation, maintenance, or inhibition of crucial cellular functions, including growth and survival, the initiation of the inflammatory response, degranulation, and chemotaxis [178, 184]. The calcium-mediated activation of mast cells was described as early as the 1970s, when histamine release was found to be dependent on calcium entry in rat peritoneal mast cells [185]. This finding was further supported by the observation that histamine release was inhibited following the use of lanthanum (La^{3+}) to block calcium entry [186]. The introduction of calcium-sensitive fluorescent dyes further elucidated the role of calcium entry in mast cell activation [187]. Responses to chemotactic stimuli such as C5a and C3a, leukotriene production, and cytokine release in mast cells were also linked to calcium entry [188–190]. Calcium entry via CRAC channels was further-

more shown to be important for mast cell activation, for cells isolated from animal models lacking Orai1 showed profound defects in degranulation and cytokine production [75]. Mast cells lacking STIM1 also showed impaired antigen-mediated activation of transcription factors such as NF-kappaB (NF-B) and the nuclear factor of activated T cells (NFAT) [191]. Pharmacological blockers of Orai1 in cells isolated from lung tissue showed reduced mast cell activation and impaired cytokine and leukotriene production [192]. Lastly, c-Fos-dependent gene expression in mast cells was revealed to depend on calcium entry via CRAC channels [3].

Understanding how mast cells are activated is of great importance. Although mast cells are known for their participation in asthma and atopic conditions in general, they have recently been shown to also contribute to the pathogenesis of other conditions, including non-atopic inflammatory conditions such as prostatitis, arthritis, and inflammatory bowel disease [193–195]. Mast cells were also shown to be associated with cancer, as a histological analysis of tumours showed an abundance of immune cells from both lineages, including mast cells [175]. The presence of mast cells within tumour tissue is, however, considered to be a poor prognostic indicator [171]. Tumour cells secrete a stem cell factor that binds to its receptor on mast cells, inducing their settlement and maturation in and around tumour cells [196]. Fibroblast growth factor 2 and the vascular endothelial growth factor were reported to be released by mast cells, thereby producing more aggressive tumour cells [197].

1.8 Leukotrienes and leukotriene receptors

CysLTs are a group of lipid mediators whose response occurs via binding with CysLT receptors. CysLTs are produced following phospholipase A2 liberation of the cell membrane phospholipid, arachidonic acid. Available arachidonic acid is oxidised by 5-lipoxygenase, which is activated by the 5-lipoxygenase-activating protein (FLAP) to 5-hydroperoxyeicosatetraenoic acid (5-HPETE), which becomes reduced to leukotriene A4. Leukotriene C4 synthase converts the unstable leukotriene A4 to leukotriene C4 [198, 199] and γ-glutamyl transpeptidase or γ-glutamyl leukotrienase prompts the conversion of LTC_4 to LTD_4. LTD_4 is then converted to the more stable leukotriene, LTE_4 , by dipeptidase [200, 201]. Calcium entry through CRAC channels is essential for the production of LTC_4 via the recruitment of cPLA2 and 5-lipoxygenase [189, 202, 203].

Agonists evoking a response through CysLT receptors have been linked to allergic conditions such as asthma and allergic rhinitis [204, 205]. CysLTs were reported to abound in secretions isolated from the upper respiratory tract of patients suffering allergic rhinitis [204]. Moreover, atopic asthmatic patients showed up to a five-fold increase in their cells' ability to produce CysLTs compared to control subjects [206]. Levels of LTE_4 isolated from urine samples of asthmatic patients correlated with the severity of asthmatic attacks [207]. In support of the role of CysLTs in the development of asthma, it was shown that using specific blockers of CysLT receptors improved the symptoms of acute asthmatic attacks [208]. CysLTs were also implicated in the pathogenesis of chronic obstructive pulmonary disease (COPD). The addition of the specific CysLT receptor blocker montelukast in the standard therapy of COPD showed symptomatic improvement in both the

short and long terms [209, 210]. The pathogenesis of chronic spontaneous urticaria was also revealed to be primarily caused by the presence of multiple inflammatory mediators, including leukotrienes [211].

CysLTs exert their effect by binding to CysLT receptors. CysLT receptors are part of the rhodopsin subfamily of GPCRs [205], a large family of receptors known for their seven distinct transmembrane-spanning domains and their association with guanosine triphosphate (GTP)-binding proteins [212]. Both receptors are linked to the Gq pathway that leads to phospholipid hydrolysis and eventual calcium rise in cells [213]. Once activated, GDP is replaced by GTP at the Gα subunit of the G protein, resulting in the dissociation of the Gα subunit and Gβγ subunits [214]. Earlier studies identified the presence of two CysLT receptors [215], although the homology between the CysLTs was reported to be only 38% [216]. The expression of the two receptors seems to vary among different tissues, with CysLT-1 expressed mainly in respiratory tissues and CysLT-2 in the conduction system of cardiac cells, vesicular cells, and respiratory tissue [216]. Both receptors were expressed in cells derived from the myeloid lineages of hematopoietic cells, including mast cells [216]. Affinities to CysLTs between the two receptors are not identical, for CysLT-1 has a higher affinity for LTD_4 than LTC_4 , while CysLT-2 shows equal affinities for both LTD_4 and LTC_4[217].

1.9 Polyphosphoinositide and phosphatidylinositol synthesis

The inositol ring of myo-inositol is composed of six hydroxyl groups that are phosphorylated at D1, D3, D4, and D5 positions, by which they yield phosphatidylinositol and the seven polyphosphoinositides (fig 1.4). Polyphosphoinositides are seven members of the same family generated from the phosphorylation of phosphatidylinositol (PI). PI is generated from the phosphorylation of myo-inositol at the D1 position of the inositol ring. The phosphate group at the D1 position of phosphatidylinositol is linked via a phosphodiester bond to the DAG backbone [218].

The synthesis of phosphatidylinositol occurs in the ER, where myo-inositol and CDP-DAG are converted to PI by the action of phosphatidylinositol synthase [219, 220]. CDP-DAG is produced from both phosphatidate cytidylyltransferase (CTP) and PtdOH by the action of CDP-DAG synthase [220].Myo-inositol comes from three sources: myo-inositol-3-phosphate synthases' action on glucose- 6-phosphate [221],the recycling of inositol produced following stimulation of the cell with an agonist linked to the PLC pathway [222] and the entry of exogenously synthesised inositol into the cell via plasma membrane inositol transporters [223–225]. Exogenously synthesised inositol gains entry into the cells through one of three known transporters: Na^+ /myo-inositol co-transporters 1 and 2 and H^+ -myo-inositol symporters [223–225]. In addition to the ER, the plasma membrane is also suggested to host PI synthesis, following the observation that PI synthesis in red blood cells isolated from turkeys occurred there [226, 227]. In support of PI

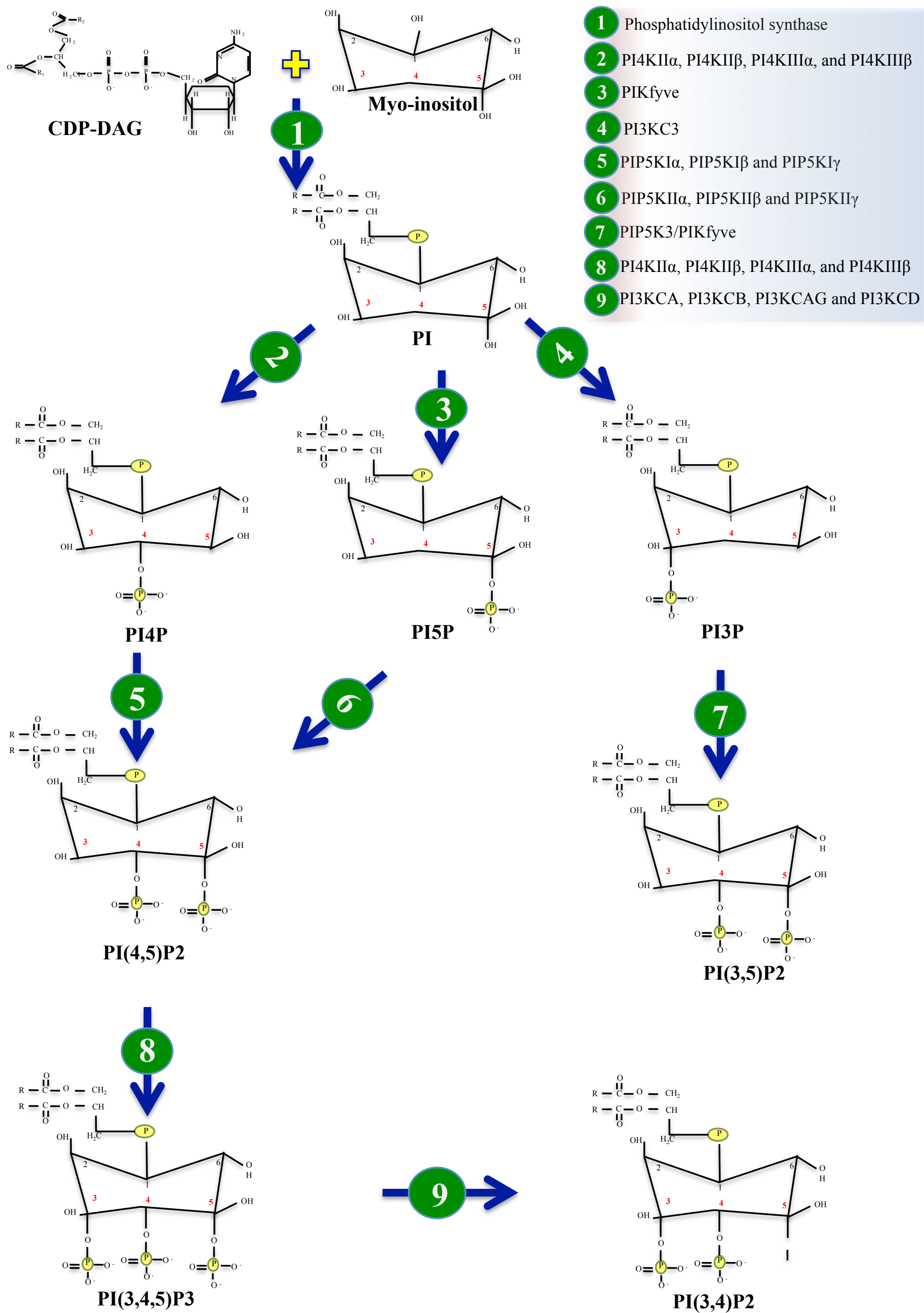

Figure 1.4. Illustration of polyphosphoinositide and phosphatidylinositol synthesis pathway and the enzymes required for each step.

synthesis in the plasma membrane, PI can be isolated from the plasma membrane of fractionated cells [228, 229]. Synthesised phosphatidylinositol depends on PI transfer proteins for their distribution within the cell [230].

The percentage and availability of phosphoinositides in relation to overall cellular phospholipids varies. The most available phosphoinositide is PI, followed by PI4P and PIP_2, which however constitute only 0.2-1% [231]. $PI(3,4,5)P_3$, and PI3P thus form a fraction of the estimated available amount of PIP_2 and PI4P [232].

1.10 PIP_2

Although PIP_2 represents only roughly 1% of all cellular phospholipids, it has been linked to an array of physiological functions within cells [231]. Part of PIP_2's versatile activity relates to its ability to either produce downstream effectors or to directly interact with multiple partner proteins and effectors. PIP_2 has been linked to numerous cellular processes and functions in different cell types, and was shown to be involved in regulating cell migration and polarity, membrane trafficking, and gene expression [233–236].

PIP_2 was also shown to directly modulate calcium levels within cells by interacting with multiple calcium-specific pumps, transporters, and channels. The plasma membrane calcium ATPase pump was shown to be regulated by the cellular levels of PIP_2 [237]. Furthermore, the sodium-calcium exchanger was also shown to be regulated both directly and indirectly by PIP_2 levels in cardiac cells [238]. Multiple voltage-gated calcium channels such as L-type channels, N-type channels, and P/Q-type channels were shown to be directly regulated by cellular PIP_2 levels

[239–241]. Nearly the entire TRP family of channels, including TRPC, TRPM, and TRPV, was shown to be either directly or indirectly regulated by PIP_2 [242]. For CRAC channels, it was reported that a reduction of PIP_2 and PI4P levels attenuated calcium influx via Orai1 [243]. The percentage of PIP_2 in detergent-resistant membranes compared to that of the detergent-solubilised membranes was suggested to govern the interactive process between STIM1 and Orai1 [244]. Pharmacological inhibition and the knockdown of PI4K were reported to inhibit CRAC channels mediating calcium influx [245]. Further research has shown that the polybasic domain of the C-terminus of STIM1 interacts with PIP_2 and that the reduction of PIP_2 levels reduces STIM1 puncta formation [246].

The role of PIP_2 in regulating immune cells such as T cells, macrophages, neutrophil granulocytes, and lymphocytes has been established [247–250]. Specifically, PIP_2 was shown to play an essential role in mast cells, whose treatment with methyl-β-cyclodextrin was shown to shift PIP_2 from one cellular domain to another, as well as to impair the activation of cells following stimulation with an antigen [251]. Furthermore, antigen-mediated calcium oscillations in RBL-2H3 cells were shown to depend on PIP_2 production via the activation of rho-GTPase CDC42 [252]. Moreover, PI4KIIα was found to mediate PIP_2 production at the plasma membrane of mast cells, which contributed to the maintenance of antigen-mediated downstream signalling [253]. Following antigen stimulation, different PIP5K1 isoforms catalysed the generation of functionally distinctive pools of PIP_2 at the plasma membrane [254]. The role of PIP_2 in exocytosis in mast cells has been thoroughly investigated, and the reduction of PIP_2 levels within the plasma membrane via an agonist signalled through the PLC pathway was

shown to be essential for exocytosis in mast cells [255]. Myristoylated alanine-rich protein kinase C substrate (MARCKS)-mediated regulation of exocytosis was proposed to occur through the sequestration of PIP_2 [256]. The interaction of the neuronal calcium sensor and PI4KIIβ was demonstrated to be important for the generation of the PIP_2 pool required for antigen-mediated exocytosis [257]. The ADP-ribosylation factor 1 (ARF1)-mediated regulation of PIP_2 levels following the activation of mast cells with an antigen was shown to mediate exocytosis [258], while a reduction in PIP5Kiβ-specific PIP_2 in mast cells isolated from knockout mice PIP5KIβ showed an augmented degranulation response and cytokine release following cross-linking of FcεRI with an antigen [259].

1.11 Phosphoinositide kinases and their cellular functions

In this section, I review the kinases involved in the synthesis of polyphosphoinositides by emphasising the ones either directly or indirectly implicated in the synthesis of PIP_2 . Depending on the site of their action, phosphoinositide kinases are classified into two groups: PI-kinases that act directly on PI, resulting in the production of PI3P, PI4P, and PI5P, and PIP kinases that induce the production of PIP_2 via PI4P and PI5P [218].

1.11.1 PI-inases

PI4Ks

PI4Ks catalyse the production of PI4P from PI and are classified into type II and type III. Both types are further subdivided into types IIα and β and types IIIα and β [260]. Most of our understanding of PI4KIIIα derives from its yeast orthologue, STT4 [261]. In eukaryotes, PI4KIIIα has been linked to the production of PI4P from PI in cultured cells and the generation of agonist-mediated calcium signalling [260, 262]. The location of PI4P synthesis was suggested first to take place in the ER and Golgi apparatus, though synthesis in the plasma membrane has been recently suggested [263, 264]. The distribution of PI4KIIIα was shown to be localised mostly in the brain [265]. PI4KIIIα was suggested to play a significant role in controlling the infectivity of hepatitis C by enhancing its replication rate [266].

The subcellular localisation of PI4KIIIβ occurs primarily in the Golgi apparatus [267] and was shown to be regulated by calcium-sensitive NCS-1 and ARF1 [268, 269]. A nuclear localisation of PI4KIIIβ was proposed, though without any clearly understood role regarding its nuclear function [267]. Similar to PI4KIIIα, PI4KIIIβ is thought to be crucial for the replication of certain RNA viruses, including hepatitis C [270].

The subcellular localisation of PI4KIIα and β is shown to vary with its association with all cellular membranes, including vesicular ones [271–273]. Due to the localisation of PI4KIIs in cellular membranes, it was suggested that both PI4KIIα and β are essential to clathrin-mediated endocytosis [274]. PI4KIIα was shown to

be involved in the regulation of epidermal growth factor receptors, and when the expression levels of PI4KIIα were reduced, receptor degradation became inhibited [275]. PI4KIIα was also shown to regulate the lysosomal delivery of β- glucocerebrosidase [276]. The activation of the two enzymes via associated partner proteins has not been investigated, although PI4KIIβ was shown to be directed towards the plasma membrane and activated through its association with Rac-GTP [272]. PI4KIIα-lacking mice were prone to the early development of degenerative central nervous system pathologies [277], though attempting to link PI4KIIα to neuronal degenerative pathologies in humans was unsuccessful [278].

PIKfyve

PIKfyve mediates the conversion of PI into PI5P by phosphorylating position 5 of the hydroxyl group on the inositol ring [279]. Having been discovered only recently, PI5P and its synthesising enzymes remain largely unexplored [280]. In fibroblasts, PI5P was shown to be involved in the osmotic response pathway, since PI5P levels changed upon cell exposure to osmotic shock [279]. T-cell auto-inhibition by the adaptor molecules Dok-1 and Dok-2 was demonstrated to be associated with and regulated by PI5P [281], while glucose metabolism via insulin was suggested to be regulated by PI5P via modified GLUT4 translocation [282, 283]. Lastly, changed levels of the inhibitor of growth protein-2 in the nucleus were associated with similar changes in PI5P levels [284].

Class III PI 3-kinase (PI3KC3)

Most of our knowledge of PI3KC3 stems from its yeast orthologue, VPS34 [285]. PI3KC3 causes the generation of PI3P upon phosphorylating the 3 position of the inositol ring on PI. PI3P is known for its association with FYVE finger proteins [286] and has accordingly been localised at early endosomes via its association with the FYVE finger domain of EEA1 [286]. Class III PI3K has been implicated in the regulation of autophagy in muscle cells [287] and, via its association with beclin 1, was suggested to play a role in suppressing tumour growth [288].

1.11.2 PIP-kinases

Unlike PI kinases, most PIP kinase activity is thought to be localised at the plasma membrane. PIP kinases are classified as either type I or type II, as detailed below [289].

Type I PIP-kinases(PIP5K1)

Type I PIP-kinases convert PI4P into PIP_2 by adding phosphate to position 5 of the inositol ring [290]. Three identified isoforms for type 1 PIP kinases are known: α, -β, and -γ. Despite being encoded by different genes, the catalytic domain is highly conserved among the three isoforms [291, 292]. Since the nomenclature of the α and -β isoforms of PI5K is not consistent between humans and rodents, the human PIP5K1α is referred to as PIP5K1β and vice versa for human PIP5K1β. Through this thesis I will be following the human nomenclature.

In addition to PIP5K1α localisation at the plasma membrane, it has also been suggested to associate with Golgi and nuclear speckles [293, 294]. Multiple functions are mediated by PIP5K1α, though its role in the regulation of phagosomes and phagocytic process has been the most investigated [290, 295–297]. The early stages of phagosome development have been shown to depend greatly on PIP5K1α and reduction in PIP5K1α levels to inhibit phagocytosis [295]. The knockout of PIP5K1α was furthermore shown to inhibit opsonisation in bone-marrow-derived macrophages [297]. Other functions such as membrane ruffling were also demonstrated to depend on PIP5K1α interaction with Rac signalling [290], and PIP5K1α was shown to contribute to calcium signalling in platelets following stimulation with thrombin [298].

Unlike PIP5K1α, PIP5K1β has been suggested to be present exclusively at the plasma membrane [290]. Moreover, PIP5K1β distribution within the body differs from that of PIP5K1α. Unlike PIP5K1α, which was mostly isolated from the spleen, PIP5K1β was highly associated with skeletal muscles [290]. The role of PIP5K1β is mostly linked to the regulation of cellular actin dynamics [299, 300] and was found to contribute to the generation and maintenance of calcium signalling and the enhancement of the degranulation process in mast cells [254, 259]. PIP5K1β also plays a role in chemotaxis [301].

The γ isoform of PIP5K is the most studied isoform of all three. Special attention to PIP5K1γ is partially attributed to its multiple splice variants. The three splice visitants shared among all mammalian species are PIP51γ87, PIP51γ90, and PIP51γ93 [292, 302, 303], though additional variants are specific to either humans or rodents [303, 304]. The distribution of PIP5K1γ seems to be widely variable

among the tissues from which is isolated, for PIP5K1γ was successfully isolated from brain, lung, and kidney tissues [292, 305]. PIP5K1γ was also suggested to serve a vital function in the organisation of focal adhesions and clathrin-mediated endocytosis [306, 307]. Similar to PIP5Kβ, PIP5K1γ was found to contribute to mediating the calcium signal in mast cells [254].

Type II PIP-kinases(PIP5K2)

Type II PIP-kinases convert PI5P into PIP_2 by adding phosphate to the 4 position of the inositol ring [280]. Generating PIP_2 by type II PIP-kinases is thought to be minimal [308]. Similar to type I PIP-kinases, type II PIP-kinases are encoded by three different genes that lead to three distinctive isoforms: α, -β, and -γ [291]. It is suggested that the -β and -γ isoforms dimerise with the highly active type II PIP-kinases α and help to target it towards subcellular domains [44, 309]. PIPKIIβ was shown to localise at both the cytoplasm and nuclear speckles with no specific organ preference [309]. By contrast, PIPKIIγ was found mostly in the epithelial cells of renal tissue [310]. As the dearth of related studies indicates, the functional role of type II PIP-kinases is poorly understood. In platelets, PIPKIIα has been shown to mediate α granule secretions [311], while PIPKIIβ was shown to interact with several receptors and to modulate their activity, including the p55 tumour necrosis factor receptor, the EGF/ErbB family of receptors, and insulin receptors [312–314].

Type III PIP kinases/PIP5K3

Type III PIP kinases act upon phosphorylate PI3P at the 5 position of the inositol ring, precipitating the production of PI(3,5)P_2 . Most of our understanding of type III PIP kinases has emerged from studies on its yeast orthologue, Fab1 [315]. Type III PIP kinases recognise PI3P via its FYVE domain [316]. The phosphatase Sac3, which reverses the action of type III PIP, thereby causing PI3P generation, was found to be necessary for the function of type III PIP kinases [317]. Type III PIP kinases were shown to regulate certain stages of epidermal growth factor receptor signalling and glucose metabolism [318, 319].

1.12 Regulation of phosphoinositide kinases by partner proteins

PIP_2 localises mostly at the cytoplasm, though other subcellular localisation has been reported at the Golgi apparatus, ER, and nucleus [320]. The spatiotemporal organisation of PIP_2 levels within cells is thought to be mediated by the subcellular localisation of PIP_2 -synthesising enzymes and/or the interaction of PIP_2 with partner proteins [321]. This dynamic is supported by the finding that multiple distinct pools of PIP_2 and PI4P synthesised by different PIP kinase isoforms were identified in the plasma membrane and Golgi apparatus [322]. Other evidence of distinctive PIP_2 pools is the enrichment in PIP_2 of certain plasma membrane areas after the cell is stimulated with an agonist that links to the PIP_2 /PLC pathway [323]. Since the subcellular localisation of PI and PIP kinases was discussed in the

previous section, this section addresses the targeted localisation of PIP_2 synthesis via PIP kinase-associated proteins.

Talin

Talin is an important protein found in focal adhesions, which are sets of scaffolding and signalling proteins that enable communication between extracellular and intracellular environments via the docking of the cellular integrin to the extracellular matrix [324]. It has been suggested that small, distinct pools of PIP_2 are synthesised via the interaction of talin and PIP5K1γ [325, 326]. A large protein, talin is composed of 2,541 amino acids [327] and has a globular head made of an atypical FERM domain composed of three subunits: F1, F2, and F3 [327]. The phosphotyrosine-binding domain of the F3 subdomain activates and recruits PIP5K1γ to focal adhesions [326], while the globular head is connected via an 80 amino acid residue segment to a C-terminal rod with binding sites for vinculin and actin [327–329]. In resting cells, talin is proposed to exist in an auto-inhibitory state in which the rod interacts with the F2 and F3 domains of the globular head (Fig 1.5). The local enrichment of PIP_2 by PIP5K1γ and talin interaction was shown to terminate this auto-inhibitory state, thus exposing all of its binding sites on both the head and rod domains [321]. PIP_2 was found to first bind the F2 subdomain of the head, followed by the F3 subdomain [330]. Though talin has been suggested to recruit PIP5K1γ to focal adhesions, thereby leading to localised PIP_2 synthesis that supports focal adhesion assembly, the opposite is proposed for calpain, which is involved in the disassembly of the focal adhesion by the cleavage of talin [321].

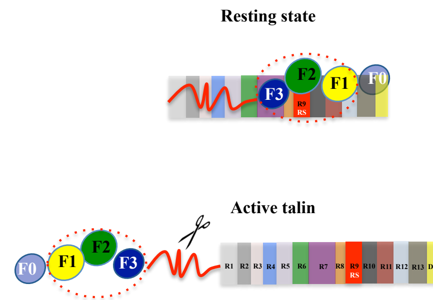

Figure 1.5. Schematic diagram showing the folding state of talin in resting conditions (upper) or once activated (lower). The linker region (marked by the scissors) is the proposed cleavage site of activated talin.

Rac1

Rac1 belongs to the Rho family of small GTPases that switch from their activation state by binding with GTP and GDP [331]. Rac1 has been suggested to play multiple roles and was demonstrated to be involved in cell proliferation and division, actin dynamics, the translocation of nuclear factors, and cancer cell migration [332–335]. The deletion of Rac1 impaired T-cell development in mice [336]. Calcium-mediated Rac1 activation was shown to be important for platelet activation via stimulation with thrombin [337]. Moreover, Rac1 activity was suggested to be modulated by the calcium sensor calmodulin via its calmodulin-binding domain, an interaction shown to be important for endocytosis [338, 339]. All three isoforms of PIP5K1 were shown to be activated by Rac1 [340]. The localisation of PI51Kα and PIP5K1β at spatially different subdomains of the cell was found to be mediated by Rac1 [331, 341].

ADP ribosylation factor

ARF is a six-member family of small GTPase shown to regulate PIP_2 production in distinct pools within the cell [321]. ARF1 and ARF6 are the most studied isoforms, both of which were shown to be involved in PIP_2 production [342]. The generation of PIP_2 within the Golgi apparatus was attributed to ARF1-mediated PIP5K1β activation [343]. In neuronal cells, PIP5K1γ recruitment to the synaptic vesicle membranes is mediated by ARF6 [344], while the recruitment of both PI51Kα and PIP5K1β in ruffling membranes is mediated by ARF6 [345].

AP-2 adaptor complexes

Adaptor proteins are part of the clathrin coats of the endocytotic vesicles [346]. The involvement of PIP5K1γ in regulating endocytosis in HEK 293 cells was suggested to occur via its interaction with AP-2 [347]. Moreover, AP-2 was found to activate PIP5K1γ in neuronal cells and mediate its recruitment towards endocytotic vesicles [348]. In the monkey kidney cell line (CV-1), clarified that recruitment of AP-2 into the membrane PIP5K1β was important for the endocytosis of transferrin receptors [349].

1.13 Measurement of PIP_2 levels

The multiple cellular functions and roles performed by PIP_2 required the development of tools for measuring and monitoring the dynamics of PIP_2 changes in cells [350].

The GFP-tagged PH domain of PLCδ-1 (PLCδ1PH-GFP) is the most popular method for tracking the changes of PIP_2 during live cell imaging or in fixed cells [350]. Pleckstrin homology (PH) domains were shown to associate and recognise multiple phosphoinositides [351], and the PLCδ1PH-GFP was found to bind PIP_2 with relatively high specificity [352]. Unless tagged with glutathione S- transferase and visualised using electron microscopy, PLCδ1PH-GFP detects the levels of PIP_2 only in the plasma membrane and endocytotic vesicles [320, 353]. The function of PLCδ1PH-GFP as PIP_2 has been widely discussed, since its affinity for IP_3 is considerably strong [354], a thought mostly based on the finding that PLCδ1PH-GFP translocation was reduced following agonist stimulation in the presence of type-I 5-phosphatase [10]. One possibility attributed to this finding is the elimination of calcium feedback on PLC [350]. The detailed analysis of PLCδ1PH-GFP translocation in response to PIP_2 and IP_3 revealed that PLCδ1PH-GFP is a reliable probe for monitoring changes in PIP_2 levels [350].

Debate over the specificity of PLCδ1PH-GFP has discouraged scientists from using it, and the need for a more specific PIP_2 reporter prompted the introduction of the tubby domain [355]. The tubby protein is a plasma membrane-bound protein associated with the PIP_2 -binding domain named the tubby domain [356]. Stimulating cells with an agonist that links to the Gq/11-PLC pathway revealed the dissociation of the protein from the plasma membrane [355]. The characterisation of the tubby protein as potentially specific to PIP_2 shows a possibly highly specific probe [357]. One identified limitation of the tubby domain, however, was later reported to be its high affinity for PIP_2 , which complicates detecting changes in PIP_2 levels in physiological conditions [354, 358].

Another method for visualising changes in PIP_2 levels uses the technique of patch-clamp electrophysiology. A family of channels shown to be modulated by PIP_2 is the inwardly rectifying potassium channels [359]. The open probability of the channel was shown to depend on the availability of PIP_2, and potassium current runs down with the reduction of PIP_2 levels [360]. The affinity of PIP_2 seems to vary and to depend on the agonist and individual potassium channel [359].

Lastly, one of the earliest methods for measuring changes of phospholipids within cells was radiolabelling with phosphorus-32 or 3H-inositol, followed by phospholipid separation by thin layer chromatography or high performance thin layer chromatography [361]. Mass spectrometry can also be used to monitor changes in cellular phosphoinositides, though a great limitation lies in its inability to differentiate PI4P from PIP_2 [362]. Though considered to be a reliable method, a few limitations are associated with radiolabelling, including its high cost compared with that of newly developed methods. Another limitation is that the information gathered is based on a population of cells and not at the level of single cells. Finally, radiolabelling facilities and TLC or HPLC are not always available [361].

1.14 Summary

Mast cells are an essential part of the innate immune system, which is the first line of defence against non-self antigens prior to the activation of the adaptive immune system. Mast cells are best known for their role in allergic responses, though have recently been shown to contribute in multiple chronic diseases and cancers. Calcium signalling in mast cells and the opening of CRAC channels were shown to be

essential to mast cell activation and participation in immune responses. The role of calcium signalling via CRAC channels in the production of PIP_2 through the interaction with polyphosphoinositide kinases has never been investigated. Accordingly, following the characterisation of CRAC channel components in the mast cell line (RBL-2H3) and their role in mediating LTC_4 calcium signalling, this thesis addresses whether calcium entry via CRAC channels is involved in PIP_2 production and thus the activation of mast cells following stimulation with lipid mediator LTC_4 .

Chapter 2

Materials and Methods

2.1 Cell culture

The RBL-2H3 cells were a kind gift from Professor Shamshad Cockcroft (University College London, London, UK). The cells were frozen in a cell-freezing medium at passage 14 when they first arrived at Professor Parekhs lab. Accordingly, multiple stocks were made and then the cells were frozen using a freezing medium and were stored at the University of Oxford, in the Department of Physiology, Anatomy and Genetics liquid nitrogen facility. Following thawing of the cells, they were passaged twice prior to being used for any work. The culturing of cells was always done in sterile conditions using a cell-culture laminar flow hood; cells were cultured every 3-4 days depending on the reached confluency. Finally, in all the experiments, no cells beyond passage 30 were used.

The cells were routinely maintained in Dulbeccos Modified Eagles Medium and

supplemented with 10% FCS and 1% penicillin-streptomycin. They were kept in an atmosphere containing 5% CO_2 maintained at 37°. For calcium-imaging experiments, patch clamp experiments and immunofluorescence experiments, the cells were passaged onto 13-mm coverslips using 0.25% trypsin and used 48 hours after passaging.

2.2 Cytoplasmic calcium measurements

Calcium measurements were performed utilising the ratiometric intracellular calcium indicator Fura-2 AM. Plated RBL-2H3 cells on coverslips were washed twice with standard external solution prior to loading with Fura-2 AM. All the experiments were performed at room temperature, and the cells were kept in the dark once loaded. RBL-2H3 was loaded with Fura-2 AM (4 μM) for 40 minutes in an external solution comprised of the following components: 145 mM NaCl, 2.8 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM D-glucose and 10 mM HEPES (pH 7.4 with NaOH). The cells were then washed three times with the same solution without Fura-2 AM and kept for 15 minutes prior to imaging for further de-esterification. The calcium-free solution composition was similar to the external solution, except that calcium chloride was substituted for 0.1 mM ethyleneglycolbis-(β-aminoethylether)-N, N, N, N-tetraacetic acid.

For calcium measurement experiments, a Nikon Eclipse TE2000-U inverted microscope equipped with an IMAGO CCD camera-based system from TILL Photonics (Grafelfing, Germany) was used. The cells were excited at 356/380 nm alternately with an exposure time of 20 ms and an acquisition every 2 s. Data were analysed

after export to IGOR Pro (WaveMetrics, Lake Oswego, OR, USA).

The number of calcium oscillations was quantified in Igor Pro (WaveMetrics, Portland, OR, USA). Oscillations were considered if the R_{peak} - R_{base} value was >0.1.

2.3 Knockdown and over-expression experiments

For both small interfering RNA (siRNA) and plasmid transfections, the Amaxa electroporation system was used. The Amaxa cell line nucleofactor kit T was used for the RBL-2H3 cells with Lonza electroporation apparatus. Approximately 100,000 cells were resuspended in 100 µl of the provided nucleofector solution in the nucleocuvette along with 1 µg of the indicated plasmid or 30-50 nM of the siRNAs and electroporated, using the X-01 program of the machine. Following this, cells were plated onto coverslips in small petri dishes in antibiotic-free medium; 12 hours later, medium was changed to an antibiotic-containing medium, and 24 hours later the cells were used. All the used siRNA are listed in table 2.1.

Gene	Source	Catalogue number
STIM1 silencer select, Pre-designed siRNA	Life Technologies	4390771
STIM2 Stelth siRNA	Life Technologies	SR508429
Orai1 siRNA duplexes	Origene Technologies	SR508429
N-TARGETplus Pip5k1c siRNA - SMARTpool	Dharmacon	L-100440-02-0005
N-TARGETplus Pip5k1a siRNA - SMARTpool	Dharmacon	L-089298-00-0005
ON-TARGETplus Talin-1 siRNA - SMARTpool	Dharmacon	L-096661-01-0005
ON-TARGETplus Talin-2 siRNA - SMARTpool	Dharmacon	L-101143-02-0005

Table 2.1. Used siRNA name and source

2.4 Immunofluorescence

The RBL-2H3 cells seeded on coverslips were transferred to 6-well tissue culture plates prior to experimentation. The cells were washed three times with phosphate buffer saline, and then fixed using 4% paraformaldehyde in phosphate-buffered saline (PBS) for 25 minutes. Fixed cells were washed three times at 5-minute intervals, then permeabilised using 0.5% Triton X-100 in PBS for 5 minutes. Following this, the cells were washed as described above. They were then blocked using SuperBlock (PBS) Blocking Buffer for one hour at room temperature; 1:200 of primary antibody in PBS was added to the cells overnight in 4°C. The following day, the cells were washed for 10 minutes at 10-minute intervals with PBS with 0.1% Tween 20 (PBS-T) buffer. Alexa 488 anti-mouse or anti-rabbit or Alexa 568 anti-rabbit conjugated antibody in 1:200 in PBS for 1 hour at room temperature was added to the cells. Finally, the cells were washed three times with PBS-T at 10-minute intervals. Next, the cells were mounted onto microscope slides 76 x 26 mm using Vectashield Antifade Mounting Medium with DAPI. Clear nail polish was applied to the outermost part of the coverslip to prevent its movement. Images were obtained using the Inverted Olympus FV1000 Confocal system equipped with a motorised stage. Images were obtained using 60 x oil objective of 1.3 numerical apparatus and excitation at 488 or 568 nM. All the images were grouped according to image size of 640*480 and step size of 4 microns along the z-axis.

Images were then opened with ImageJ and the multiple stacks were opened in a single window using the z-project tab [363]. Fluorescence intensities were analysed for each treatment and normalised to the maximum measured fluorescence.

Gene	Primer- sense (5'3')	Primer- antisense (5'3')	Amplification length
Orai1	AGTCCTCACCTTCCCACTGG	GCCTCTTCCTTCCACACTCTG	133
Orai2	ACGCATCACAAGAGCCACAG	TGCGAGGACAGGAGTATGGAG	82
Orai3	GCAACATCCACAATCTCAACTCTG	ACCCAGCCCACCAAAACAAAC	92
STIM1	TGGAGCTGGCACAGTATCAG	TGATTGTCCCGAGTCAACAG	181
STIM2	TAAGCTGTCTCGCTGCTTCA	TGATTGTGGCGAGTCAAGAG	506
Pip5k1α	TACCTTTCACCTGGAGCTGG	TCACTCTCCTCCCAAACACC	130
Pip5k1β	GCTGCTTCTATTCATGGGCA	CACAAGAGCTTTCCAGGAGTG	133
Pip5k1γ	GGGAGGCCATTGAGACAGAT	GCCACGCCCTTTCTTAGATG	278
Talin-1	TCGGAAGTGGCTTGTGTAGT	GAGAACGCCCGAACTAAACG	122
Talin-2	GTGGCAGCTAGAGAAACAGC	GGCTTCTTGGATGAGCATGG	153
β-actin	TTGTAACCAACTGGGACGATATG	GATCTTGATCTTCATGGTGCTAGG	764

Table 2.2. RT-PCR primers

2.5 Reverse transcriptase polymerase chain reaction

A QIAshredder was used for homogenisation of the cell lysate. Following this, using the commercial RNA extraction kit provided by Qiagen, RNeasy Mini Kit, total cellular RNA was extracted. The amount of RNA per sample was determined by absorbance at 260 nm; this was measured using a NanoDrop Lite spectrophotometer (Thermo Scientific). The process of reverse transcription of 1 µg RNA was achieved using an iScript cDNA synthesis kit. The produced cDNA was amplified utilising the GoTaq Green Master Mix. The product of the polymerase chain reaction was separated by electrophoresis on 2% agarose gel. Ethidium bromide staining was used for visualisation of the PCR products when placed under an ultraviolet light. The nucleic acid sequence of the used primers and the cycling conditions are listed in Tables 2.2 and 2.3 respectively. 2.2 and 2.3 respectively.

Gene	Initial Denaturation	Initial Denaturation	Annealing	Extension	Final Extension	Hold
Orai1	95°C /300s/One cycle	95°C 30s 30 cycles	55°C 30s 30 cycles	72°C 30s 30 cycles	70°C 180s 1 cycle	4°C Forever
Orai2	95°C 300s 1 cycle	95°C 30s 30 cycles	54°C 30s 30 cycles	72°C 30s 30 cycles	70°C 180s 1 cycle	4°C Forever
Oria3	95°C 300s 1 cycle	95°C 30s 30 cycles	55°C 30s 30 cycles	72°C 30s 30 cycles	70°C 180s 1 cycle	4°C Forever
STIM1	95°C 300s 1 cycle	95°C 30s 30 cycles	55°C 30s 30 cycles	72°C 30s 30 cycles	70°C 180s 1 cycle	4°C Forever
STIM2	95°C 300s 1 cycle	95°C 30s 30 cycles	55°C 30s 30 cycles	72°C 30s 30 cycles	70°C 180s 1 cycle	4°C Forever
Pip5k1α	95 C 300s 1 cycle	95°C 30s 30 cycles	60°C 30s 30 cycles	72°C 30s 30 cycles	70°C 180s 1 cycle	4°C Forever
Pip5k1β	95°C 300s 1 cycle	95°C 30s 30 cycles	60°C 30s 30 cycles	72°C 30s 30 cycles	70°C 180s 1 cycle	4°C Forever
Pip5k1γ	95°C 300s 1 cycle	95°C 30s 30 cycles	60°C 30s 30 cycles	72°C 30s 30 cycles	70°C 180s 1 cycle	4°C Forever
Talin-1	95°C 300s 1 cycle	95°C 30s 30 cycles	60°C 30s 30 cycles	72°C 30s 30 cycles	70°C 180s 1 cycle	4°C Forever
Talin-2	95°C 300s 1 cycle	95°C 30s 30 cycles	60°C 30s 30 cycles	72°C 15s 30 cycles	70°C 180s 1 cycle	4°C Forever
β-actin	95°C 300s 1 cycle	95°C 60s 30 cycles	56°C 30s 27 cycles	72°C 45s 27 cycles	70°C 180s 1 cycle	4°C Forever

Table 2.3. RT-PCR cycling conditions

2.6 Electrophysiological experiments

Pipettes were coated with Sylgard and fire-polished. The DC resistance of filled pipettes ranged from 3 to 5 MΩ. Pipettes were filled with an internal solution containing 145 mM potassium glutamate, 8 mM NaCl, 1 mM $MgCl_2$, 0.10 mM EGTA and 10 mM HEPES (pH 7.2 with KOH). The external solution was composed of 120 mM NaCl, 20 mM KCl, 2 mM $CaCl_2$, 2 mM MgCl2, 10 mM HEPES and 10 mM D-glucose (pH 7.4 with NaOH). The inwardly rectifying K^+ current was measured at 0 mV at voltage ramps spanning - 100 to + 100 mV in 50 ms. The measured current was normalised to cell size by dividing by the cell capacitance. All recordings were performed using the perforated patch clamp technique using amphotericin B. Amphotericin B stock was mixed with DMSO yielding a final concentration of 60 mg/mL and sonicated in a water-bath sonicator for 20 minutes. The pipette tip was backfilled with 0.30 mg/mL amphotericin B followed by placing the pipette tip into amphotericin B-free standard internal solution for 5-7 seconds to prevent leaking of amphotericin B into the external solution prior to sealing. Once a gigaohm sealing was achieved, access resistance was monitored until it reached between 15 and 20 MΩ and stabilised for 15 minutes. Once resistance was stabilised, recording started.

2.7 Western blots

Cells were washed gently three times with phosphate-buffered saline. The washed cells were scraped using a cell scraper after the application of 70 µl of lysis buffer. The lysis buffer was composed of RIPA buffer supplemented with protease inhibitor cocktail, .01% Triton X-100, 10 mM sodium metavanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF). In cells pre-treated in calcium-free conditions, cOmplete Mini EDTA-free tablets were added to the lysis buffer. The concentration of protein per sample was identified using the DC protein assay kit as per the manufacturers protocol. Using a heating block for 2 minutes at 95°C, 30 µg of protein per sample was quantified and denatured; 30 µl of sample mixed with 2x Laemmli sample buffer 1:1 along with 5 µl of PageRuler molecular weight marker was loaded into 10% SDS-PAGE gel that was made 30 minutes prior to running the gel. The gel ran for 90 minutes at 90v. Protein was next transferred into nitrocellulose membrane using a semi-dry protein transfer apparatus provided by Biorad; 5% non-fat dry milk in phosphate-buffered saline was used to block the membrane for 1 hour at room temperature with mild agitation. The blocked membrane was then washed once with a washing buffer and incubated with the primary antibody 1:2000 for 2 hours at room temperature. Following this, the membrane was washed three times for 10 minutes per wash and then the secondary antibody 1:4000 in 5% non-fat dry milk in phosphate-buffered saline solution for 2 hours at room temperature. Finally, the membrane was washed again three times with 10 minutes for each wash. Visualisation was accomplished by the use of enhanced chemiluminescence plus the Western blotting detection system. The films were scanned using a conventional HP office scanner and the relative

band intensities were analysed using ImageJ [363]. All the bands were normalised to the correspondingβ-actin levels, and all the values were normalised to the maximum intensity.

2.8 Statistical analysis

All experiments were performed on three independent occasions unless otherwise specified in the text. Independent sample groups were first assessed for normality and equality of variances. To compare single-group treatment, an unpaired t-test or Mann-Whitney U test was used (StatsDirect v2.6.2, Sale, UK). Differences were considered significant at $p < 0.05$. All normal and log-normal data were presented in the text and figures as mean ± SEM, * denotes $P<0.05$, **$P<0.01$, ***$P<0.001$ and **** $P<0.0001$.

2.9 Materials

2.9.1 Cell culture

Item	Manufacturer	Catalogue Number	Use/Purpose
DMEM Dulbecco's Modified Eagle Medium	Life Technologies	41965-039	Maintain cultured RBL-2H3 cells

Penicillin-streptomycin	Life Technologies	15140-122	Supplement for cell culture medium
Fetal Bovine Serum	Life Technologies	10270-098	Supplement for cell culture medium
Cell Freezing Medium-DMSO	Sigma	C6164-50ML	Stocking of cells
Trypsin	Life Technologies	25300-054	Cells dissociation during cell culture passaging
DISH Petri 35X10MM easy-grip N-TR PS ST	VWR International Ltd	25373-041	Tissue culture
Corning 100mm TC-treated culture dish	Scientific Laboratory Supplies Limited	430167	Tissue culture

Falcon Disposable Petri Dishes, Sterile, Corning (60 x 15 mm)	VWR International Ltd	25373-085	Tissue culture
Pipettor tip Finntip 10 x 96 rack 1000µL	Thermo Scientific	10399310	Tissue culture
Finntip 250 Universal Sterile Pipette Tips, 0.5 to 250 µL, 10x96/rack	Thermo Scientific	25001-65	Tissue culture
Finntip Pipet Tips 0.2 to 10uL; Pink	Thermo Scientific	9400310	Tissue culture
Centrifuge Tube 15ml	Scientific Laboratory Supplies Limited	SLS 352097	Tissue culture
Centrifuge Tube 50ml	Scientific Laboratory Supplies Limited	734-0448	Tissue culture

2.9.2 Calcium measurement

Item	Manufacturer	Catalogue Number	Use/Purpose
Fura-2-AM	Life Technologies	F1201	Ratiometric calcium indicator
Amaxa Cell Line Nucleofector Kit T	Lonza	VCA-1002	RBL-2H3 cells transfection
Universal Negative control	Life Technologies	AM4611	Transfection of control cells
Coverslip 13mm Diameter	Scientific Laboratory Supplies Limited	12392128	Preparation of cells for imaging

2.9.3 Immunofluorescence

Item	Manufacturer	Catalogue Number	Use/Purpose
SuperBlock (PBS) Blocking Buffer	Cell signalling	37515	Blocking of cells for IHC

Anti-ORAI1 antibody produced in rabbit	Santa Cruz Biotechnology	Sc68895	Primary antibody
Anti-STIM2 antibody produced in rabbit	Cell signalling	49175	Primary antibody
Anti-STIM1 antibody produced in rabbit	Cell signalling	4916	Primary antibody
Anti-Pip5k1c Antibody MAO-R1	Proteintech Europe	15713-1-AP	Primary antibody
Anti-Talin 1 antibody [8D4]	Abcam	ab157808	Primary antibody
Goat anti-Mouse IgG Secondary Antibody, Alexa Fluor 488 conjugate	Life Technologies	A-11029	Secondary antibody
Goat anti-Rabbit IgG Secondary Antibody, Alexa Fluor 488 conjugate	Life Technologies	A-11008	Secondary antibody

Goat anti-Rabbit IgG Secondary Antibody, Alexa Fluor 568 conjugate	Life Technologies	A-11036	Secondary antibody
VECTASHIELD Antifade Mounting Medium with DAPI	Euromex	PB.5150	Microscope slides
SEVENTEEN Lasting Fix Nail Polish	Boots	10088316	Coverslips fixation

2.9.4 Reverse transcriptase polymerase chain reaction

Item	**Manufacturer**	**Catalogue Number**	**Use/Purpose**
RNeasy Mini Kit	Qiagen	74104	RNA extraction
QiaShredders	Qiagen	79654	Homogenisation of cells
iScript cDNA synthesis kit	Biorad	170-8890	RNA reverse transcription
GoTaq Green master mix	Promega	M712	cDNA amplification
GenElute Mammalian Genomic DNA Miniprep	Sigma	G1N10-1KT	Genomic DNA isolation
Hyperladder IV	Bioline	BIO-33056	Molecular weight marker

Ethidium bromide staining	Sigma	E1510	Nucleic acid stain
Agarose	Merck Millipore	193983	Agarose gel

2.9.5 Electrophysiological experiments

Item	**Manufacturer**	**Catalogue Number**	**Use/Purpose**
Glass pipettes	Hilgenberg, Germany	—	Patch clamp
SYLGARD 184 Silicone Elastomer Kit	Dow Corning	—	Coating of Glass pipettes
Amphotericin B	MP Biomedicals	0219504380	Perforated patch initiation

2.9.6 Western blots

Item	**Manufacturer**	**Catalogue Number**	**Use/Purpose**
DC Protein Assay	Biorad	500-0112	Quantification of protein content

RIPA buffer	Sigma	R0278	Cells lysis buffer
Sodium orthovanadate	Sigma	P7626-250MG	Supplement for lysis buffers
Pierce Phosphatase Inhibitor Mini Tablets	Life Technologies	88667	Supplement for lysis buffers
Protease Inhibitor Cocktail	Sigma	P8340	Supplement for lysis buffers
COmplete Mini, EDTA-free, 25 tablets	Roche Diagnostics	11836170001	Supplement for lysis buffers
Triton X-100	Sigma	T8787-100ML	Supplement for lysis buffers
Skimmed milk powder	Sigma	70166-500G	Blocking agent
40% Acrylamide/Bis Solution	Biorad	161-0148	Enhancement of molecular weight protein separation
Nitrocellulose Transfer Membrane- 0.45 um	Cambridge Bioscience Ltd	AC2106	Transfer of protein in Western blot

Extra Thick Blot Paper, 7.5 x 10 cm, 60 sheets	BioRad	170-3965	Transfer of protein in western blot
Tris-Glycine Transfer Buffer	Cell signalling	12539	Transfer buffer
Tris-Glycine SDS Running Buffer	Cell signalling	4050	Running buffer
Tris Buffered Saline	Cell signalling	12498	Washing buffer
Tween 20	Sigma	P7949	Supplement in washing buffer
Anti-Talin 1 antibody [8D4]	Abcam	ab157808	Primary antibody
Anti-ERK2 antibody	Santa Cruz	Sc-154	Primary antibody
Goat anti-mouse HRP-conjugated	Santa Cruz	sc-2005	Secondary antibody
Goat anti-rabbit HRP-conjugated	Santa Cruz	sc-2301	Secondary antibody
PageRuler molecular weight marker	Life Technologies	26619	Protein marker

2.9.7 General chemicals and reagents

Item	**Manufacturer**	**Catalogue Number**
Phosphate buffered saline tablets	Sigma	P4417
Ethanol (Molecular biology grade)	Sigma	E4884
Ethylenediaminetetraacetic acid (EDTA)	Sigma	E4884
D-(+)-Glucose	Sigma	G8270
Calcium chloride	Sigma	21115
Hydrochloric acid	Sigma	318949
2-mercaptoethanol	2-mercaptoethanol	31350-010
10x Phosphate Buffered Saline	Biorad	161-0780
Methanol	Sigma	322415
2-APB	Tocris	1224
Calpain inhibitor II	Sigma	AV6060
Ethylene glycol-bis(2-aminoethylether)-N,N,N,N-tetraacetic acid	Sigma	E3889
Potassium chloride	Sigma	63020
Magnesium chloride	Sigma	63020
HEPES	Sigma	H3375
Wortmannin	Sigma	W1628
1-Oleoyl-2-acetyl-sn-glycerol (OAG)	Sigma	06754

Synta	GSK	—
YM201636	Cayman Chemical	13576
Caesium chloride	Sigma	C4036
Methyl--cyclodextrin	Sigma	C4555
BTP2	Tocris	3939
Gadolinium(III) chloride	Sigma	G7532
Lanthanum(III) chloride	Sigma	449830
LTC_4	Merck Millipore	434692
PIP_2 diC8 (PI(4,5)P2 diC8)	Echelon Biosciences	P-4508
Unlabelled Shuttle PIP Carrier 3	Echelon Biosciences	P-9C3
Phosphatidylinositol 4-phosphate diC8 (PI(4)P diC8)	Echelon Biosciences	P-4008
Sodium chloride	Sigma	S7653
Thapsigargin	Merck Millipore	586005
Sodium hydroxide	Fluka	C990R69

Chapter 3

Characterisation of CRAC channels components

3.1 Introduction

The main aim of this chapter is to characterise the molecular components of CRAC channels in RBL-2H3 cells and their involvement in supporting calcium signals to the physiological agonist LTC_4. Understanding how calcium influx contributes to the maintenance of calcium signals and the activation of mast cells has markedly improved over the past several years, aided by the identification of the stromal interaction molecules, STIM1 and STIM2, and the Orai family of calcium-selective store-operated channels.

The two STIM proteins share significant homology, with the majority of differences appearing in the amino and carboxyl terminals, which are discussed in detail in

Chapter One. In comparison to STIM1, STIM2 has a lower affinity for calcium, mediates a lower level of calcium entry and its connection to Orai1 is hindered by cytoplasmic calmodulin. It has been suggested that STIM2 primarily functions during basal calcium entry in response to external stimuli. In contrast, STIM1 activation necessitates a considerable reduction in the stores' contents [42, 364–366].

It has been consistently shown that endogenous Orai1 mediates calcium entry via CRAC channels in multiple cell types [367–369]. Accordingly, Orai1 has been considered the key component in mediating calcium entry via CRAC channels. While focus has been on Orai1, Orai3 has recently received noteworthy consideration. Although Orai3 plays a role in mediating SOCE, there has been no observable effect on calcium signalling when co-expressing Orai3 with STIM1 in HEK293 cells; however, the calcium signal was successfully rescued in cells deficient in Orai1 [64]. In several reports, it was proposed that Orai3 mediates calcium entry in breast cancer cells, the cell lines of haematological cancers and in allergy and inflammation responses [81, 86, 89], all of which will be discussed below

Studies have found that a knockdown of Orai3 reduced the proliferation and differentiation of cancer cells and attributed this to the cells' failure to progress beyond G1 [85, 86, 370]. Contrary to the observations in breast cancer cells, activation of Orai3 has led to apoptosis in acute myeloid leukaemia (AML) and multi-myeloma cell lines. The anti-AML drug, tipifarnib, acts to induce calcium entry via Orai3, and this finding is supported by the fact that 2-APB synergistically increases the effect of tipifarnib, while gadolinium chloride inhibits its actions [87]. Furthermore, the role of Orai3 has been implicated in neointimal hyperplasia, and Orai3

may be up-regulated in the synthetic phenotype of vascular smooth muscle cells [88]. Orai3 has also been found to mediate calcium influx in store-independent pathways, when stimulated with thrombin [88]. The described effect of thrombin is consistent with earlier descriptions of Orai3 being an integral sub-unit in arachidonate-regulated Ca^{2+} (ARC) channels [84, 371].

Pertaining to the current thesis, research suggests that Orai3 influences the inflammation response. Knockdown of Orai3 in human mast cells, which were isolated during a post-surgical resection of lung tissue, significantly reduced degranulation to levels comparable to Orai1 knockdown [89]. Further evidence supporting the involvement of Orai3 in allergic and inflammatory responses reveal how Orai3 maintains the function of effector T-helper lymphocytes when exposed to reactive oxygen species at the site of inflammation. It has been suggested that reactive oxygen species directly inhibit Orai1 channels by interacting with the cysteine residue located at position 195, which is a process absent in Orai3 [81]. Additionally, Orai3 expression levels are noted to be higher in effector cells when compared to progenitors, and knockdown of Orai3 results in a marked reduction of calcium influx [81].

The family of canonical transient receptor potential (TRPC) channels was introduced as a possible player in maintaining store-operated calcium entry in different cell types [372–376]. When the channel is ectopically expressed, the evidence for the influence of TRPC channels on SOCE appears to depend on the channel's subfamilies and the origins of the cells [377–382]. In terms of placing the findings in a physiological context, few studies have investigated whether TRPC channels contribute to the development of calcium oscillations. In a human submandibular

gland cell line, TRPC1 channels support carbachol-induced calcium oscillations, along with Orai1 [383]. In HEK cells, it was suggested that TRPC3 maintains methacholine-mediated calcium oscillations when Orai1 was blocked [134, 135].

Activation of G protein-coupled cysteinyl leukotriene (cysLT1) receptors on the surface of mast cells with the pro-inflammatory factor LTC_4 leads to the release of calcium through linking to phospholipase C. LTC_4 -mediated calcium oscillations are characterised by early all-or-none repetitive calcium spikes on a low baseline. The opening of CRAC channels is crucial in maintaining LTC_4 -mediated calcium signalling [384]. CysLTs have been linked to bronchospasm, excessive mucus production and improper T helper 2 cells response in asthmatic patients [385, 386].

The rat basophilic leukaemia (RBL-2H3) mast cell line is considered to be an excellent model system for the study of allergy responses [180]. It has been suggested that the functional and structural features of RBL-2H3 cells are observed in differentiated mucosal mast cells [387, 388]. Multiple studies have researched how calcium activation mediates inflammatory responses [187, 389–392]. Only a few studies have addressed the role of SOCE-mediated calcium entry in RBL-2H3 cell activation [74, 89, 382, 393]. These studies looked exclusively at the roles of STIM1/Orai1 or Orai2 in mediating SOCE by using various physiological and pharmacological agonists. This chapter describes the profile of CRAC channel components in RBL-2H3 cells and the roles these components play in mediating LTC_4 -induced calcium signalling. The possible contribution of TRPC3 channels to calcium oscillations, when challenging mast cells with LTC_4, will also be examined. By utilising the calcium-sensitive fluorescent dye Fura-2, pharmacological blockers and small interfering RNA techniques, this study concludes that only STIM1 and

Orai1 contribute to SOCE in RBL-2H3 cells and, therefore, the maintenance of LTC_4 -mediated calcium oscillations.

3.2 Results

3.2.1 Expression of Orai and STIM protein transcripts in RBL-2H3 cells

CRAC channel components have never been fully investigated in RBL-2H3 cells. The expression levels of STIM and Orai proteins were investigated by utilising the RT-PCR technique (Fig 3.1). Both STIM proteins were expressed. The expression of STIM1 and STIM2 was expected, as the proteins act as calcium sensors and are able to detect the depletion of the endoplasmic reticulum calcium stores [34, 35]. The expression levels of Orai1, 2 and 3 were robust in RBL-2H3 cells (Fig 3.1).

3.2.2 Cytosolic calcium changes in response to physiological stimulation of RBL-2H3 cells and the importance of CRAC channels in maintaining oscillatory calcium signals

In a physiological context, the pro-inflammatory LTC_4 binds to CysLT1 receptors on the surface of mast cells and links to the PLC pathway, generating an oscillatory calcium response [394]. In a calcium-containing external solution, Fura-2 loaded RBL-2H3 cells, responded to LTC_4 by generating all-or-none repetitive cal-

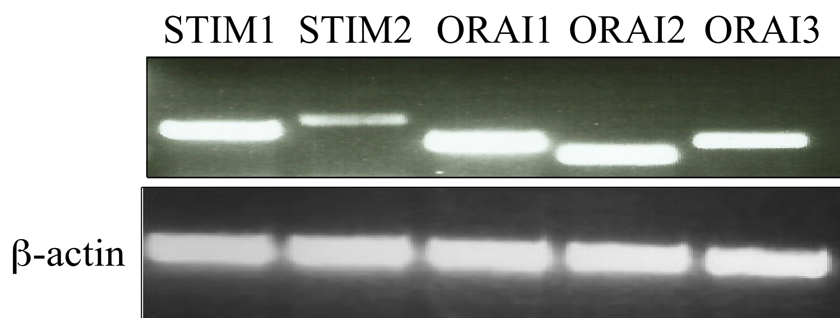

Figure 3.1. RT-PCR shows the presence of CRAC channel components in RBL-2H3 cells
Representative experiment showing the expression levels of STIM and Orai protein transcripts in RBL-2H3 cells. The lower panel shows control β-actin loading.

cium spikes (on a low baseline). The number of oscillations increased in a dose-dependent manner (Fig 3.2A-H).

When cells were stimulated with a submaximal dose of LTC_4, which was found to be 160 nM (Fig 3.2I), oscillations were retained throughout the experiment, which typically lasted for $\approx$ 800 seconds (Fig 3.3A). This response is typical of the LTC_4-mediated calcium signal observed in other mast cell lines [395]. To understand the importance of calcium entry in the calcium oscillations of RBL-2H3 cells, the cells were stimulated with the same dose of LTC_4, while bathed in a calcium-free solution. In contrast, cells stimulated in the absence of external calcium showed a rapid rundown of calcium oscillations (Fig 3.3B). To confirm the role of calcium entry in the oscillatory calcium response induced by LTC_4, the cells were pre-treated for 3 minutes with 10 μM of the calcium channel blocker La^{3+}. La^{3+} is a non-specific calcium channel blocker that block CRAC channels, TRPC,

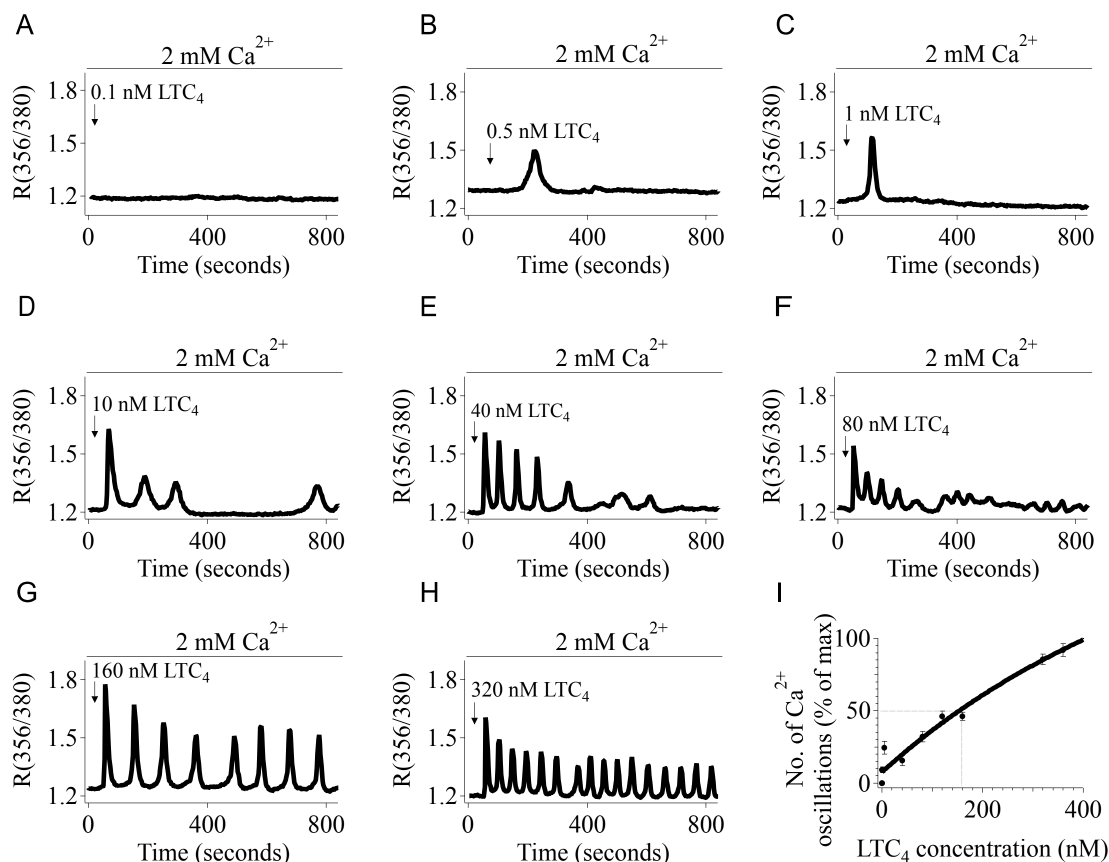

Figure 3.2. LTC_4 dose response curve
(A-L) Representative experiments showing the application of the indicated LTC_4 dose in cells in the presence of external calcium. (M) the number of oscillations was quantified from more than 25 cells from two independent experiments and values were fitted with a Hill-type equation, yielding an EC50 value of 160 nM.

TRPV and both L-type and T-type channels [118]. Research has shown that, in the presence of external calcium, micro-molar concentrations of the trivalent cation La^{3+} blocked CRAC channels [396]. To determine the IC_{50} values for La^{3+}, cells were stimulated with 2 µM of thapsigargin, which is a non-competitive inhibitor of SERCA, in the absence of external calcium [397]. After calcium release, readmission of external calcium resulted in a cytosolic calcium rise due to store-operated

calcium entry. Pre-treatment with La^{3+} for 3 minutes resulted in a dose-dependent reduction in the calcium entry signal (Fig 3.3C). Data were analysed and fitted to yield an IC_{50} value of 0.45 μM (Fig 3.3D).

Application of LTC_4 to La^{3+} pre-treated cells resulted in calcium oscillations that ran down with a time course similar to that observed in a calcium-free solution, despite the presence of external calcium (Fig 3.3E). Compared to cells stimulated in a calcium-containing external solution, there was a 53.1% $\pm$ 5.5% reduction in the number of oscillations in the La^{3+} pre-treated cells during an 800 second recording period; this percentage is similar to the 44.9% $\pm$ 2.3% reduction observed when the cells were stimulated in a calcium-free solution. A reduction in the number of oscillations was noticed 200 seconds after the cells began to oscillate, and no statistical difference in the number of oscillations was observed at earlier time points. Although calcium entry is required for maintaining oscillations, this requirement can be bypassed as it has been found that pre-treating cells with a milli-molar dose of La^{3+} will prevent calcium extrusion by blocking the plasma membrane Ca^{2+} ATPase pump and thus maintain repetitive calcium oscillations [394]. Accordingly, when the cells were stimulated with a submaximal dose of LTC_4, in the absence of external calcium, calcium oscillations were maintained because the released calcium was actively transported back to the ER via sarco-endoplasmic reticulum calcium transport ATPase (SERCA) pumps to be available for the next IP3-mediated oscillatory calcium response (Fig 3.3F). Aggregate data from 3 independent experiments are shown in in Fig 3.3G. Although oscillations can be maintained without any calcium influx, under these non-physiological conditions, it is important to note that it is not the calcium oscillations but the spatially

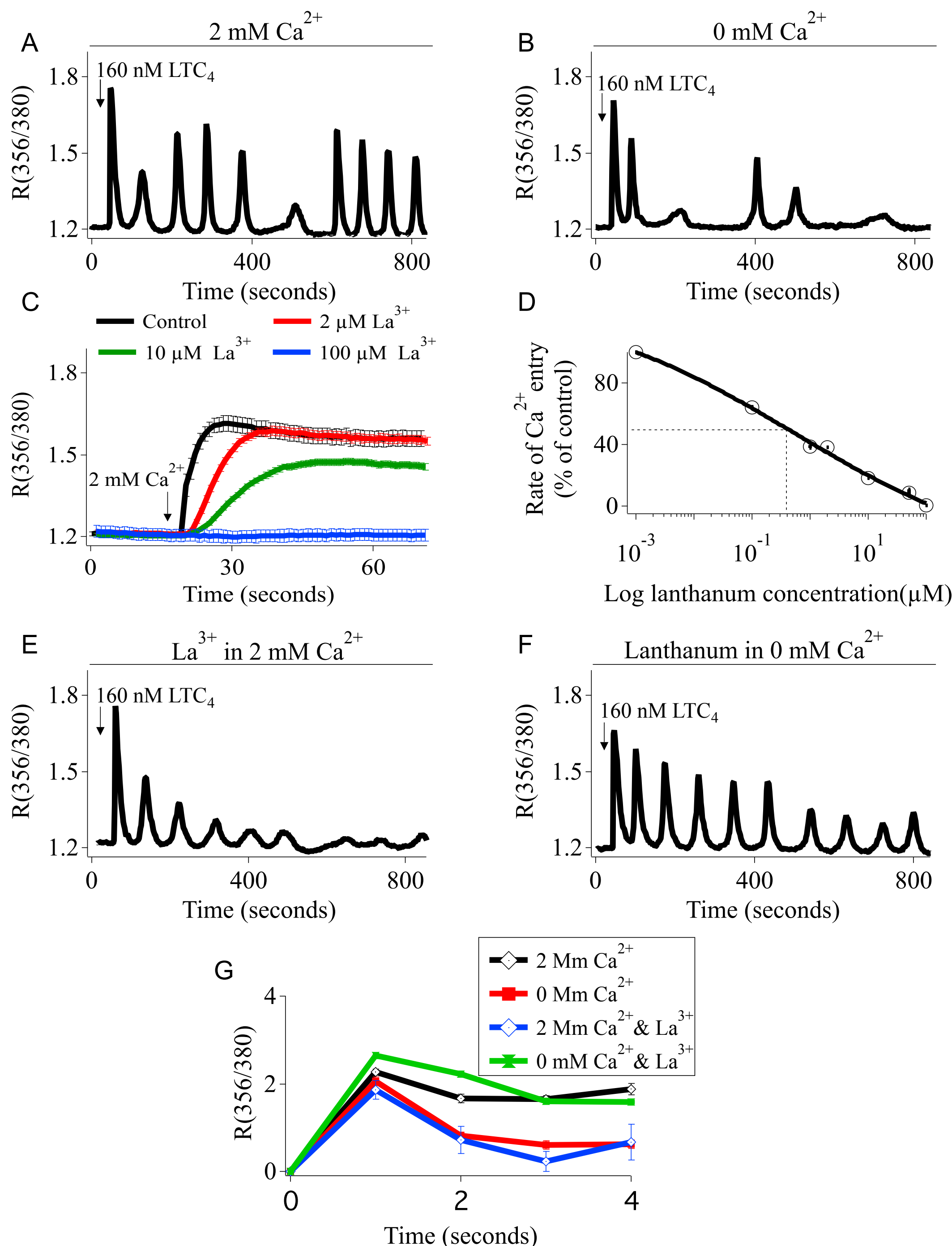

Figure 3.3. Cytosolic calcium changes in response to physiological stimulation and the importance of CRAC channels in maintaining oscillatory calcium signals

Representative calcium oscillations in RBL-2H3 cells (A) following the application of 160 nM LTC_4 in the presence of external calcium. (B) The same concentration of agonist was used, but calcium was absent from the bath solution. (C) CRAC-mediated calcium entry was blocked in a dose-dependent manner, following pre-treatment with lanthanum, and the CRAC channel was activated with thapsigargin with the addition of calcium. (D) Lanthanum dose inhibition curve. (E) Oscillations in cells stimulated with LTC_4 in the presence of external calcium but pre-treated with 10 µM lanthanum for 3 minutes in order to block CRAC channels. (F) Cells were pre-treated with 1 mM of lanthanum for 3 minutes to prevent calcium extrusion, by blocking the plasma membrane calcium ATPase pump and stimulating with an LTC_4 in a calcium-free solution. (E) Collective data from more than 50 cells for each treatment are shown from three independent experiments. Each bin number represents a period of 200 s.

restricted calcium signals adjacent to the open CRAC channels that activate gene expression in response to multiple transcription factors [398].

3.2.3 Orai3 does not contribute to the maintenance of the oscillatory calcium response in RBL-2H3

To explore whether Orai3 contributes to calcium entry via CRAC channels in RBL-2H3 cells, Fura-2 imaging experiments were carried out. Single-molecule photo-bleaching studies have reported that stimulation with 2-APB activates the channel in its dimeric state [399]. 2-APB is believed to potentiate Orai3 activity by increasing the channel's pore size thus promoting calcium influx without store depletion [82, 115]. To test the potential involvement of Orai3 in LTC_4 -driven responses, 2-APB was applied to RBL-2H3 cells bathed in an external solution to activate Orai3 (Fig 3.4A). A large calcium rise occurred, demonstrating the functional presence of Orai3 in these cells, consistent with the RT-PCR data of Fig 3.1. To see if the Orai3-dependent response exhibited a different pharmacology to that of Orai1, I turned to Synta. This molecule is an effective inhibitor of Orai1 [89, 125]. Consistent with this, pre-treatment with Synta for 5 minutes effectively reduced the observed rise in cystolic calcium signalling, following the readmission of calcium to cells pre-treated with thapsigargin, when compared to the control group (Fig 3.4B). Aggregate data from 3 independent experiments are shown in Fig 3.4C. In contrast to the Orai1-mediated calcium signal, the calcium signal observed following 2-APB application was not affected by the incubation of cells with Synta for several minutes prior to the activation of Orai3 (Fig 3.4D). Data from 3 independent experiments are shown in Fig 3.4E.

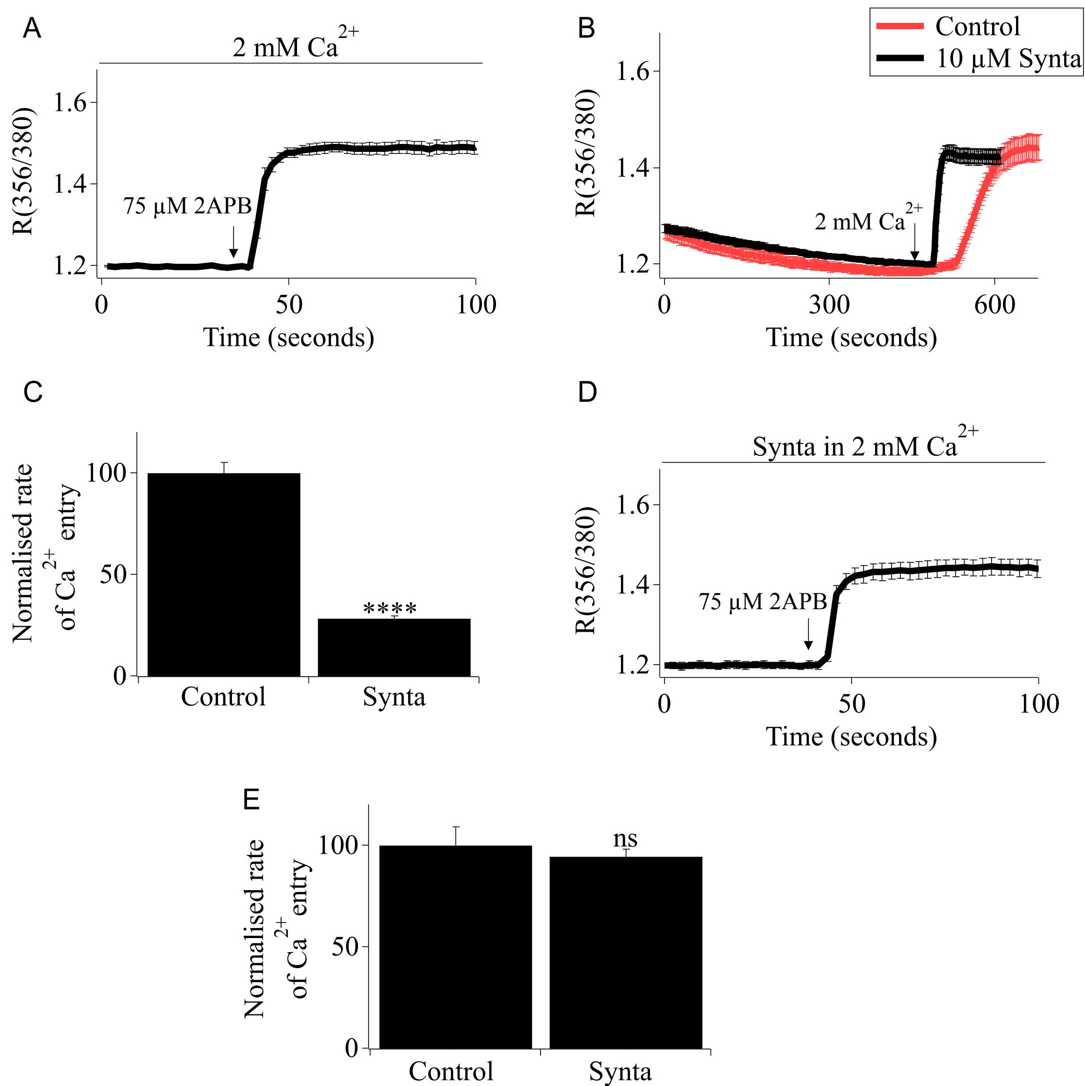

Figure 3.4. The specific Orai1 blocker, Synta, has no effect on Orai3 activation
(A) Representative experiment in RBL-2H3 cells comparing the rate of Ca^{2+} entry following the application 75 µM 2-APB in the presence of external calcium. (B) The effect of Synta on calcium entry via Orai1 is compared in control cells and cells pre-treated with Synta for several minutes following depletion of internal store by thapsigargin and calcium application. (C) The rate of Ca^{2+} entry was quantified from three independent experiments and shown in the histogram. (D) Representative experiment showing the rate of Ca^{2+} entry following the application of 75 µM 2-APB in cells pre-treated with 10 µM Synta. (E) The rate of calcium entry was quantified from three independent experiments and shown in the histogram.

To explore the possibility of Orai3 playing a role in LTC_4 -mediated calcium oscillations, cells were pre-treated with Synta for five minutes prior to stimulation with 160 nM of LTC_4 in the presence of external calcium (Fig 3.5A). The pattern of the obtained calcium signal was comparable to that observed when control cells were stimulated in the absence of external calcium, which resulted in a reduction in the number of oscillations $\approx$ 200-300 seconds after the cells began to oscillate (Fig 3.5B). The number of oscillations from 3 independent experiments were quantified, and there were no statistically significance differences between cells stimulated with LTC_4 and bathed in either a calcium-containing external solution combined with Synta or in a calcium-free solution (Fig 3.5C). From this set of experiments, it is clear that Orai3 does not contribute to the oscillatory calcium signal activated by LTC_4 . Instead, Orai1 appears to be essential.

3.2.4 The role of Orai1 in maintaining LTC_4 -mediated calcium oscillations in RBL-2H3

To investigate further the role of Orai1 expression on LTC_4 -driven calcium oscillations, I turned to an siRNA knockdown approach. Protein levels were measured using an immunocytochemistry technique. Analysis of the results showed a reduction in protein expression of $\approx$ 60%, following knockdown of Orai1 (Fig 3.6A). A summary of the aggregate data from 3 independent experiments, which included 40 cells, is shown in the histogram in Fig 3.6B. The rate of rise in the cytoplasmic calcium signal was measured following the application of calcium to cells pre-treated with thapsigargin and bathed in a calcium-free solution. The rate of calcium entry in Orai1 knockdown cells was reduced by 55%, when compared to control cells

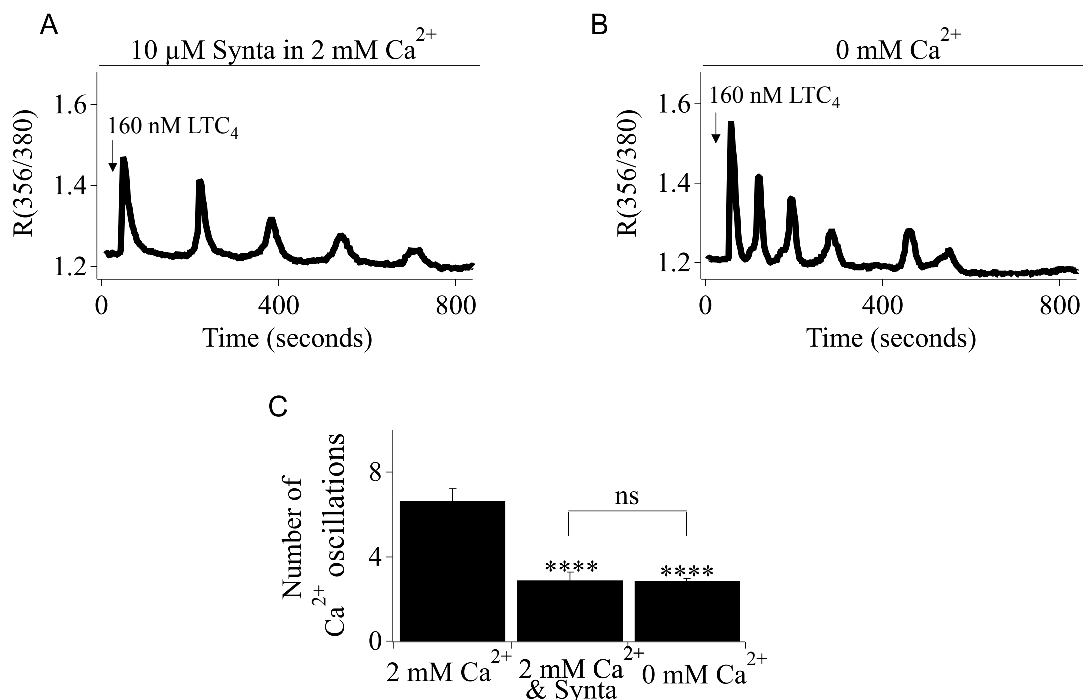

Figure 3.5. Orai3 has no role in maintaining the oscillatory calcium response in RBL-2H3

Representative calcium oscillations in RBL-2H3 cells (A) following the application of 160 nM of LTC_4 in the presence of external calcium in cells pre-treated with 10 mM of Synta. (B) Control cells were treated with the same concentration of LTC_4 But with no Ca^{2+} in the external solution. (C) The number of LTC_4 -mediated calcium oscillations from three independent experiments were quantified over a period of 800 seconds and shown in the histogram.

transfected with scrambled siRNA (Fig 3.6C, D).

The application of 160 nM LTC_4 in Orai1 knockdown cells induced repetitive oscillatory calcium signals that were similar to mock-transfected cells, with the exception that they ran down more rapidly and yielded a smaller number of total oscillations (Fig 3.7A). Aggregate data from 3 independent experiments are shown in the histogram in Fig 3.7B. The observed rundown of calcium oscillations could not be attributed to changes in store calciuum content, because the calcium signal induced by thapsigargin in Orai1 knockdown cells was comparable to the calcium

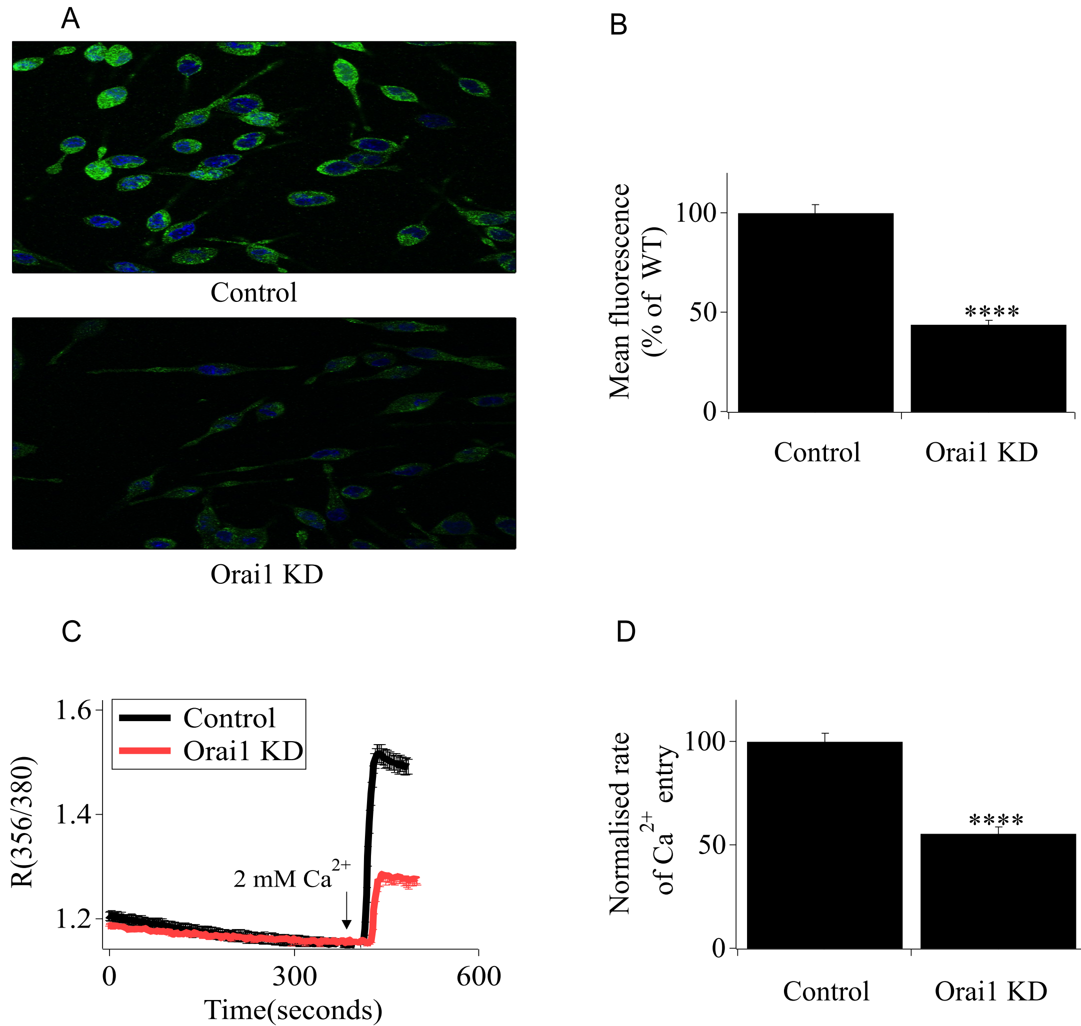

Figure 3.6. Knock down of Orai1 in RBL-2H3 cells

(A) Orai1 protein expression images obtained using a confocal microscope in mock-transfected cells and in those cells in which Orai1 had been knocked down (B) Histogram shows aggregate data from three independent experiments (each bar is the average from 50 cells). (C) Representative experiment showing the average rate of Ca^{2+} release and entry in mock-transfected cells and in those in which Orai1 had been knocked down. Data from three independent experiments were quantified and shown in the histogram in panel D.

signal in mock-transfected cells (Fig 3.7C). Additionally, the number of calcium oscillations induced by LTC_4 in Orai1 knockdown cells stimulated in an external solution that lacked calcium was similar to the number seen in mock-transfected cells (Fig 3.7D, E). Therefore, one can conclude that the quicker rundown of calcium oscillations in Orai1 deficient cells is due to the loss of calcium entry. These observed findings are consistent with observations of the RBL-1 cell line, in which it has been found that Orai1 is responsible for mediating LTC_4 -induced calcium oscillations and NFAT-driven gene expression [395]. Studies have also shown that Orai1 is responsible for calcium influx in RBL-2H3 cells or human lung mast cells stimulated with antigen [89, 393].

3.2.5 LTC_4 -mediated calcium oscillations are maintained by STIM1 in RBL-2H3

To complete the profile of CRAC channels in supporting the oscillatory signal of LTC_4 , the roles of STIM1 and STIM2 were investigated. First, an siRNA approach was used to knockdown the proteins, and expression levels were assessed by immunocytochemistry (Fig 3.8A,B). A reduction in protein expression of 50% was achieved for both STIM1 and STIM2, when compared to mock-transfected cells as shown in the corresponding histograms in Fig 3.8A and B.

When cells were bathed in a solution containing calcium and stimulated with a submaximal dose of LTC_4 , there were fewer calcium oscillations in STIM1 knockdown cells (Fig 3.9A and B). In contrast, knockdown of STIM2 appeared to have no effect on the oscillatory signal and number of oscillations, which were compa-

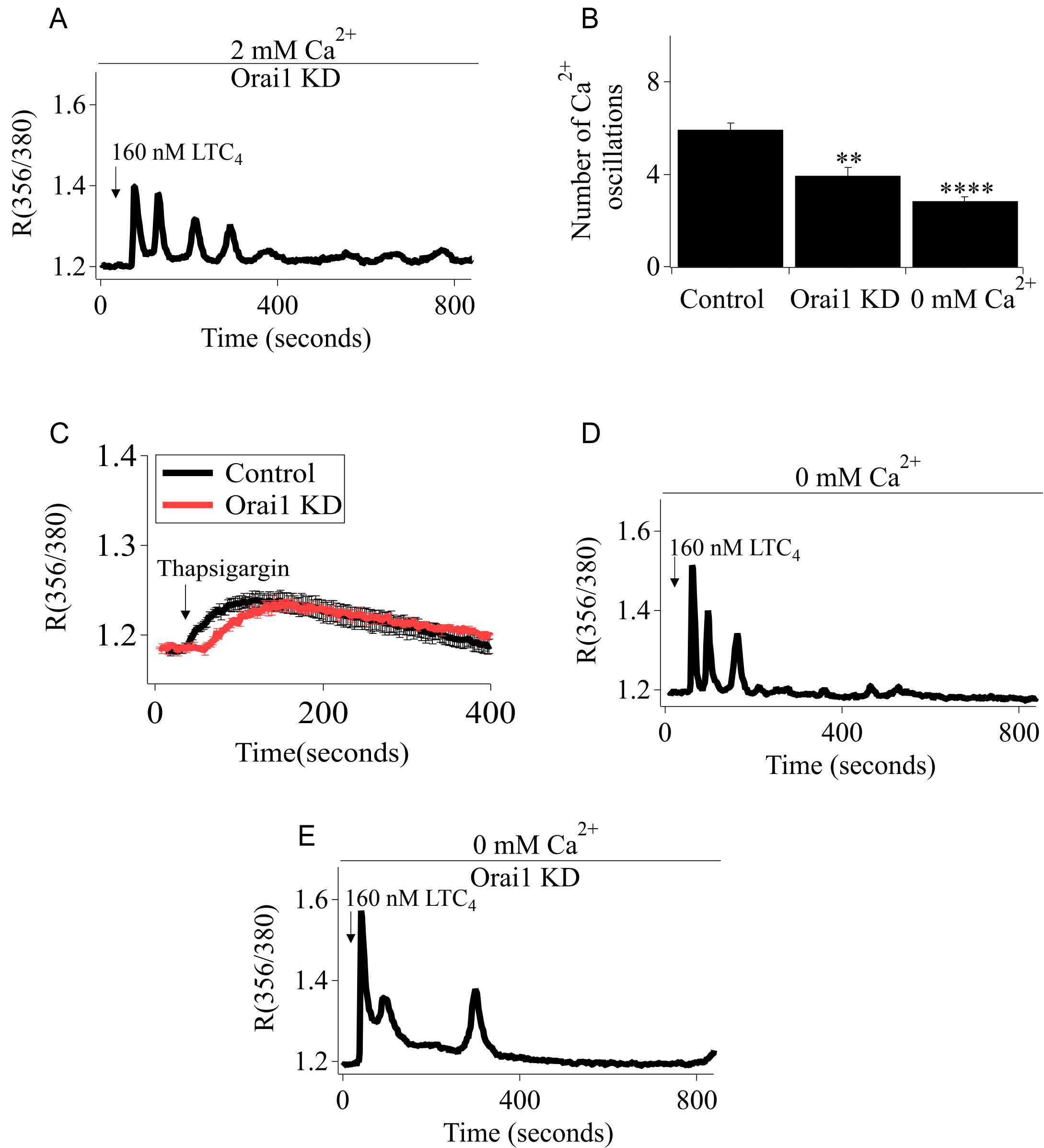

Figure 3.7. Role of Orai1 in LTC_4 -mediated calcium oscillations in RBL-2H3 cells Representative calcium oscillations in RBL-2H3 cells following stimulation with 160 nM LTC_4 in Orai1 knock down cell. Aggregate data from three independent experiments are shown in B. (C) Rate of calcium release induced by 2 μM thapsigargin in wild type cells and in Orai1 KD cells. (D) Representative calcium oscillations in the absence of external calcium in control cells and (E) Orai1 knock down cells.

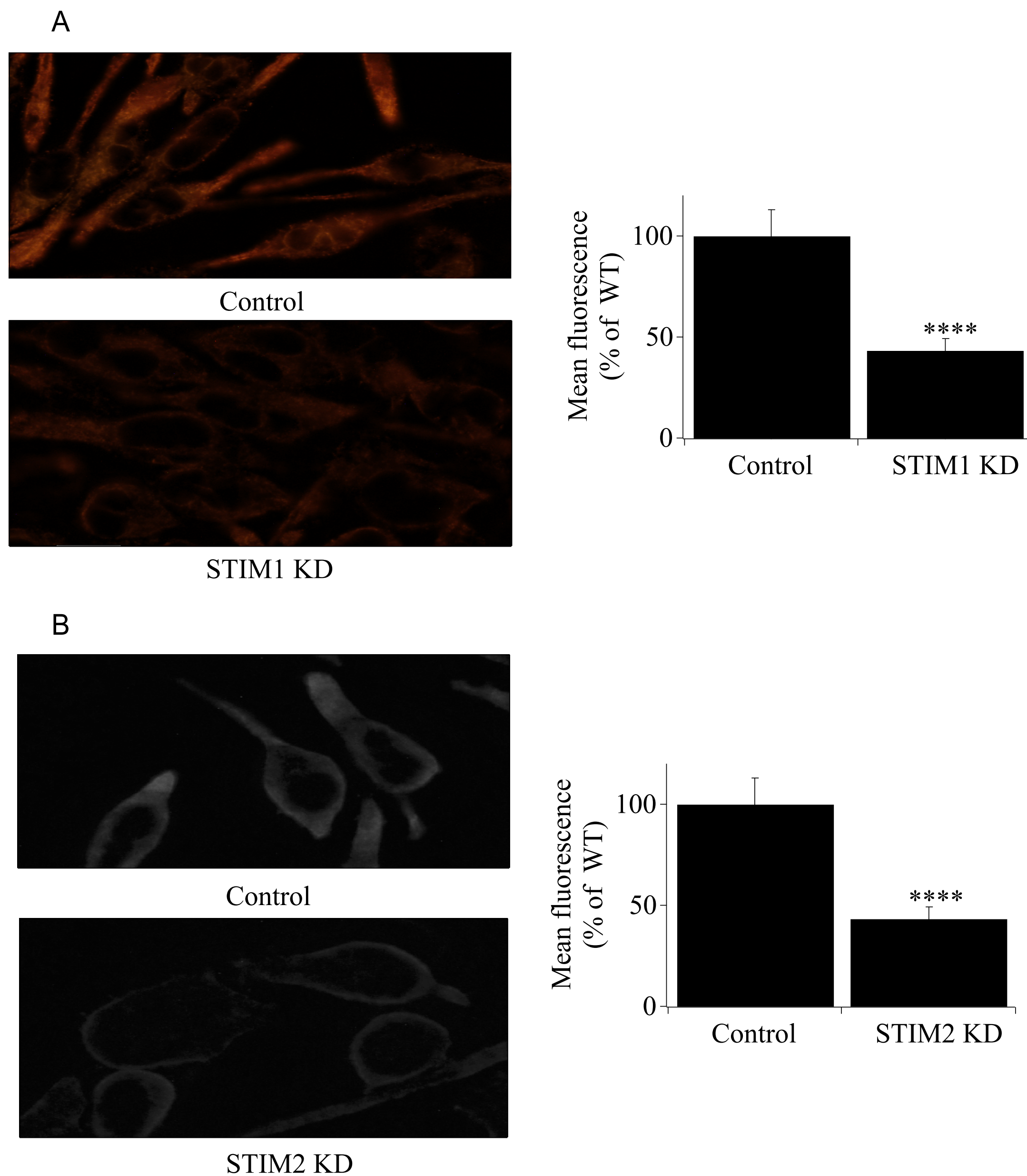

Figure 3.8. Knocked down of STIM proteins in RBL-2H3 cells

(A) Representative images of Immunohistochemical staining of mock-transfected cells or knock-down cells of STIM1 and STIM2 measured by fluorescence microscopy. Quantitative measurements of mean florescence from two independent experiments for STIM1 are shown in B and for STIM2 as shown in C.

rable to those seen in mock-transfected cells (Fig 3.9C and D). It is important to note that the reduction in oscillations seen in STIM1 knockdown cells cannot be attributed to changes in intracellular stored content because knockdown of STIM1 had no effect on the calcium signal when the cells were stimulated in the absence of external calcium (Fig 3.9E).

3.2.6 TRPC3 channels do not maintain the oscillatory calcium response in RBL-2H3

Since the role of CRAC channels in supporting calcium oscillations induced by LTC_4 has been established, whether other plasma calcium permeable channels could imitate Orai in supporting the calcium signal induced by LTC_4 was examined. Members of the TRPC family of channels were originally sought as potential candidates for SOCE, prior to the discovery of STIM and Orai proteins [400, 401]. Based on multiple functional and structural properties, TRPC are divided into 2 broad categories. The first category contains TRPC1, 4 and 5, while TRPC3, 6 and 7 form the second category [402]. The second category is believed to be sensitive to diacylglycerides and not dependent on store depletion [403, 404].

TRPC3 channels were overexpressed in RBL-2H3 cells. To activate TRPC3, cells were exposed to a cell membrane permeable analogue of DAG, namely OAG [403]. In cells bathed in a calcium free solution, 100 µM of OAG was applied, followed by the addition of calcium to the solution. The application of OAG did not induce calcium release when external calcium was absent, but the addition of calcium to the bath solution caused a prominent calcium rise (Fig 3.10A). Following the same

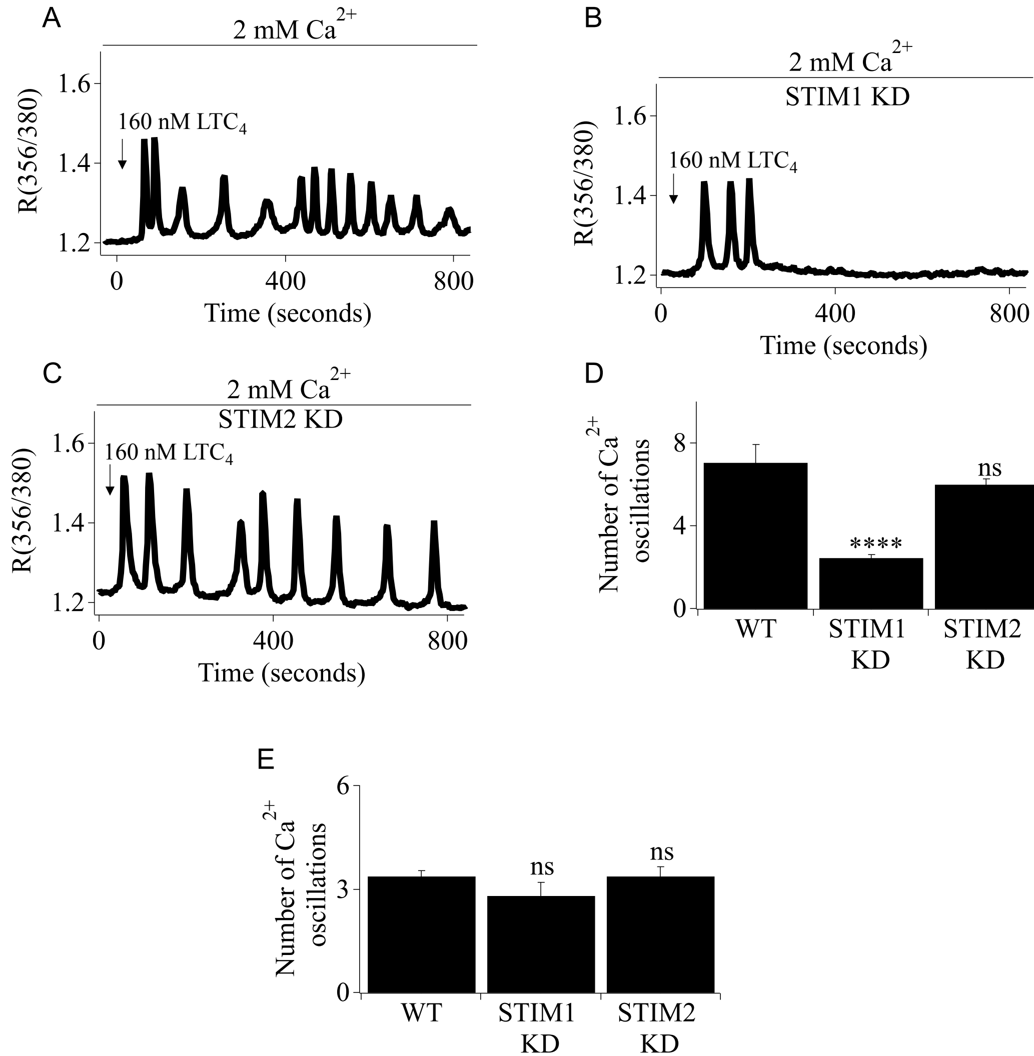

Figure 3.9. Role of STIM proteins in maintaining the oscillatory calcium signals Representative calcium oscillation in a cell stimulated with a submaximal dose of LTC_4 in the presence of 2 mM external calcium in (A) mock- transfected cell, (B) STIM1 knock down cell or (C) STIM2 knockdown cell. Data from two independent experiments are analysed and shown in D. Data from two independent experiments where mock transfected cells, STIM1 or STIM2 knockdown cells were stimulated with LTC_4 in the absence of external calcium, are analysed and shown in the histogram in E.

protocol but for non-transfected cells, the addition of calcium failed to induce any rise in the calcium signal (Fig 3.10A). When calcium was directly applied, without prior application of OAG, to TRPC3-transfected cells, no calcium rise was recorded (data are not shown). To test the contribution of TRPC3 to calcium oscillations, a blocker that effectively blocks Orai1 with no effect on TRPC3 needed to be found. Using the above protocol, OAG was added to the cells bathed in a calcium-free solution and calcium was readmitted, but following pre-treatment with various calcium channel blockers. The rate of calcium entry was quantified from multiple independent experiments and is shown in Fig 3.10B. The specific Orai1 blocker, Synta, but not La^{3+} or Gd^{3+} , failed to alter the rate of the TRPC3 mediated calcium entry, as shown in Fig 3.10C. To test if TRPC3 can maintain and rescue LTC_4 -mediated calcium oscillations in RBL-2H3 cells, following pre-treatment with Synta to block Orai1, TRPC3 over-expressing cells were bathed in a calcium containing solution, and Synta was added for five minutes prior to stimulation with LTC_4 . Oscillatory calcium responses were transient, and an early rundown of the oscillations was clearly observed (Fig 3.10D).

Consistent with multiple reports, TRPC3 channels are permeable to calcium when activated by OAG [203, 403, 405]. In this study, TRPC3 failed to support the calcium signal when the cells were stimulated with LTC_4 .

3.3 Key findings of this chapter

- All CRAC channels components are expressed in RBL-2H3 cells.
- Challenging CysLT1 receptors on the surface of mast cells with a submaximal

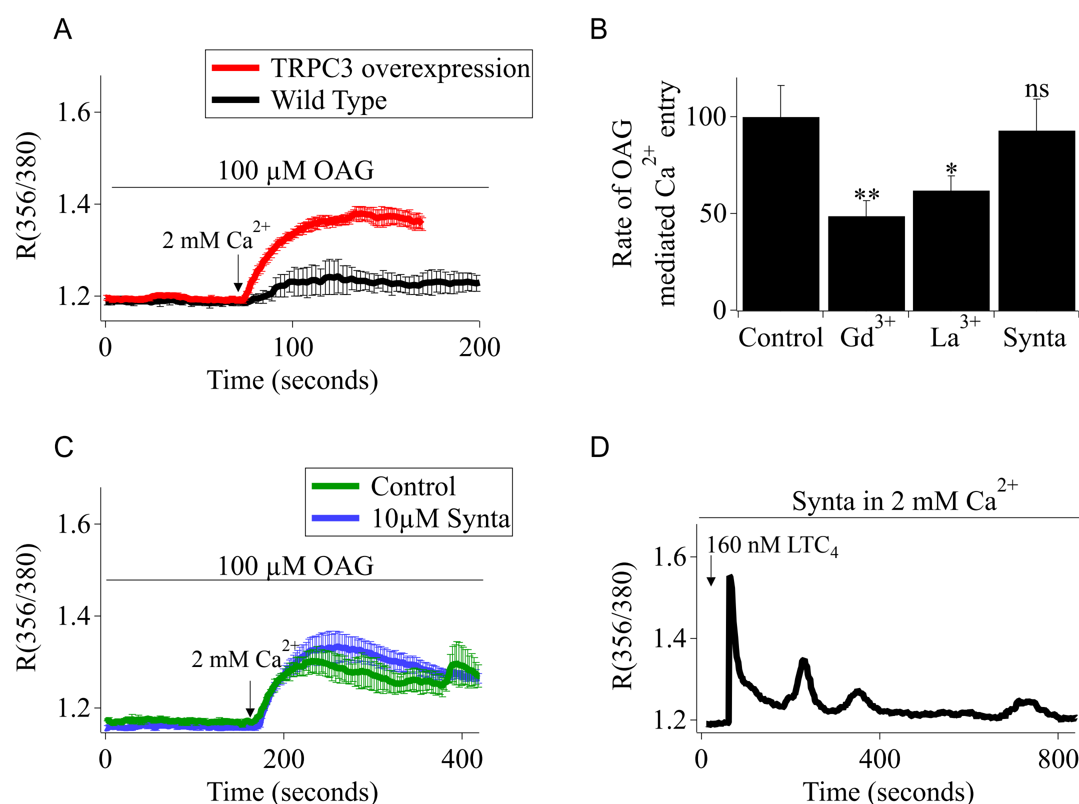

Figure 3.10. TRPC3 do not maintain the oscillatory calcium response in RBL-2H3 (A) Ca^{2+} influx signals in response to OAG for wild type cells and for cells expressing recombinant human TRPC3 channels. (B) Aggregate data from two independent experiments showing the effect of gadolinium, lanthanum and Synta effect on TRPC3-mediated calcium (C) Synta has no inhibitory effect on Ca2+ influx signals in response to OAG for cells expressing recombinant human TRPC3 channels when compared to Synta un-treated cells. (D) Blocking CRAC channels with Synta in cells expressing TRPC3 in the presence of external calcium did not rescue the oscillatory calcium response when cells were stimulated with LTC_4 .

dose of LTC_4 leads to the generation of calcium oscillations in RBL-2H3 cells.

- Maintenance of LTC_4 -driven calcium oscillations is dependent on calcium entry via CRAC channels in RBL-2H3 cells.

- Calcium oscillations were only dependent on calcium entry via Orai1 but not Orai3 or other plasma membrane calcium permeable channels.

- Gating of STIM1 but not STIM2 to Orai1 was shown to be essential for the

continuity of LTC_4 -driven calcium oscillations.

3.4 Discussion

Mast cells respond to variety of stimuli and have been used as a model to investigate IP_3 -mediated calcium oscillations with a particular focus on CRAC channels role in supoorting these oscillations [369]. Although calcium entry via CRAC channels was shown to be essential for mutiple stimuli in RBL-2H3 cells [74, 89, 382, 393], here I focus focus primarily on the role of CRAC channels components in LTC_4 driven calcium oscillations.

Orai3 was expressed in both mast and T-cells, and is believed to mediate SOCE in both human lung mast cells and marrow-derived mast cells [74, 81]. Vasopressin-induced calcium oscillations in hepatocytes were shown to be partially dependent on Orai3 [406]. Moreover, Orai3 was shown to contribute to the development of calcium oscillations in HEK 293 cells when stimulated with an agonist that links to the M3 muscarinic receptors [407]. These oscillations were shown not to be solely dependent on Orai3 as Orai1 was suggested to be required in order to form a functional calcium permeable pore by interacting with Orai3. My results point towards no role of Orai3 in the LTC_4 driven calcium signal (Fig 3.5).

Orai1 was shown to mediate SOCE in mast cells and, when knocked down, significantly reduce SOCE in response to thapsigargin, which is a non-competitive inhibitor of SERCA pumps, or the cross-linking of antigens to type 1 Fc epsilon receptors [75, 191]. Accordingly, I went on to examine the role of Orai1 in supporting these calcium oscillations in RBL-2H3 cells, and measurements of the cyto-

plasmic calcium signal were taken, in response to LTC_4 coupling to its receptors in RBL-2H3 cells (Fig 3.7). I first utilised a pharmacological approach using Synta. Synta is believed to be a potent Orai1 blocker [89, 125] and is compound 66 from the WO2005/009954 A2 patent (3-fluoropyridine-4-carboxylic acid (2,5-dimethoxy biphenyl-4-yl)amid). Consistent with the results here, Synta has been reported to successfully block Orai1 in mast cells and other immune cells [125, 408]. The role of calcium entry via Orai1 in supporting LTC_4 -driven calcium oscillations was further confirmed by knockdown approach (Fig 3.7) and was in agreement with the results obtained using synta. My findings are here are in agreement with what with what has been reported for LTC_4 driven calcium oscillations in orai1 knockdown cells in RBL-1 cell line [395].

Despite the fact that both STIM proteins are recognised as an ER calcium sensors, it was proposed that they function differently in resting conditions and in response to different physiological stimuli [395, 409]. Expression levels of STIM2 in RBL-2H3 cells were lower than those of STIM1 (Fig 3.1). This finding is consistent with with those described by Oh-Hora et al, where lower levels of expression of STIM2, in comparison to STIM1, have also been observed in other immune cells [99]. Challenging cysLT1 receptors induced calcium oscillations that recruited STIM1 (but not STIM2) to open CRAC channels and support the continuity of the calcium oscillations (Fig 3.8). These findings are consistent with previous findings, as knockdown of STIM2 had no effect on the pattern or number of calcium oscillations in HEK293 when stimulated with a muscarinic receptors agonist [364]. On the contrary, knockdown of STIM1 was shown to induce oscillations that were transient and comparable to those observed when cells were stimu-

lated in calcium-free conditions [364]. More specifically, in RBL-1 cells, where LTC_4 induced calcium responses via gating of STIM1 to Orai1, it was proposed that for CysLTs , unlike agonists that couple to the tyrosine kinase-coupled FcεRI receptors, LTC_4 mediated calcium oscillations were not mediated through recruitment of STIM2 [395]. This finding was confirmed in various cell types, and the agonist type was proposed as the main determinant of STIM protein recruitment [410].

TRPC3 channels are activated by DAG generated by PLC activation [411]. Overexpression of TRPC3 in RBL-2H3 cells failed to maintain the oscillatory calcium signal when Orai1 was blocked (Fig 3.10). Two previous reports from the same research group have successfully maintained the oscillatory calcium signal induced by methacholine in TRPC3-overexpressing HEK cells [134, 135]. One possibility for the observed discrepancy between my results and and those reported is that the experimental protocol used in both studies differs to the one followed here; the calcium blocker gadolinium was added more than 30 minutes after stimulation with methacholine, whereas in my study, Synta was added prior to stimullation with LTC_4 . Another explanation is that in contrast to LTC_4 , methacholine is believed to be a potent agonist, thus possibly generating more active PLC [135]. Moreover, the subcellular localisation of PLC is known to be different depending on the tissue and agonist type [412–415]. Finally, the observed calcium rise in methacholine stimulated HEK cells was attributed to ARC channels, which are believed to induce ten-fold higher levels of the PLC when compare to CRAC channels [416, 417].

RBL-2H3 cells express Orai2 (Fig 3.1). In agreement with this, Orai2 expression

at the level of mRNA was previously observed in immune cells, such as B cells, neutrophils and monocyte [418]. Orai2 is believed to play a minor role in the SOCE of immune cells with two exceptions: (1) Orai2 was shown to be the primary mediator in dendritic cells whose calcium entry was mediated by CRAC channels [419], and (2) Antigen-mediated exocytosis of secretory granules is shown to be dependent on Orai2 [420]. One limitation of the work described in this chapter is omitting to examine the physiological role of Orai2. Unfortunately, I'm not alone in disregarding Orai2 when examining CRAC channels' function as Hoth et al has shown that Orai2 has the lowest number of publications that investigate its role when compared to the other Orai homologues and referred to it as the neglected CRAC channel protein [73]. This was attributed to several reasons: one, is that no specific antibodies or pharmacological activator of Orai2 have been described to date, making it difficult to work with [75]. Moreover, early knockdown studies of Orai2 that showed no major functional contribution to calcium entry via CRAC channels in conjunction with the lack of identified physiological role in most cell types and bodily tissues made it less attractive for many researchers to pursue [70, 73].

In this chapter I have investigated the physiological function of CRAC channels' components in RBL-2H3 cells. Measurement of cytoplasmic calcium concentrations, in response to stimulation of cysteinyl leukotriene type I receptors, suggest that store-operated calcium entry is maintained via CRAC channels in RBL-2H3 cells. STIM2 does not maintain calcium oscillations, which supports previous reports and strengthens the theory that STIM2 functions as a housekeeping protein in resting conditions or in the presence of weak stimuli. Findings from small inter-

fering RNA, in conjunction with pharmacological blockers of Orai1 and activation of Orai3, suggest that Orai3 also plays no role in the physiological pattern of calcium oscillation in RBL-2H3 cells. Finally, the calcium signal induced by Orai1 could not be mimicked by overexpression of TRPC3 channels. This chapter concludes that calcium oscillations are therefore critically dependent on Orai1 and other plasma membrane calcium channels can not substitute it. My findings here serve as a solid platform to go on and investigate the precise role of Orai1 in supporting calcium oscillations.

Chapter 4

Role of calcium influx through CRAC channels in phosphoinositide production

4.1 Introduction

In the previous chapter, I found that the maintenance of calcium oscillations in RBL-2H3 cells was dependent on calcium entry via Orai1 channels. The aim of this study is to better understand how CRAC channels are involved in receptor-driven calcium responses; I investigate whether the opening of CRAC channels regulate the production of cellular phosphoinositides.

Calcium oscillations in non-excitable cells are initiated following IP_3-mediated calcium release from the internal stores, which are mainly the endoplasmic retic-

ulum [421]. Two models for the generation of calcium oscillations have been suggested: (i) IP_3 levels are constant but positive, and negative feedback of calcium on the IP_3 receptor will induce the opening and inactivation of the IP_3 receptor; (ii) IP_3 levels oscillate via positive and negative feedback of calcium levels on phospholipase C [422, 423]. The opening of CRAC channels is an important modulator of calcium oscillations, as there is a need for calcium entry to refill the internal stores, as well as to provide a local increase of calcium signals, which are subsequently relayed to the nucleus to initiate calcium-dependent gene expression via calcium-dependent transcription factors [394].

PIP_2 availability is crucial to the function of many ion channels, including CRAC channels [243–246, 359, 372, 424, 425]. PIP_2 has been linked to cytoskeletal structure, secretion and exocytosis, trafficking of vesicles and gene expression [426]. Moreover, PIP_2 has been linked to and was suggested to have an essential role in mediating allergies and inflammation [247–250]. It has also been linked to the proliferation of lymphocytes following antigen interaction and in oxygen reactive species production and chemotaxis in neutrophils [427]. PIP_2 has also been found to play a role in mast cell function at multiple stages and pathways of the inflammatory process [252–254, 259].

PIP_2 is the most abundant phosphoinositide, constituting more than 1% of the total plasma membrane phospholipids [428]. Most of the PIP_2 present in the cell is produced by the phosphorylation of phosphatidylinositol 4-monophosphate (PI4P) by type I phosphatidylinositol-4-phosphate 5-kinases (PIP5KIs) [291, 292, 429]. The phosphorylation of phosphatidylinositol 5-monophosphate (PI5P) via the catalytic action of type II phosphatidylinositol-5-phosphate 4-kinases (PIP4KIIs) con-

tributes to the production of PIP_2 to a much lesser extent [280, 430, 430]. Furthermore, the dephosphorylation of PI(3,4,5)P_3 by phosphatase and tensin homologues on chromosome 10 (PTEN) also leads to the production of PIP_2 [431]. PIP_2 and its precursors are all members of the seven polyphosphoinositides family. All the other polyphosphoinositides, including PIP_2 , come from phosphatidylinositol. Phosphatidylinositol is produced in the endoplasmic reticulum where its two precursor synthesising enzymes are located: phosphatidylinositol synthase (PIS) and CDP-diacylglycerol synthase (CDP-DAG synthase) [432]. Phosphatidylinositol's two precursors are myo-inositol and CDP-DAG. Phosphatidate cytidylyltransferase (CTP) with PtdOH leads to the production of CDP-DAG by CDP-DAG synthase [220]. Myo-inositol can be produced by glucose-6-phosphate via myo-inositol-3-phosphate synthase [221]. Myo-inositol can also be recycled from the produced inositol following the linking of agonists to phospholipase C [433].

In this chapter I find that LiCl treatment leads to a gradual inhibition of calcium signalling, which is a consequence of reduction of PIP_2 and thus IP_3 levels. LiCl is believed to inhibit two important mediators in the inositol re-synthesis cycle, inositol polyphosphate 1-phosphatase (IPPase) and inositol monophosphatase (IMPase) (Fig 4.1). IPPase catalyses the conversion of inositol 1,4-bisphosphate (IP_2) into inositol monophosphate (IP), and IMPase catalyses the conversion of IP into myo-inositol [433, 434]. Surprisingly, the application of myo-inositol in LiCl-pre-treated cells rescued calcium oscillations only when there was an influx of calcium. This simple manipulation of PIP_2 levels has enabled me to directly link CRAC channelss opening to regulation of the PI pathway. To confirm my findings, in the last section of this project, the measurement of PIP_2 levels was at-

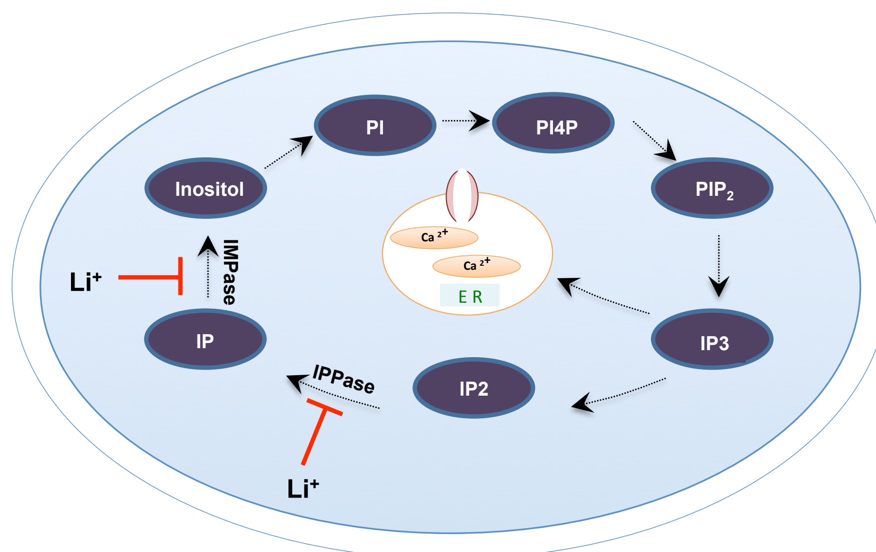

Figure 4.1. The molecular action LiCl
The proposed molecular action site of LiCl within the inositol synthesis cycle.

tempted. The results here reveal a novel role for CRAC channel-mediated calcium influx in the replenishment of the cellular PIP_2 pool, leading to prolonged calcium signalling through the maintenance of calcium oscillations.

4.2 Results

4.2.1 Role of PIP_2 in agonist-mediated calcium oscillations

To understand the role played by PIP_2 in the oscillatory calcium signals generated by LTC_4 , I hypothesised that pre-treating cells with LiCl should lead to a reduction of PIP_2 levels within cells. This hypothesis is based on reports that LiCl interferes with the phosphoinositide turnover mechanism, that is, the inhibition of IPPase and IMPase by LiCl during the oscillatory response will lead to less inositol being

available to PI4K and PI5K for conversion to PIP_2 [435]. To test this, cells were first exposed to 10 mM LiCl for 90 min (less than the IC50 for LiCl in RBL-2H3) see figure Fig 4.2 below. This dose is based on the initial protocol described by Berridge et al, in the study where the inositol depletion hypothesis of LiCl was first suggested [421]. Stimulating LiCl-pre-treated cells with 160 nM LTC_4 resulted in an accelerated rundown of calcium oscillations (Fig 4.2A), with a reduction of $\approx$35% in the number of oscillations in an 800-s recording period compared to the control cells. In order to identify the IC_{50} for LiCl, RBL-2H3 cells were pre-treated with 1 mM of La^{3+} to block the plasma membrane Ca^{2+} ATPase pump and were stimulated with 160 nM LTC_4 following pre-treatment with different doses of LiCl. Pre-treating cells with LiCl for 90 minutes resulted in a reduction in the number of oscillations in a dose-dependent manner. Aggregate data are shown in Fig 4.2B. Data were fitted with the Hill-type equation, which yielded an IC_{50} value of 16 mM (Fig 4.2C). To rule out direct effects of LiCl on calcium entry or calcium release, cells were stimulated with a maximum dose of thapsigargin, in a calcium-free solution, and the rate of calcium release was monitored. Following this, a calcium influx through CRAC channels was initiated by the readmission of 2 mM calcium to the external solution. This suggests that LiCl did not affect the processes of calcium entry or release (Fig 4.2D). Stimulation of cells pre-treated with LiCl (50 mM) with thapsigargin led to calcium release and influx that were similar to those of the control cells (Fig 4.2D). Finally, to exclude a possible effect of the increase in osmolarity of the solution with LiCl, the number of LTC_4-mediated oscillations was compared between cells in 50 mM of LiCl in a standard external solution containing 155 mM of NaCl with those in a 105 mM NaCl external solution. No difference was observed (Fig 4.2E).

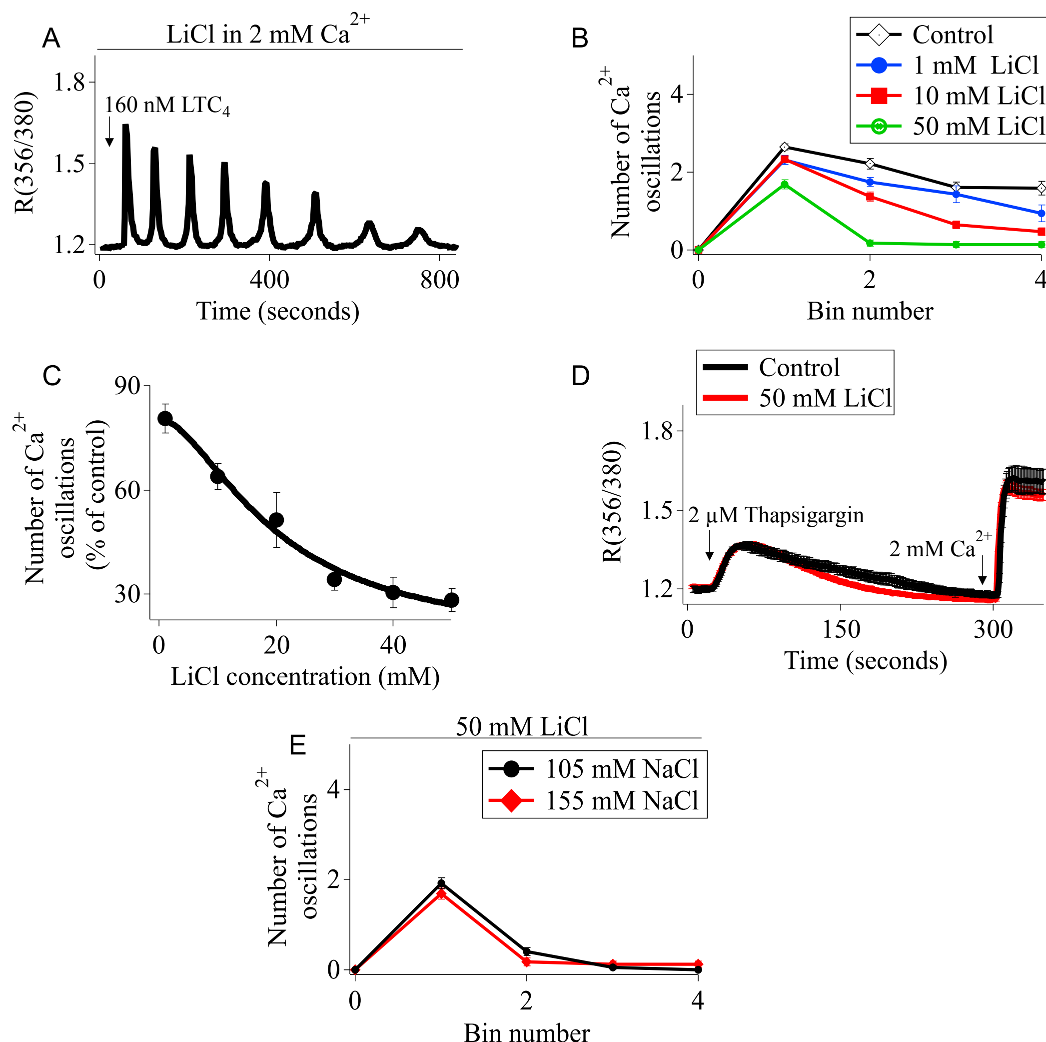

Figure 4.2. Role of PIP2 in agonist-mediated calcium oscillations
Representative calcium oscillations following the application of 160 nM of LTC_4 in (A) RBL-2H3 cells pre-treated with 10 mM LiCl in the presence of external calcium. (B) RBL-2H3 cells (n >25) pre-treated with 1 mM of lanthanum and stimulated with LTC_4 in the absence or presence of multiple doses of LiCl. Each bin number represents a period of 200 s. (C) Number of oscillations was quantified and normalised to the number of oscillations observed in control cells. Values were fitted with a Hill-type equation, yielding an IC50 value of 16 mM. (D) Representative experiment showing the average values for the rate of calcium release and entry in control cells and in cells pre-treated with 50 mM of LiCl for 90 minutes. (E) Aggregate data from more than 25 cells for each group (N = 2) showing the number of oscillations with 50 mM of LiCl in normal standard external solution containing 155 mM of NaCl and 105 mM of NaCl. Each bin number represents a period of 200 s.

4.2.2 Ruling out other sites of LiCl action on calcium signal

Pre-treatment with 15 mM of LiCl for 90 min resulted in an accelerated rundown of calcium oscillations when the cells were stimulated with LTC_4 in the presence of external calcium (Fig 4.3A). The rundown was similar to that seen in control cells stimulated with LTC_4 in the absence of external calcium (Fig 4.3B). The number of oscillations in cells pre-treated with 15 mM LiCl in a calcium-containing external solution was reduced by 48.8%±4.07% in an 800-s recording period, which is comparable to that seen when cells were stimulated with LTC_4 in a calcium-free solution (44.9%±2.3% reduction; Fig 4.3B).

These results would be consistent with the idea that the accelerated rundown of the oscillations in LiCl is due to depletion of PIP_2 levels. However, I considered alternative possibilities: (i) that is due to LiCl interference with the initial PIP_2 pool, thus affecting the ability of LTC_4 to activate PLC. To test this, I compared the rate of rundown of calcium oscillations between LiCl-pre-treated and untreated cells when stimulated with 160 nM LTC_4 in the absence of external calcium (Fig 4.3C, D). (ii) It has been suggested that LiCl interferes with G proteins [436], However, the results here clearly suggest no interference (Fig 4.3C and D) . Furthermore, the response cannot be attributed to the effect of LiCl on calcium levels within the internal calcium stores, as no difference in calcium release was seen between the control cells and those pre-treated with LiCl following application of 1 μM ionomycin (Fig 4.3E). Aggregate values from three independent experiments showed that there was no significant difference between the two groups (Fig 4.3F).

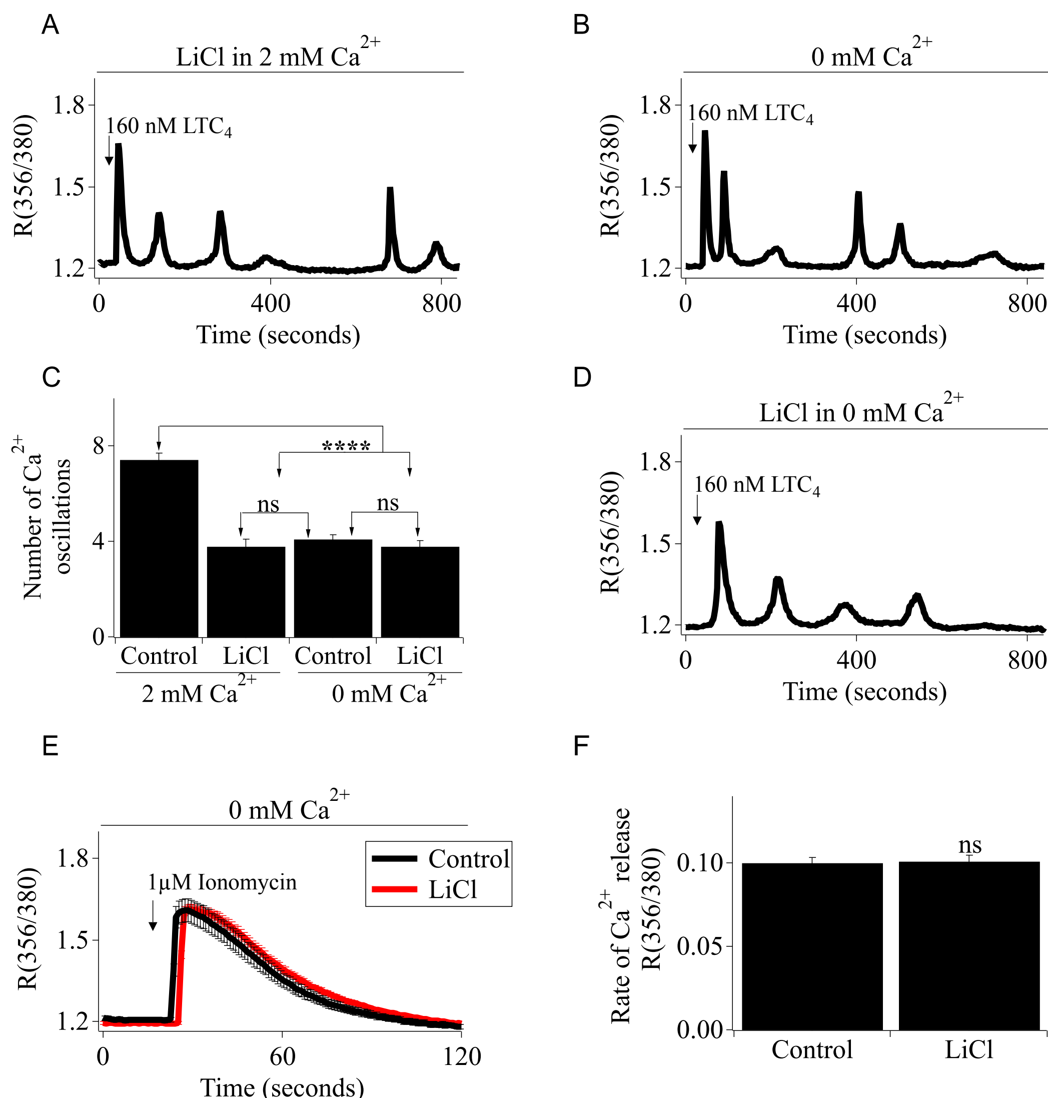

Figure 4.3. Role out other sites of LiCl action on the calcium signal

(A) Representative oscillatory calcium response following stimulation with 160 nM of LTC_4 in cells pre-treated with 15 mM of LiCl for 90 min. (B) Control cell stimulated with LTC_4 in the absence of external calcium. (C) Aggregate data from multiple experiments quantifying the number of total calcium oscillations in 800 s with LTC_4 treatment in the presence or absence of external calcium in the control cells and cells pre-treated with LiCl. The data presented in the figure represent more than 50 cells from four independent experiments. (D) Representative cell showing LTC_4 induced calcium oscillations following pre-treatment with LiCl in a calcium free solution. (E) Rate of ionomycin-induced calcium release in control cells and cells pre-treated with 15 mM of LiCl for 90 min. Data from three independent experiments were quantified and shown in the histogram in F.

4.2.3 Rescue of agonist-mediated calcium oscillations in lithium chloride pre-treated cells by treatment with inositol

If the rundown of oscillations to LTC_4 in LiCl-treated cells is indeed due to PIP_2 depletion, the addition of inositol should rescue the response. To test this, I applied 15 mM inositol for 3 minutes prior to LTC_4 stimulation in the presence of external calcium and 15 mM LiCl . I found that 15 mM consistently rescued the oscillations (Fig 4.4A). Aggregate data comparing the number of oscillations from four independent experiments are shown in Fig 4.4B. Surprisingly, inositol failed to rescue the oscillations in LiCl-treated cells when there was no calcium influx (Fig 4.4C). Aggregate data from three independent experiments are shown in Fig 4.4D. It is important to note that inositol availability constitutes a rate-limiting step, as the application of inositol in cells resulted in a 56.9%±5.5% increase in the number of oscillations over an 800-s recording period when compared to the control cells (collective data from 28 cells from three independent experiments) (Fig 4.4E).

4.2.4 Importance of calcium entry for PIP_2 replenishment

The failure of inositol to rescue the calcium oscillations when there was no calcium entry prompted us to examine the role of calcium entry in PIP_2 production. I blocked CRAC channels using La^{3+} in the presence of external calcium. The complete block of CRAC channels with 10 μM of La^{3+} resulted in a rundown of oscillations in cells pre-treated with LiCl and stimulated with LTC_4 in the presence of external calcium and inositol (Fig 4.5A). To further support the findings

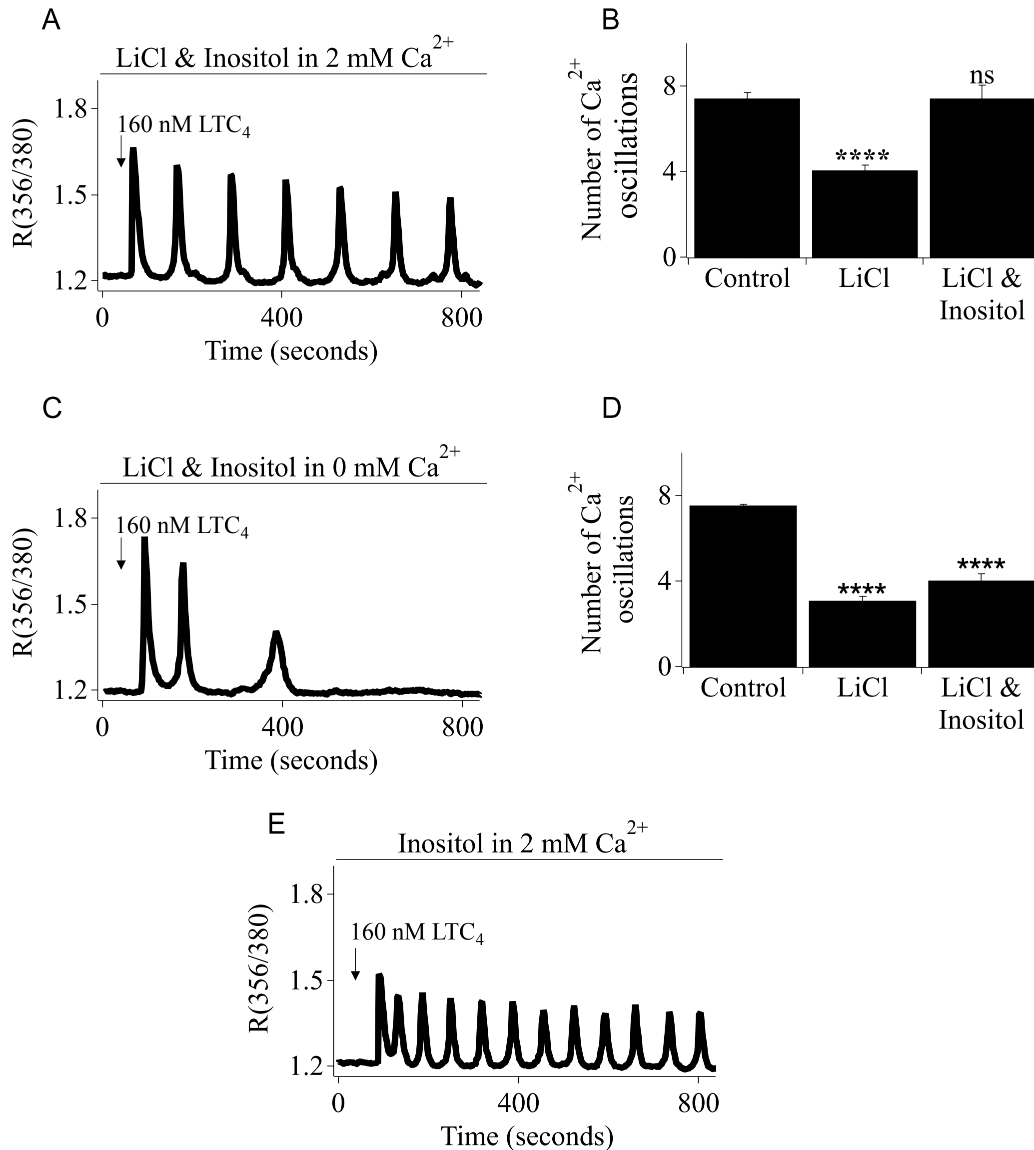

Figure 4.4. Rescue of agonist-mediated calcium oscillations in lithium chloride pre-treated cells by treatment with inositol

(A) Representative calcium oscillations in RBL-2H3 cells following stimulation with 160 nM of LTC_4 in the presence of LiCl and 15 mM of myo-inositol. (B) Aggregate data from four or more experiments quantifying the number of LTC_4 -induced calcium oscillations in 800 s. (C) A dose of 15 mM inositol failed to rescue the LTC_4 -mediated calcium oscillations in the absence of external calcium in cells pre-treated with 1 mM lanthanum and LiCl. (D) Aggregate data from four or more experiments are presented. (E) Inositol pre-treatment in control cells increased the number of oscillations by more than 50%.

here, the specific CRAC channel blocker BTP2 was used. BTP2 is a pyrazole derivative (YM-58483) that has been shown to effectively block CRAC channels without affecting the contents of the internal calcium stores. [122, 437]. First, BTP2's effect on calcium entry via CRAC channels was tested, where cells were incubated with BTP2 for three minutes in the absence of external calcium. Following this, a maximum dose of thapsigargin was applied, then calcium was added to the bath solution. The rate of calcium entry in cells pre-treated with BTP2 when compared to the control cells showed a reduction of ≈86%. Aggregate data from two independent experiments are shown in Fig 4.5B. Consistent with that observed when using La^{3+} to block CRAC channels, incubation with 20 μM of BTP2 prevented the rescue of calcium oscillations by inositol (Fig 4.5C), therefore inositol is not sufficient to rescue the oscillations and needs calcium entry through CRAC channels. Aggregate data from four independent experiments are shown in Fig 4.5D.

4.2.5 Measurement of PIP2 levels

To further confirm that the observed results is due to changes in PIP_2 levels, I attempted to measure PIP_2 levels directly.

Inwardly rectifying potassium channels

The inwardly rectifying potassium channels channel (IRK) was examined and characterised in both RBL-2H3 and RBL-1, and a robust current was observed in patch clamp recordings [38,39]. The activation of IRK channels is dependent on the lev-

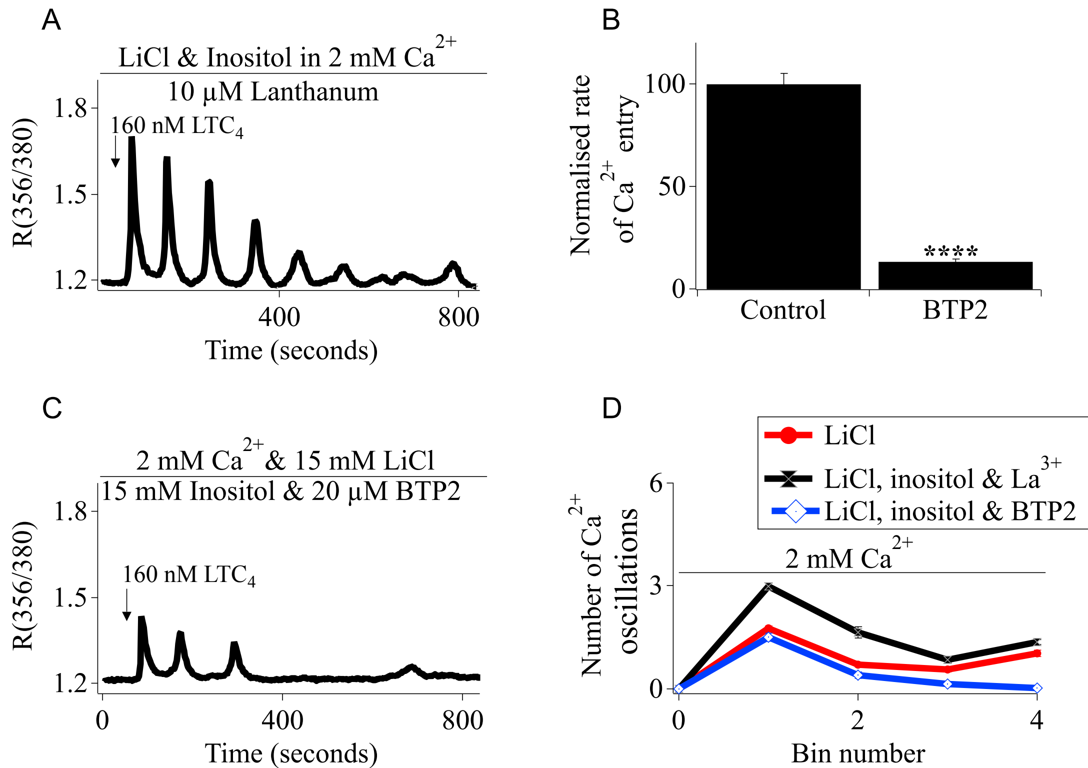

Figure 4.5. Importance of calcium entry for PIP2 replenishment

(A) Blocking of CRAC channels with 10 µM of lanthanum resulted in the failure of inositol to restore the LTC_4-mediated calcium response when cells were pre-treated with LiCl in the presence of external calcium. (B) Histogram quantifying the rate of calcium entry where CRAC-mediated calcium entry was blocked following pre-treatment with 20 µM of BTP2 and CRAC channel activation by thapsigargin and the addition of Ca2. (C) 20 µM of BTP2 resulted in the failure of inositol to restore the LTC_4-mediated calcium response in a representative cell in the presence of external calcium and LiCl pre-treatment . Collective data from four experiments (n >50) are shown in D.

els of phospholipids in the cell membrane [40]. PIP_2 is believed to activate the channel by bringing the transmembrane domain closer to the cytoplasmic domain where the phosphorylated head group and the fatty acid chain of PIP_2 bind the cytoplasmic and transmembrane domains, respectively [41]. The approach of the experimental protocol here was identical to that published [438]. The cells were bathed in 20 mM potassium, and a potassium-based internal solution was used. The current was measured at 0 mV for 50 ms at voltage ramps spanning - 100 to + 100 mV. To confirm that the observed current under these conditions is the IRK current, application of 10 mM cesium to the external solution led to a quick and complete inhibition of the current (Fig 4.6A). Cesium is considered to be a potent and thoroughly investigated blocker of the IRK channel [439–441]. The application of LTC_4 failed to induce any changes in the measured current, as shown in Fig 4.6B (N=8).

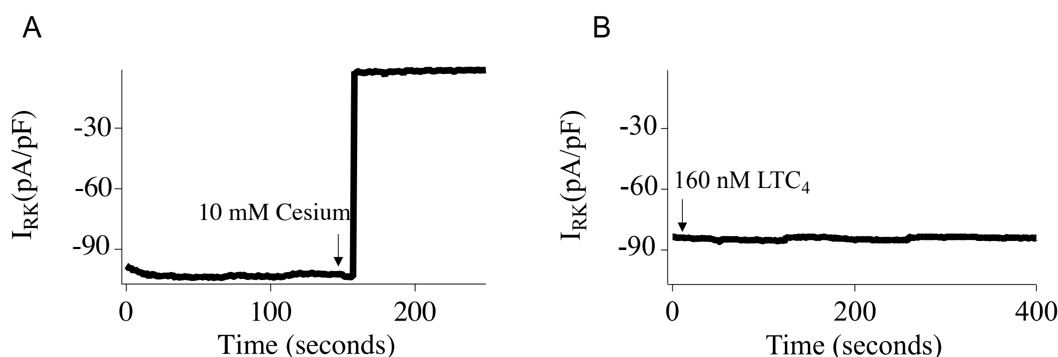

Figure 4.6. Measurement of the inwardly rectifying potassium current (IR_K) in rat basophilic leukaemia (RBL-2H3) cells

(A) Representative patch clamp recording showing the blocking of the observed current following the local application of cesium to the external bath. (B) Representative patch clamp recording following the stimulation of RBL-2H3 cells with 160 nM LTC_4.

The tubby domain

PIP_2 levels can be monitored using the PIP_2-binding C-terminus of the tubby construct. This protein was first identified in the tubby mouse, where it was found that defects in the tub gene led to late-onset obesity. The protein is bound to the membrane via binding to PIP_2 through its tubby domain [356]. The C-terminus of the tubby domain binds to PIP_2, PI(3,4)P_2 and PI(3,4,5)P_3. Unlike the PLCdelta-PH domain, tubby translocation is not affected by the downstream products of PIP_2 hydrolysis [355]. Quinn et al. first developed the cYFP-R332H mutant by conservatively mutating one of the head groups that binds PIP_2. CYFP-R332H is believed to be highly sensitive to PIP_2 and reversibly translocates in response to many agonists [356]. RBL-2H3 cells were transfected with the (YFP)-tagged R332H-tubby construct. In un-stimulated cells, the tubby domain showed a clear cell membrane localisation with some cytoplasmic fluorescence (Fig 4.7A). Stimulating the cells with 160 nM LTC_4 in the presence of external calcium had no effect on florescence localisation (Fig 4.7B). Despite tubby's high affinity to PIP_2, it was reported that minor changes in PIP_2 levels could not be detected, therefore I was unable to measure change in PIP_2 levels using tubby [354].

The GFP-PLCδ1-PH domain

Considering the need for a probe with a lower PIP_2 affinity so that minor changes in PIP_2 levels could be detected, I turned to the PLCδ1-PH domain construct [442, 443]. Similar to the tubby domain, in resting conditions the probe localised to the plasma membrane (Fig 4.8A). When LTC_4 was applied in the presence of external

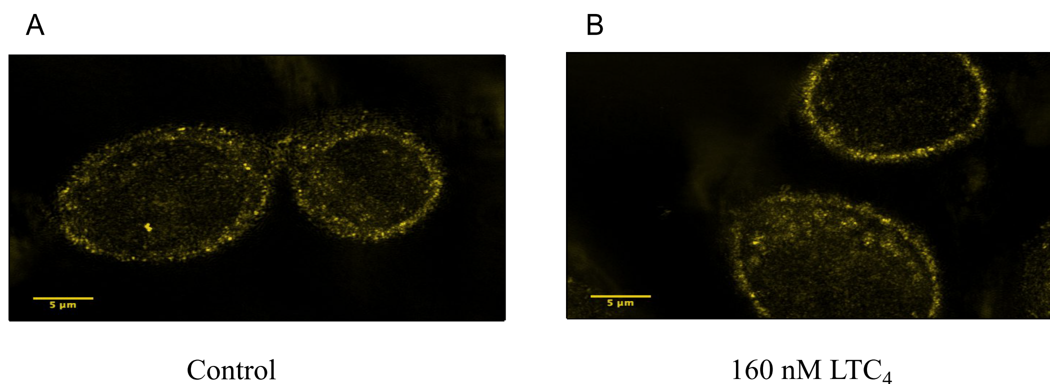

Figure 4.7. Monitoring the localisation of the tubby domain in rat basophilic leukaemia (RBL-2H3) cells

(A) Representative control cells showing the localisation of the tubby domain obtained using a confocal microscope. (B) Stimulation with LTC_4 in the presence of external calcium had no effect on the localisation of the tubby domain up to 20 minutes following stimulation.

calcium, there was a noticeable increase in cytosolic fluorescence following the translocation of GFP-PLCδ1-PH from the membrane to the cytoplasm (Fig 4.8B). When cells were pre-treated with LiCl, either weak or no translocation of the domain was observed when cells were stimulated in the presence of external calcium (Fig 4.8C). Consistent with the calcium imaging results above, the addition of 15 mM inositol successfully rescued the signal, in that translocation of the domain was observed in LiCl pre-treated cells when 160 nM LTC_4 was applied in the presence of external calcium (Fig 4.8D). Blocking CRAC channels with 10 μM La^{3+} prevented the rescue of PIP_2 synthesis by inositol, as no translocation was observed following stimulation with LTC_4 (Fig 4.8E).

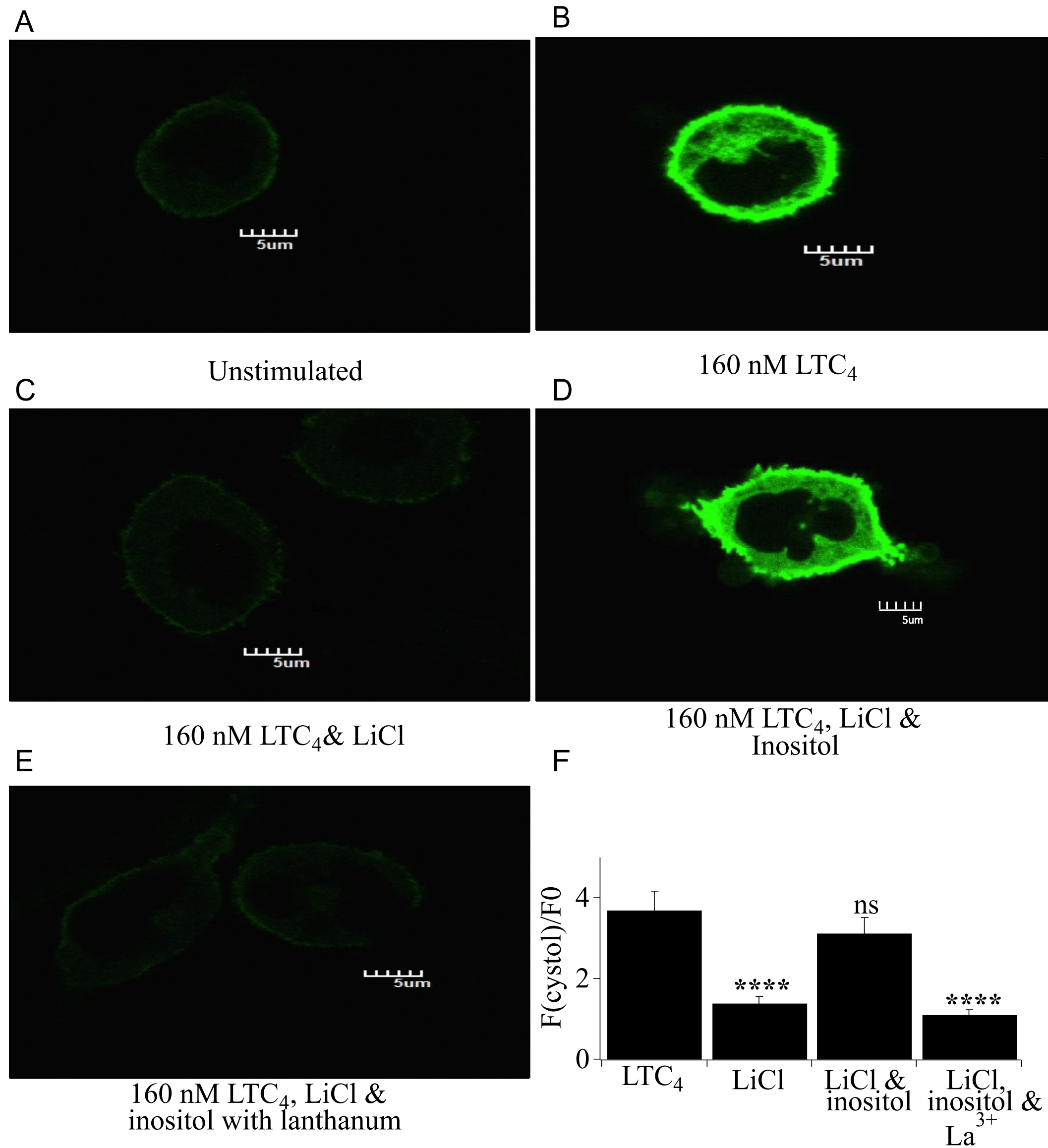

Figure 4.8. PIP2 monitoring in RBL-2H3 cells using the PLCδ1-PH domain
(A) The probe binds to PIP2 at the membrane. (B) Increase in cytosolic fluorescence following translocation of GFP-PLCδ-PH from the membrane to the cytoplasm. (C) LiCl blocked the re-synthesis of PIP2 and no translocation was seen. (D, E) The addition of 15 mM of inositol led to PIP2 re-synthesis and GFP-PLCδ-PH translocation only when there was an influx of calcium. Cytoplasmic florescence quantified from 40 cells for each treatment is shown in the histogram.

4.3 Key findings of this chapter

- Pre-treatment of RBL-2H3 cells with LiCl led to the rundown of calcium oscillations evoked by CysLT1 receptors.
- The inhibitory effect of LiCl is attributed to its action on phosphoinositide levels and showed no interference with the initial PIP_2 pool within cells or calcium levels within the internal calcium stores.
- Inositol rescued the oscillatory calcium signal induced by LTC_4 in LiCl pre-treated cells.
- The action of inositol was dependent on calcium entry via CRAC channels.
- The GFP-PLCδ1-PH construct proved to be sensitive enough to minor changes in PIP_2 levels and confirmed the key findings of this chapter.

4.4 Discussion

The hydrolysis of PIP_2 into IP_3 and DAG is crucial for cell surface GPCRs and tyrosine kinase coupled ones that link to PLC, to relay an intracellular response [433, 444, 445]. PIP_2 can be depleted by LiCl [446]. The molecular action of LiCl has been well investigated since the early work of Allison et al. showing that treatment of rats with LiCl led to a reduction in inositol levels with a build-up of inositol monophosphate [446, 447]. The interference of LiCl with the inositol phosphate metabolism led Berridge et al. to develop an inositol depletion hypothesis of LiCl, proposing that LiCl action was brought of out by inhibiting IP_3-mediated

calcium signalling [433]. Further research showed the build-up of d-Ins1P in cells isolated from the cerebral cortex of rats treated with LiCl [448]. The last finding is considered a key one in supporting the theory of LiCl's effect on the inositol cycle, as d-Ins1P is the product inositol of the PLC pathway (recycled inositol) [449, 450]. Finally, in cultured cells, it was found that pre-treatment with LiCl and stimulation of the cells with a strong agonist that links to the PLC pathway significantly reduced cellular phosphoinositides [449, 451]. Thus, the observations in this chapter where LiCl led to the rundown of calcium oscillations (Fig 4.2A and B) and prevented the translocation of PLCδ1-PH into the cytoplasm (Fig 4.8C) further support the theory of the inositol-depletion hypothesis of LiCl.

As LiCl blocks the last step in inositol synthesis, the addition of exogenous inositol into the culture medium reverses the inhibition [451, 452]. In agreement with this, I have found that the addition of 15 mM inositol into the bath solution rescued the oscillations in LiCl pre-treated cells (Fig 4.4A). The short time needed for inositol pre-treatment in order to rescue the oscillations was surprising (3 minutes). Inositol gains entry into the cells via three transporters, Na^+ /myo-inositol co-transporter 1 and 2 and H^+ -myo-inositol symporter [223–225]. As discussed previously, myo-inositol and CDP-DAG will lead to the production of phosphatidylinositol. This process takes place in the endoplasmic reticulum or, as it has been suggested recently, in highly mobile endoplasmic reticulum-derived organelles [432]. A few reports have suggested the possibility of two pools for the synthesis of phosphatidylinositol, one, in the endoplasmic reticulum and one in the plasma membrane. Phosphatidylinositol synthesis in red blood cells isolated from turkeys were found to be completely in the cell membrane [226, 227]. Furthermore,

following cell fractionation, isolation of phosphatidylinositol synthase from plasma membrane and ER fractions was successful [228, 229, 453]. My findings here might point towards a plasma membrane synthesis of phosphatidylinositol, although a more detailed examination of the expression levels and activity of the transporters and kinetics of inositol transfer in mast cells is needed.

The hypothesis that calcium entry is necessary to replenish phosphoinositide is based on (i) the failure of inositol to rescue the oscillatory calcium signal induced by LTC_4 in the absence of calcium entry in cells exposed to La^{3+} (Fig 4.4C and D) and (ii) lack of rescue after blocking of calcium entry with a micromolar dose of La^{3+} (Fig 4.5A) in cells bathed in calcium and LiCl-containing solution. The possibility that La^{3+} might enter the cell and interfere with LiCl function has been considered. In particular, the lanthanide cation, gadolinium, has been reported to compete with magnesium for the metal binding sites on IMPase [454, 455]. However, there are four pieces of evidence against this: (i) When a hundred-times higher dose of La^{3+} (1 mM) was used in the calcium-free medium, LTC_4-induced calcium oscillations were observed over a period of 800 s, which indicates that even if La^{3+} were able to enter the cells and compete with magnesium over the metal binding site, the effect was weak. (ii) IMPase cannot be co-inhibited by La^{3+} and LiCl, as the mechanism by which LiCl inhibits IMPase is different from that of lanthanide cations, and LiCl forms a stable complex between the enzyme, phosphate and lithium after hydrolysis of the substrate at the second metal site. If the first metal binding site is occupied by lanthanide cations, hydrolysis or unbinding of IP is unlikely. Consequently, no phosphate head will be available for lithium binding [456]. (iii) No La^{3+} was detected within mast cells, assessed

through binding to Fura-2 over the recording period of 800 s when the 1 mmol dose was used [203]. (iv) Substituting La^{3+} with the specific CRAC channel blocker BTP2 to block calcium entry produced similar results (Fig 4.5C).

My data provide new evidence that the replenishment of PIP_2 levels requires calcium entry via CRAC channel. The effect of calcium on polyphosphoinositides has been reported before in the regulation of PI4KB by the neuronal calcium sensor-1 (NCS-1) [457]. PI4KB is one of four enzymes mediating the conversion of phosphatidylinositol into PI4P [271]. NCS-1 is a member of the calcium-binding protein family of neurons [458]. It is believed that NCS-1 affinity for calcium is high and is partially activated at resting conditions [459]. NCS-1 is shown to be activated and to regulate PI4KB at ≈400 nM of intracellular calcium, which is suggested to be the threshold where NCS-1 overturns the inhibitory effect of calneurons [460]. It has been reported that unlike PI4KA, PI4KB has no role in supporting the PI4P in the plasma membrane and is localised to the Golgi complex [460, 461]. The results of this project introduce new evidence implicating calcium in regulating the levels of polyphosphoinositides, but only with a substantial elevation of calcium levels and in different cellular micro domains.

Only a limited understanding of phosphoinositides has been derived from the direct intracellular measurement of these lipids [361]. This is due to two main reasons; first, the low levels within the cells in comparison to the total cellular phospholipid make it difficult to separate them from other components [462]. Second, the most accurate methods in measuring phosphoinositide require radio-labelling followed by high-performance liquid chromatography or isolation of intracellular organelles and cellular membranes followed by fractionation of the lipid extracts [361]. All of

these laboratory techniques are considered laborious and time consuming, and do not provide information at the level of an individual cell. Three of the main modern approaches to measuring phosphoinositides have been attempted in this project [424]. I first monitored the PIP_2 sensitive inwardly rectified potassium current [463]. The application of LTC_4 in the external bath solution failed to reduce the current over the 10-minute recording period (Fig 4.6B). LTC_4 is believed to cause modest calcium store depletion suggesting weak action of the PLC pathway [394]. Accordingly, failure of LTC_4 to inhibit the inwardly rectifying potassium (Kir) channels was not totally surprising, as it has been reported that a certain threshold of PIP_2 hydrolysis must be reached for the channel to close [424]. Moreover, the results here are in agreement with other reports which failed to detect any changes in the channels' conductance [464, 465]. Stimulating neurons with bradykinin failed to inhibit the channel [464]. Furthermore, in cardiac cells, endothelin-1 application induced no recordable changes in the channel's conduction [465].

Similar to LTC_4 's effect on IRK, the application of LTC_4 in cells transfected with the tubby protein had no effect on the localisation of the protein (Fig 4.7A, B). This could be attributed to tubby's reported higher affinity for PIP_2 and therefore the requirement for strong PLC activation with a significant PIP_2 hydrolysis [354, 358]. Moreover, failure of tubby to translocate was not a unique phenomenon of LTC_4 . It was reported that a physiological dose of AngII stimulation failed to stimulate the tubby domain to translocate or induced a slower and partial translocation [354]. Failure of YFP-tubby to translocate to the cytoplasm was also found in neurons following bradykinin application, unless the B2 receptors were exogenously overexpressed [358].

PLCδ1PH-GFP proved to be sensitive and successful translocation of the domain was reported upon stimulation with LTC_4 , AngII and bradykinin as well as here [354, 358, 466]. There is one limitation that has been widely discussed regarding PLCδ1-PH. Since the interaction of PLC1PH-GFP and PIP_2 occurs via the phosphorylated inositol head group, IP_3 could compete with PIP_2 , hence reducing its specificity as a PIP_2 probe. This was discussed in detail by Varnai et al., with the conclusion that under physiological conditions, PLCδ1-PH is a reliable PIP_2 probe [350]. Furthermore, differences were noted between PLCδ1PH-GFP translocation and IP_3 levels when measured concurrently [467]. Because I have used cells with endogenous levels of receptors and have applied a sub maximal dose of LTC_4 , my conditions matched those seen physiologically, and therefore support the use of PLCδ1PH-GFP as a measure of PIP_2 .

The results in this chapter identified a new role for CRAC channels in mast cell activation. By using cytoplasmic calcium ion imaging and specific fluorescence PIP_2 binding probes, I found that the inhibition of the cellular phosphoinositide pool by lithium chloride led to the loss of the oscillatory calcium signal. Adding the substrate inositol rescued the signal, but only when external calcium was present. Pharmacologically blocking CRAC channels with a low concentration of lanthanum chloride or BTP2 prevented the recovery of oscillations and translocation of PLCδ1-PH in LiCl-treated cells even when inositol was present. Therefore, I conclude that the opening of CRAC channels and subsequent calcium entry is required for PIP_2 production and thus the maintenance of agonist-mediated calcium signalling.

Chapter 5

The conversion of PI4P into PIP_2 requires calcium entry via CRAC channels

5.1 Introduction

In the previous chapters, it was established that calcium entry through CRAC channels contributed to PIP_2 production and hence supported the oscillatory calcium signals induced by LTC_4 in RBL-2H3 cells. This chapter aims to further examine the mechanism by which calcium entry replenishes the amount PIP_2 . Using specific pharmacological blockers and small interfering RNA, levels of PI4P, PI5P and PIP_2 will be manipulated, and the impact on calcium oscillations will be investigated. I further aim to pinpoint the specific PIP5K1 isoforms responsible for the production of these polyphosphoinositides and to confirm the importance

of calcium entry via CRAC channels in their function. Finally, with the goal of understanding the spatial profile of PIP_2 synthesis, I examined whether the LTC_4-mediated calcium signal is dependent on a local or global membrane PIP_2 pool.

To investigate the possible site of calcium action on phosphoinositide kinases, a thorough understanding of this complex set of enzymes is needed. Phosphoinositide kinases are divided into two main categories: PI-kinases and PIP kinases. PI-kinases are the enzymes responsible for the conversion of PI into PI4P and PI5P. PIP kinases are further divided into type I and type II PIP kinases. PIP kinases are responsible for the conversion of PI4P and PI5P into PIP_2 [218].

Phosphatidylinositol 4 kinases (PI4Ks) are the enzymes involved in the conversion of PI into PI4P. There are four mammalian homologues of PI4K: types II α and II β and types III α and III β [271, 273, 468, 469]. The conversion of PI into PI5P is believed to be mediated by PIKfyve [279, 470, 471]. The function in immune cells and the cellular localisation of these enzymes have been discussed in great detail in chapter one of this thesis.

Type 1 PIP kinases are encoded by three different genes yielding three distinctive isoforms: α, -β and -γ. The three isoforms are 80% identical in their catalytic domain [292, 472]. All three isoforms are involved in mediating the conversion of PI4P into PIP_2 [290]. PIP5K1γ has three splice variants: PIP5K1γ87, PIP5K1γ90 and PIP5K1γ93 [292, 302, 303]. PIP51γ93 is out of the scope of this thesis, as it is considered a neuronal-specific splice variant [302]. Two additional isoforms have been described to be specific in humans, and one isoform is specific in rodents [303, 304], The cellular localisation of PIP5K1γ splice variants is variable and covers the areas in which PIP5K1α and γ are suggested to be sub-localised in

[473]. The subcellular localisation and the expressing organs/tissues of the three isoforms of PIP5K are believed to be different. PIP5K1α localises to the Golgi and nuclear speckles and is believed to be the most abundant isoform in the spleen [293, 294]. PIP5K1β is believed to be the most abundant isoform in the plasma membrane and is seen mostly in the skeletal and cardiac muscles [290, 294, 474]. PIP5K1γ splice variants function and localise differently, but in general, PIP5K1γ has been associated with focal adhesions and has been seen in brain, lung and kidney tissues [292, 305].

By overexpression and knockdown studies, type 1 PIP kinases have been implicated in several cellular functions and various activities. The role of PIP5K1 isoforms in different cell types seems to be specific, and no isoform can compensate for the loss of the others [297, 298, 430]. PIP5K1α is involved in cytoskeleton dynamics, cell polarity, membrane ruffling and initiating process of phagosome formation [290, 295]. PIP5K1β is involved mainly in actin polymerisation and reorganisation [299, 300]. PIP5K1γ is implicated in the formation of clathrin-coated vesicles, integrin-mediated adhesion and actin assembly [306, 307].

The role of Type 1 PIP kinases in mediating immune function is well known. PIP5K1α is suggested to play a role in phagocytosis by modifying F-actin levels in mouse leukemic monocyte-macrophages [296]. Bone marrow-derived macrophages showed impairment in opsonisation and the engulfment of particles when the PIP5K1α or γ was knocked out [297]. More specific to mast cells, PIP5K1β-deficient mast cells isolated from mice showed an exaggerated degranulation following stimulation of the Fc epsilon R1 receptor. This was accompanied systematically by cutaneous anaphylaxis [259]. In contrast to that observed in PIP5K1β-deficient

mast cells, when PIPKIγ was knocked out, mast cells showed a reduced response when stimulated with antigens [254]. Furthermore, the inhibition of PI5Ks in mast cells resulted in a reduced response to antigens by multiple mechanisms [253].

Here, I will investigate the polyphosphoinositides' pathway involved in supporting the calcium signal induced by LTC_4. By the pharmacological inhibition of phosphatidylinositol 4 kinases and PIKfyve, I identified that LTC_4-mediated calcium signalling is dependent on PI4P specific pools. The conversion of PI4P into PIP_2 by Type 1 PIP kinases required calcium entry through CRAC channels. Small interfering RNA towards PIP5K1α and PIPKIγ showed that both supported the calcium signal induced by LTC_4 and provided an overlapping function. Finally, the spatial profile of PIP_2 synthesis in mast cells will be examined.

5.2 Results

5.2.1 Effect of pharmacological inhibitors of PI4K and PIKfyve on calcium oscillations generated following LTC_4 stimulation

PI4P has long been identified as a PIP_2 precursor, and its role in intracellular signalling has been well documented [218, 475]. PI5P is the most recently identified member of the polyphosphoinositides family [280]. Since PI5P discovery, it has been implicated in several intracellular pathways and its levels have shown to change upon stimulation with histamine, thrombin and insulin [476]. Moreover, PI5P was shown to play a role in T-cell activation [281]. Accordingly, to examine

the site where calcium entry through CRAC channels is needed in the synthesis of PIP_2 , I started by examining the role played by its two direct precursors, PI4P and PI5P, in supporting the calcium signal mediated by LTC_4 .

The use of pharmacological blockers to investigate the synthesis of phospholipids and to characterise their recycling dynamics has been limited due to the lack of specific blockers [477]. One of the few specific blockers available is the PIKfyve inhibitor YM201636. At the nano-molar range, it was shown that PIKfyve preferentially inhibited PI5P production [478, 479]. Pre-treating cells with 160 nM YM201636 for 30 minutes prior to stimulation with a submaximal dose of LTC_4 had no effect on the calcium signal when compared to the control cells (Fig 5.1A and B). Collective data from three independent experiments are shown in Fig 5.1D. Pre-treatment with a micro-molar concentration of Wortmannin is suggested to be a potent PI4K inhibitor [480]. Compared to control cells (Fig 5.1A), pre-treatment with 10 µM Wortmannin for 10 minutes resulted in $48.5\% \pm 6.3\%$ reduction in the number of oscillations when cells were stimulated with 160 nM LTC_4 in the presence of external calcium (Fig 5.1C). The observed reduction cannot be attributed to a reduction in the rate of calcium entry, as the addition of calcium to the bath solution in cells pre-treated with 10 µM Wortmannin following stimulation with 2 µM thapsigargin was comparable to the rate of calcium entry seen in control cells (Fig 5.1E and F).

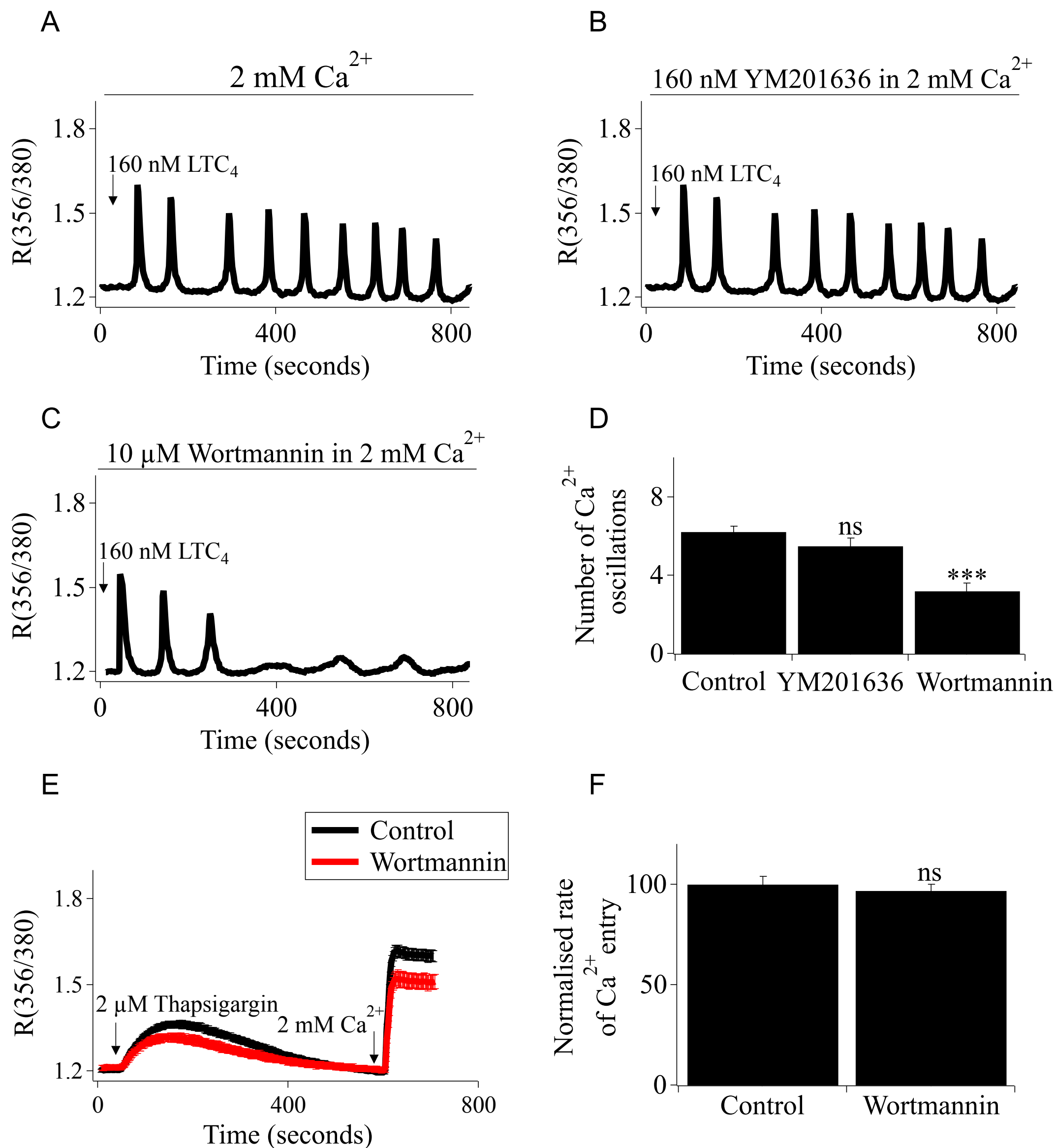

Figure 5.1. The role of PI4K and PIKfyve in generating PIP_2

(A) Representative calcium oscillations in RBL-2H3 cells following the application of LTC_4 in the presence of external calcium. (B) Representative cell showing the oscillatory calcium response following the application of LTC_4 in cells pre-treated with YM201636 for 30 minutes prior to stimulation. (C) Calcium oscillations in a representative cell following the application of Wortmannin for 10 minutes. (D) Collective data from three independent experiments quantifying the number of LTC_4 -mediated calcium oscillations. (E and F) Representative experiment showing the average rate of both calcium release and calcium entry in control and Wortmannin pre-treated cells. Rates of calcium release from three independent experiments were quantified and shown in the histogram.

5.2.2 The conversion of PI4P into PIP_2 requires calcium entry

Since the preceding pharmacological data indicate that the LTC_4-mediated calcium oscillation in RBL-2H3 is mediated by the Phosphatidylinositol/PI4P pathway, I decided to first examine a potential role for calcium entry in the last step in PIP_2 synthesis. Accordingly, I tested whether the conversion of PI4P into PIP_2 requires calcium entry via CRAC channels. To do this, cells were first treated with 15 mM LiCl for 90 minutes and consistent with that observed earlier in Chapter Four, there was a reduction in the number of oscillations following stimulation with 160 nM LTC_4 in the presence of external calcium. The number of calcium oscillations was reduced by 55.5% $\pm$3.7% (Fig 5.2A, B and G). The addition of 70 µM of the water-soluble, short-chain diC8 PI4P pre-mixed with histone H3 carrier to facilitate its delivery into the cells seven minutes prior to stimulation with 160 nM LTC_4 successfully prevented rundown of oscillations in the presence of LiCl, as seen in Fig 5.2C. Collective data from three independent experiments are shown in Fig 5.2G. The addition of H3 histone carriers to control cells had no inhibitory or stimulatory effect in cells stimulated with 160 nM of LTC_4, as the number of calcium oscillations was statistically comparable to that observed in the control cells (Fig 5.2D). Moreover, the application of histone in LiCl pre-treated cells failed to rescue the oscillations (Fig 5.2E). Aggregate data from three independent experiments are shown in Fig 5.2G. Blocking CRAC channels with 20 µM BTP2 prevented PI4P from rescuing the oscillations in LiCl pre-treated cells when stimulated with 160 nM LTC_4 in the presence of external calcium. The number of calcium oscillations was reduced by 58.4%$\pm$2.2% when compared to

the application of 160 nM of LTC_4 in control un-treated cells (Fig 5.2F).

To further strengthen a role for calcium entry in converting PI4P to PIP_2, control cells were bathed in a solution that lacked calcium and then pre-treated with 1 mM La^{3+} to prevent the extrusion of calcium by the SERCA pump. Adding LTC_4 to the external solution led to the generation of oscillations that lasted 800 seconds despite the absence of calcium entry (Fig 5.3A). Pre-treating the cells with 15 mM LiCl for 90 minutes resulted in rundown of calcium oscillations, as expected, and was consistent with earlier results in Chapter Four (Fig 5.3B). The addition of 70 μM PI4P pre-mixed with the histone H3 carrier failed to rescue the oscillations in cells pre-treated with 1 mM La^{3+} and pre-incubated with LiCl for 90 minutes (Fig 5.3C). The reduction in the number of calcium oscillations in LiCl pre-treated cells alone or following the addition of PI4P, was $66.1\% \pm 3.3\%$ and $67.4\% \pm 3.4\%$, respectively. Collective data from three independent experiments were analysed, and the average number of calcium oscillations is shown in the histogram in Fig 5.3D. The results here localise the site step the phosphoinositide cycle where calcium entry is required via CRAC channels.

5.2.3 The expression levels of PIP5K1 isoforms in the rat basophilic leukaemia (RBL-2H3) cell line

To further investigate how calcium entry is involved in the conversion of PI4P into PIP_2, the expression levels of PIP5K1 isoforms in RBL-2H3 cells were examined. The expression levels were measured by RT-PCR . PIP5K1α and γ were robustly expressed (Fig 5.4 upper panel). It was somewhat surprising that PIP5K1β was

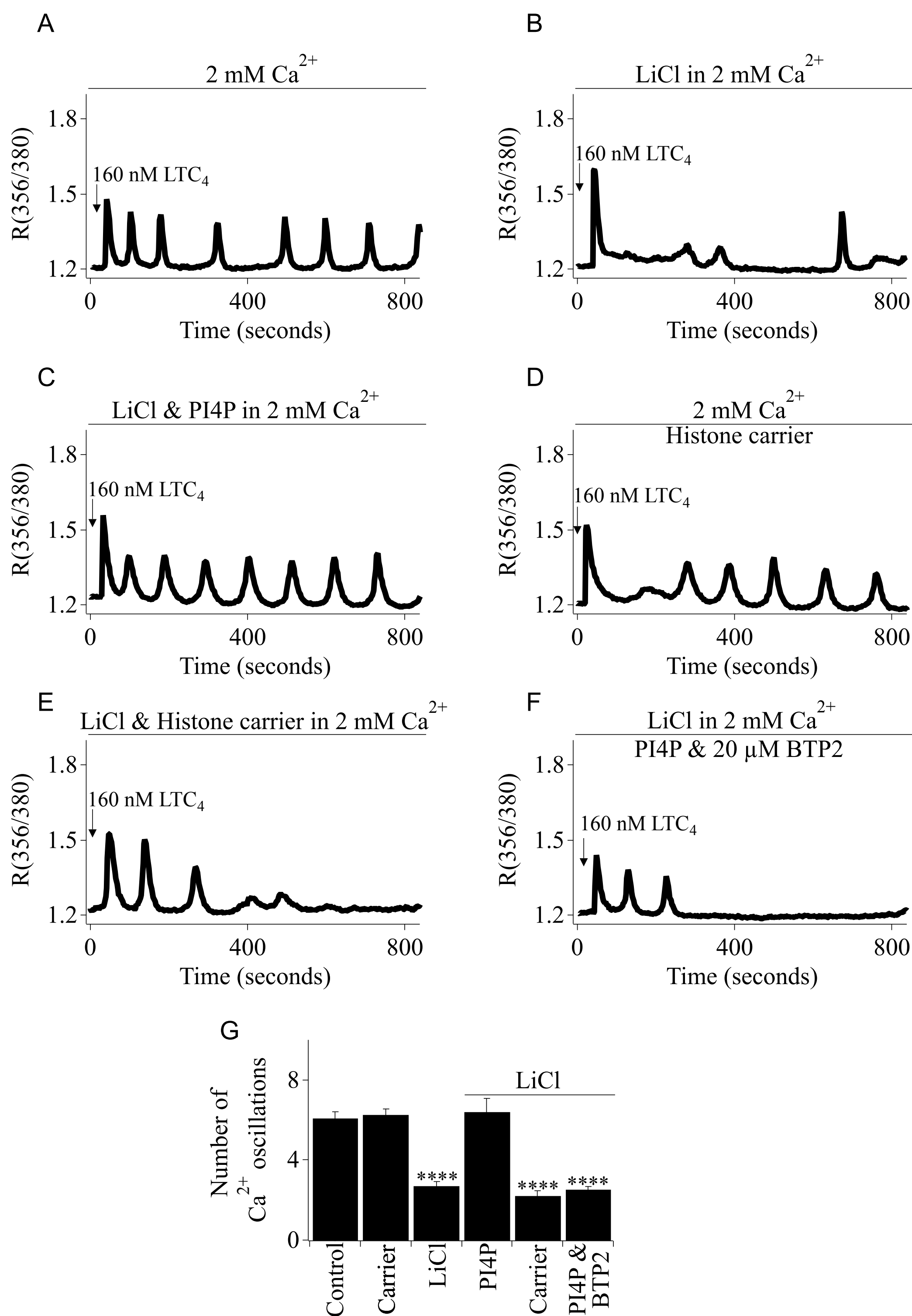

Figure 5.2. The conversion of PI4P to PIP_2 requires calcium entry
Representative calcium oscillations in (A) control cells or (B) cells pre-treated with LiCl following the application of 160 nM LTC_4 in the presence of external calcium. (C) Pre-treatment with 70 µM PI4P with carrier rescued the oscillations in cells pre-treated with LiCl. (D) The application of the H3 histone carrier had no effect on control cells. (E) H3 histone carrier failled to rescue the oscillatory calcium signal when stimulating the cells with LTC_4 in LiCl-pre-treated cells. (F) Failure of PI4P to rescue the oscillations in LiCl pre-treated cells when CRAC-mediated calcium entry was blocked using 20 µM BTP2. Collective data from three experiments (n >30) are shown in G.

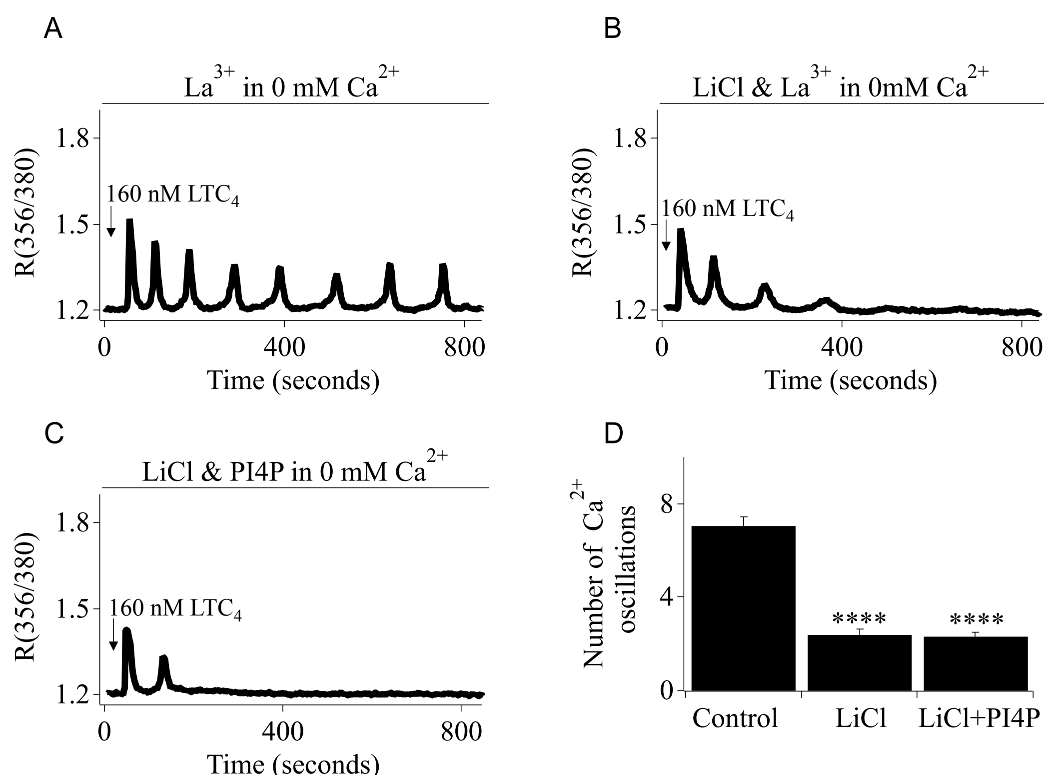

Figure 5.3. Failure of the conversion of PI4P into PIP_2 when there is no calcium entry
(A) Representative calcium oscillations in RBL-2H3 cells were pre-treated with 1 mM of lanthanum to prevent calcium extrusion by blocking the plasma membrane calcium ATPase pump and then stimulated with LTC_4 in a calcium-free solution. (B) Pre-treatment with 15 mM of LiCl led to the early rundown of calcium oscillations. (C) Pre-treating the cells with 70 mM of PI4P failed to rescue the oscillation in LiCl pre-treated cells. Aggregate data from three experiments are shown the histogram in (D).

not expressed in RBL-2H3 cells specifically because isoform β was suggested to be involved in the antigen-mediated response in RBL-2H3 cells [254]. The conclusion in the study by Vasudevan et al. was based on using RBL-2H3 cells, as an over-expression system of active and catalytically mutated PIP5K1β, and endogenous levels were not examined. Nevertheless, this prompted us to further confirm my finding. Same set of primers were used to detect PIP5K1β at both genomic DNA and cDNA levels. As seen in Fig 5.4 (lower panel), the levels of PIP5K1β at the

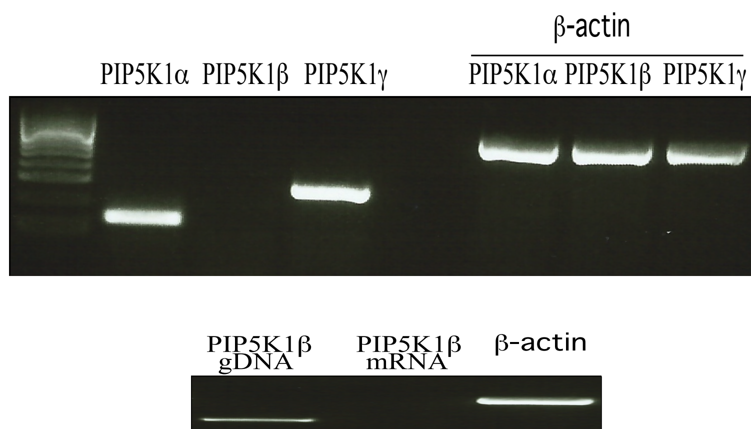

Figure 5.4. PIP5K1 isoforms in RBL2H3 cells
RT-PCR showing the expression of PIP5K1 isoforms in RBL-2H3 cells. The lower panel shows purified genomic DNA of PIP5KIβ in RBL-2H3.

genomic DNA level were robust but not expressed at the mRNA level.

5.2.4 Knockdown of PIP5K1 isoforms in the rat basophilic leukaemia (RBL-2H3) cell line

To identify which of the two isoforms expressed in RBL2-H3 is involved in mediating the calcium signal induced by LTC_4, an exogenous siRNA was introduced into the cells to obtain a transient reduction in protein expression. Thirty-six hours after transfecting the cells with the siRNA, the protein expression levels of both PIP5K1α and γ were examined by the use of confocal microscopy following fixing and staining the cells using immune-histochemical techniques. A reduction in protein expression of $\approx 50\%$ for both isoforms was obtained, as seen in Fig 5.5A and B. Data collected from three independent experiments are shown in the corresponding histogram.

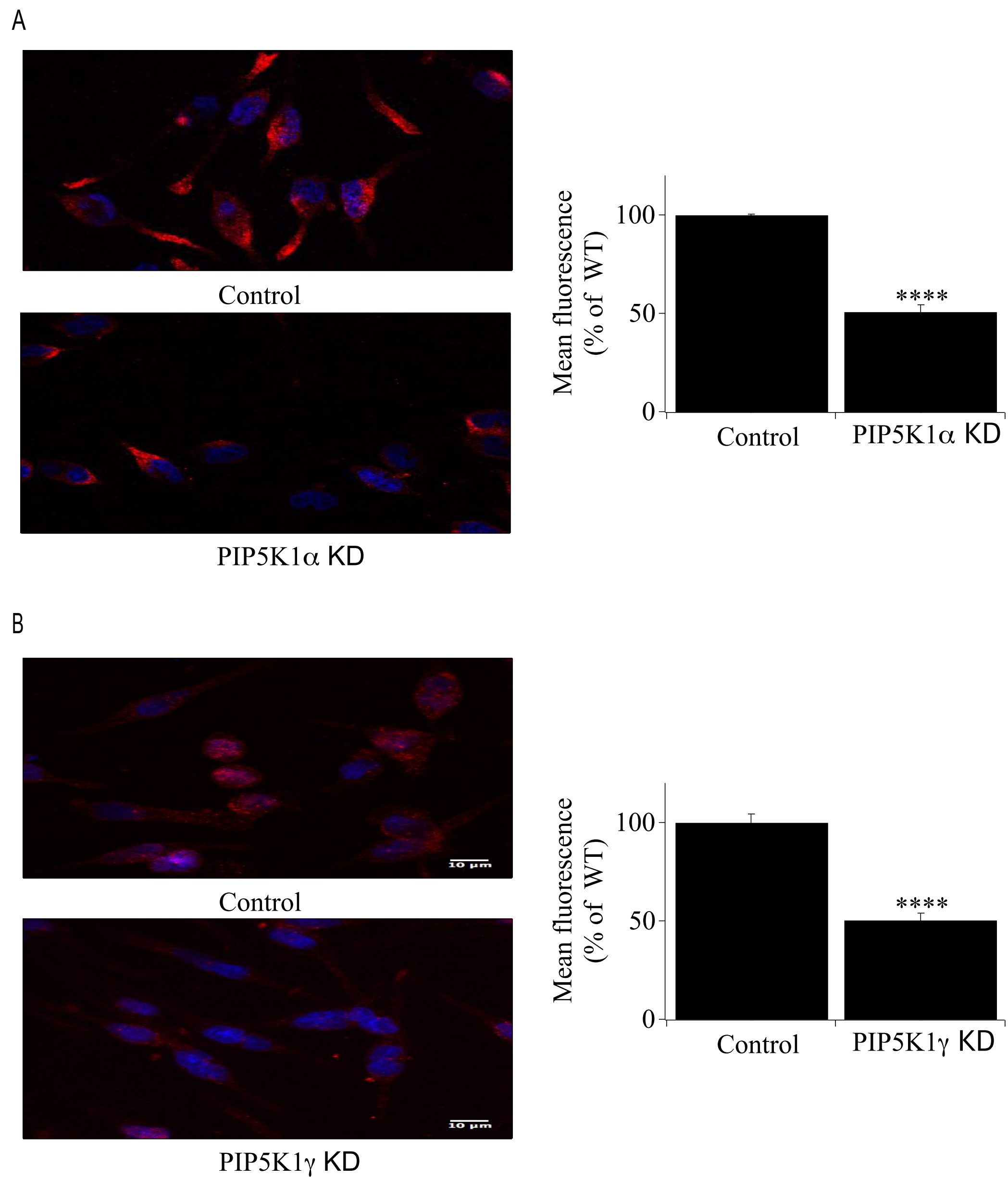

Figure 5.5. Knockdown of PIP5K1 isoforms in RBL-2H3 cells
Confocal microscopy images showing the expression of (A) PIP5K1α and (B) PIP5K1γ protein levels in mock-transfected cells and knockdown cells. The corresponding histograms represent a collection of data from 40 cells for each group.

5.2.5 Effect of PIP5K1α and γ knockdown on the calcium signal

To determine which PIP5K1 isoforms contributed to the PIP_2 pool that is hydrolysed by the LTC_4 /PLCβ pathway, I measured the calcium signal evoked by LTC_4 in either mock-transfected cells or in those where PIP5K1α or γ was knocked down. Compared to mock-transfected cells, the knockdown of PIP5K1α resulted in rapid rundown of calcium oscillations induced by LTC_4 when cells were stimulated in the presence of external calcium (Fig 5.6A and B). The number of calcium oscillations in PIP5K1α was reduced by $74.0\% \pm 2.3\%$ when compared to mock-transfected cells. Surprisingly, PIP5K1γ was also recruited to sustain the calcium signal because a rundown of calcium oscillation was also prominent when the cells were stimulated with 160 nM LTC_4 in the presence of external calcium (Fig 5.6C). The knockdown of PIP5K1γ resulted in a decrease in the number of calcium oscillations by $60.4\% \pm 8.6\%$ when compared to the ones observed in mock-transfected cells. Collective data from three independent experiments are shown in Fig 5.6D. The effect of the knockdown of either PIP5K1α or PIP5K1γ on calcium oscillations in the absence of external calcium was mild but significant (Fig 5.7A-D). PIP5K1α and PIP5K1γ resulted in $\approx 20\%$ and $\approx 30\%$ reductions in the number of calcium oscillations, respectively, when compared to mock-transfected cells.

The striking reduction in the number of calcium oscillations following PIP5K1α or PIP5K1γ knockdown is most likely related to the limited availability of PIP_2 for hydrolysis by PLC rather than impaired calcium entry. To confirm this I compared the rate of calcium entry and release in mock-transfected cells and in those

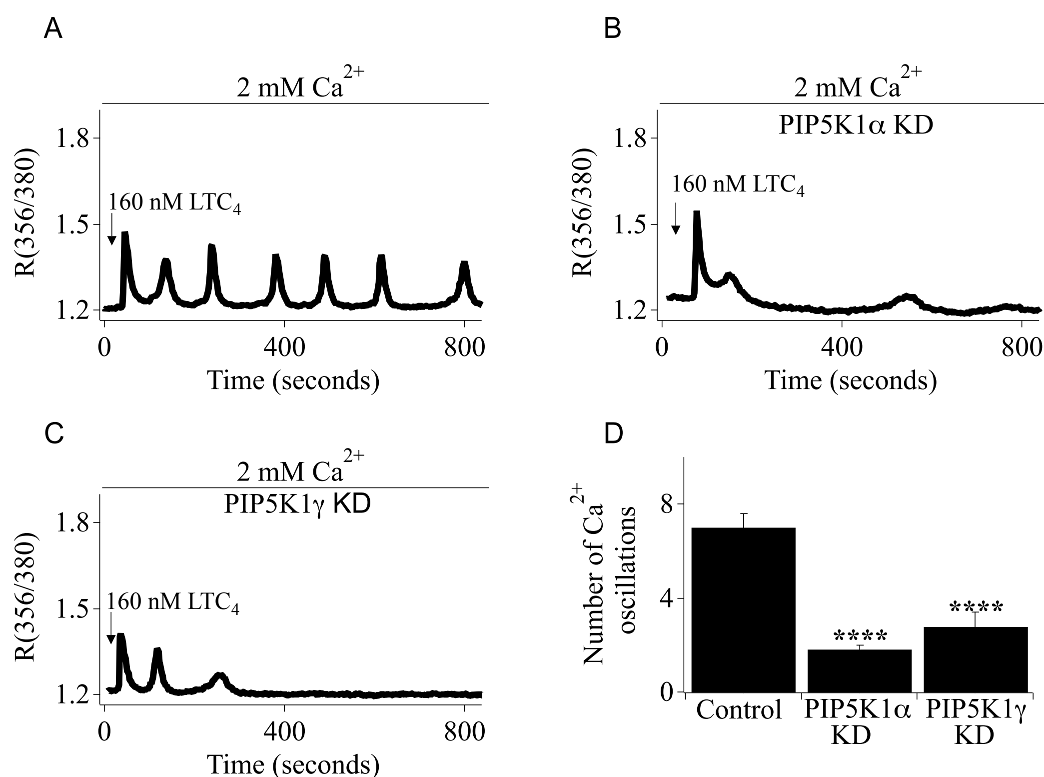

Figure 5.6. The effect of PIP5K1 on the calcium signal generated by LTC_4 in the presence of external calcium

Representative cell showing the oscillations of (A) mock-transfected, (B) PIP5K1α KD or (C) PIP5K1γ cells following the application of 160 nM in the presence of external calcium. (D) Histogram showing the average number of calcium oscillations from three independent experiments.

where PIP5K1α and PIP5K1γ were knocked down. The cells were bathed in a calcium-free solution, and 2 μM thapsigargin was added to the external bath solution followed by the addition of 2 mM calcium when the calcium signal (due to release) had returned to the baseline. Although the contents of calcium stores were slightly reduced, this reduction was not significant (upper and lower error bars are shown in Fig 5.7E. The rate of calcium entry was comparable between PIP5K1α, PIP5K1γ and mock-transfected cells, as seen in Fig 5.7E. Although the peak signal of PIP5K1α was higher than the control cells, the average rate of

calcium entry from three independent experiments is shown in Fig 5.7F.

To further confirm that the observed reduction in calcium oscillations in PIP5K1α, and PIP5K1γ knockdown cells is attributed to the limited availability of PIP_2, the cells were bathed in a LiCl containing solution and PI4P was added (Fig 5.8A-C). Unlike the results shown in Fig 5.2C and Fig 5.2G, PI4P was unable to rescue the oscillatory calcium signal when LTC_4 was added.

5.2.6 Spatial profile of PIP_2 synthesis in RBL-2H3

Since the discovery of these PIP5K1 isoforms, it was proposed that different isoforms functioned in different ways. Here I showed that at the functional level, both PIP5K1α and PIP5K1γ support the calcium signal induced by LTC_4. On the question of why different PIP5K1 isoforms are needed, it was proposed that different PIP5K1 isoforms localise to different micro domains near the cell membrane to generate specialised pools of PIP_2 [290]. my finding of both isoforms contributing to the calcium signal mediated by LTC_4 does not support the hypothesis of localised PIP_2 micro domains mediated directly by PIP5K1s isoforms. LTC_4 - mediated calcium signalling in RBL-1 was shown to be dependent on lipid rafts [481]. Accordingly, both isoforms have to be in lipid rafts. To examine this hypothesis, cells were pre-treated with 10 mM MβCD for 30 minutes to disrupt the lipid rafts. MβCD pre-treatment had no effect on the localisation of the leukotriene receptors, as shown in Fig 5.9A and B. Compared with the control cells, the calcium signal in MβCD pre-treated cells was abolished in cells stimulated with 160 nM LTC_4 in the presence of external calcium (Fig 5.9C). Aggregate data from three independent experiments is shown in the histogram in Fig 5.9D. The pre-treatment

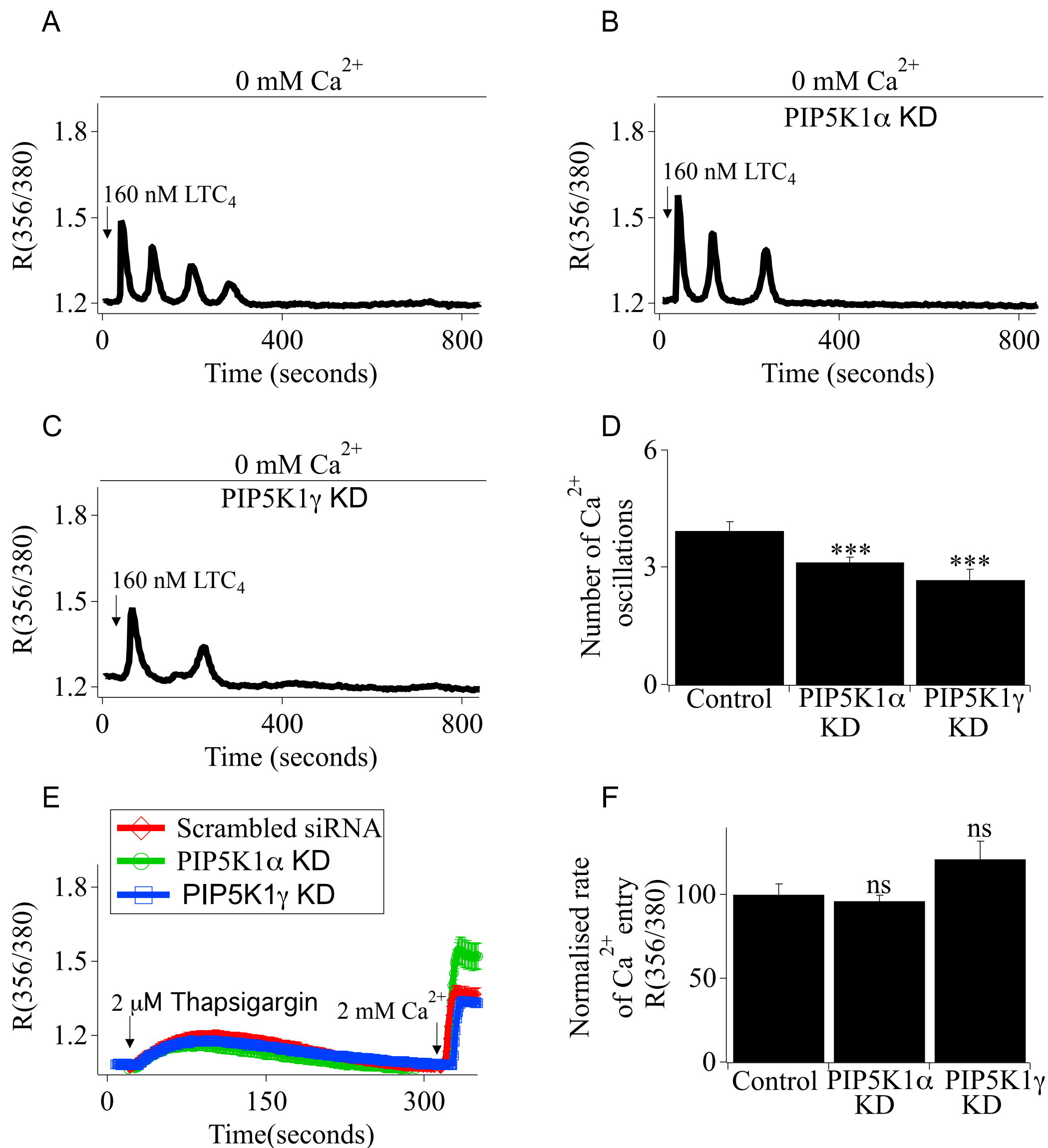

Figure 5.7. The effect of PIP5K1 isoforms knockdown on the calcium signal generated by LTC_4 in the absence of external calcium

Representative graph showing the oscillations of (A) mock-transfected, (B) PIP5K1α knockdown or (C) PIP5K1γ knockdown cells following the application of 160 nM LTC_4 in the absence of external calcium. (D) Histogram showing the average number of calcium oscillations from three independent experiments. (E and F) representative experiment comparing the average rate of calcium release and entry between mock-transfected, PIP5K1α knockdown and PIP5K1γ cells. Collective data from three independent experiments are shown in the histogram.

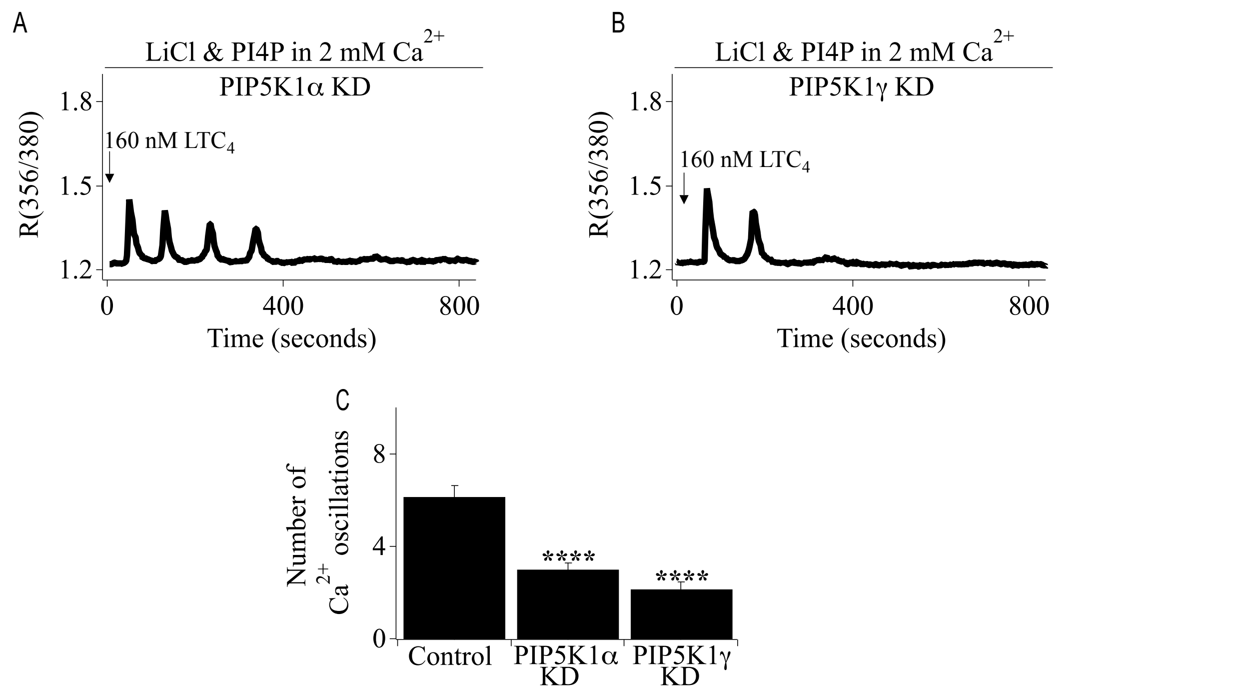

Figure 5.8. Failure of PI4P to rescue the oscillatory calcium signal in PIP5K1α and PIP5K1γ knockdown cells

Stimulation with 160 nM LTC_4 after the application of 70 μM PI4P in 15 mM LiCl pre-treated (A) PIP5K1α or (B) PIP5K1γ knockdown cells. Collective data from three independent experiments are shown in the histogram in C.

of MβCD had no effect on the calcium signal mediated by the P2Y receptor when activated with ATP (Fig 5.9E), indicating the localisation of the P2Y receptor to a spatially different micro domain other than that of the leukotriene receptor (possibly non-lipid rafts). The intact ATP-mediated calcium signal provided evidence that both PIP5K1α and PIP5K1γ support calcium signalling in both lipid rafts and non-lipid raft domains of the plasma membrane. One possibility is that PIP5K1 isoforms localise to a specific domain of the plasma membrane and a global PIP_2 pool is produced that is accessible by other receptors. To examine this, cells were pre-treated with 15 mM LiCl for 90 minutes to deplete the cellular PIP_2 levels. Following this, cells were stimulated with LTC_4 . In Chapter Four, I established that the early rundown of calcium oscillations in cells pre-treated with LiCl and stimulated with LTC_4 is related to the depletion of PIP_2 levels. Accordingly, once the cell stopped oscillating, ATP was applied at a concentration of 100 µM . Surprisingly, ATP induced a calcium signal comparable to that observed in the control cells (Fig 5.10A and D). It is clear that receptors do not share the same general pool of PIP_2 and once the local PIP_2 pool accessible to a receptor is depleted, access to the global pool is restricted. Since the site of inhibition of LiCl is early in the inositol cycle, the observed signal could be related to different PI4P pools accessible by the P2Y receptor [322]. Accordingly, LiCl pre-treated cells were pre-treated with 10 µM Wortmannin for 10 minutes followed by stimulation with LTC_4 , and once the oscillations stopped as a result of PIP_2 depletion, cells were stimulated with 100 µM of ATP Fig 5.10B. Again, no effect on the calcium signal induced by ATP was observed when the PI4P/PIP_2 pathway was inhibited. To investigate whether the observed signal might be related to the PI5P/PIP_2 pathway, LiCl pre-treated cells were pre-treated with 160 nM of the PIKfyve inhibitor YM201636 Fig 5.10C.

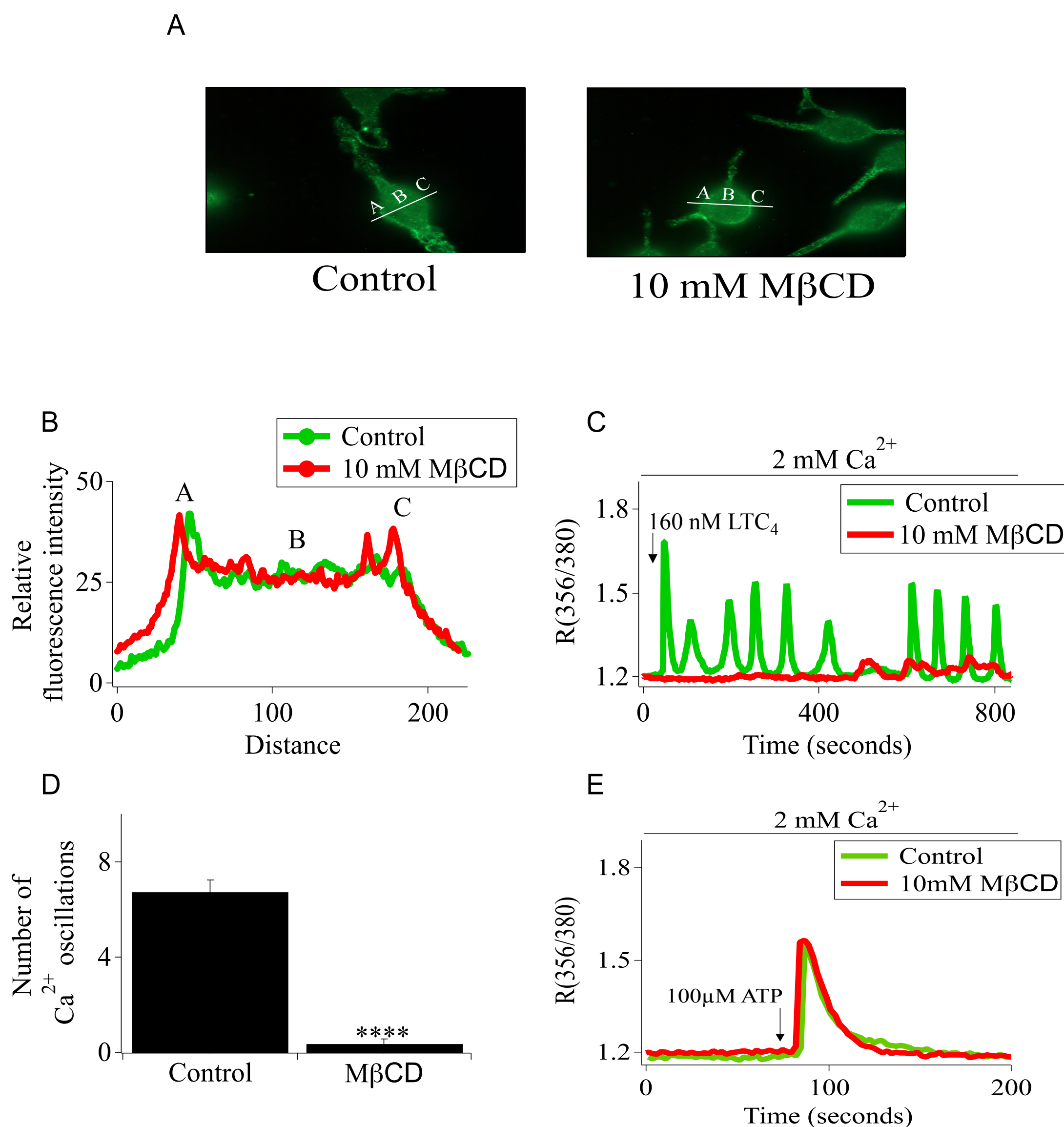

Figure 5.9. Subcellular localisation of the leukotriene receptor and the P2Y receptor (A) Images obtained using confocal microscopy showing the localisation of FLAG-tagged CysLT1 following stimulation with 160 nM LTC_4 in control cells and in cells treated with 10 mM MβCD. The fluorescence profiles from the line scans are shown in (B). (C) Representative calcium oscillations following the stimulation of LTC_4 in control cells and in cells pre-treated with 10 mM MβCD. Aggregate data analysing the number of oscillations from three independent experiments (30 cells) are shown in the histogram in D. (E) Representative calcium signal following the application of ATP in control cells and cells pre-treated with 10 mM MβCD for 30 minutes.

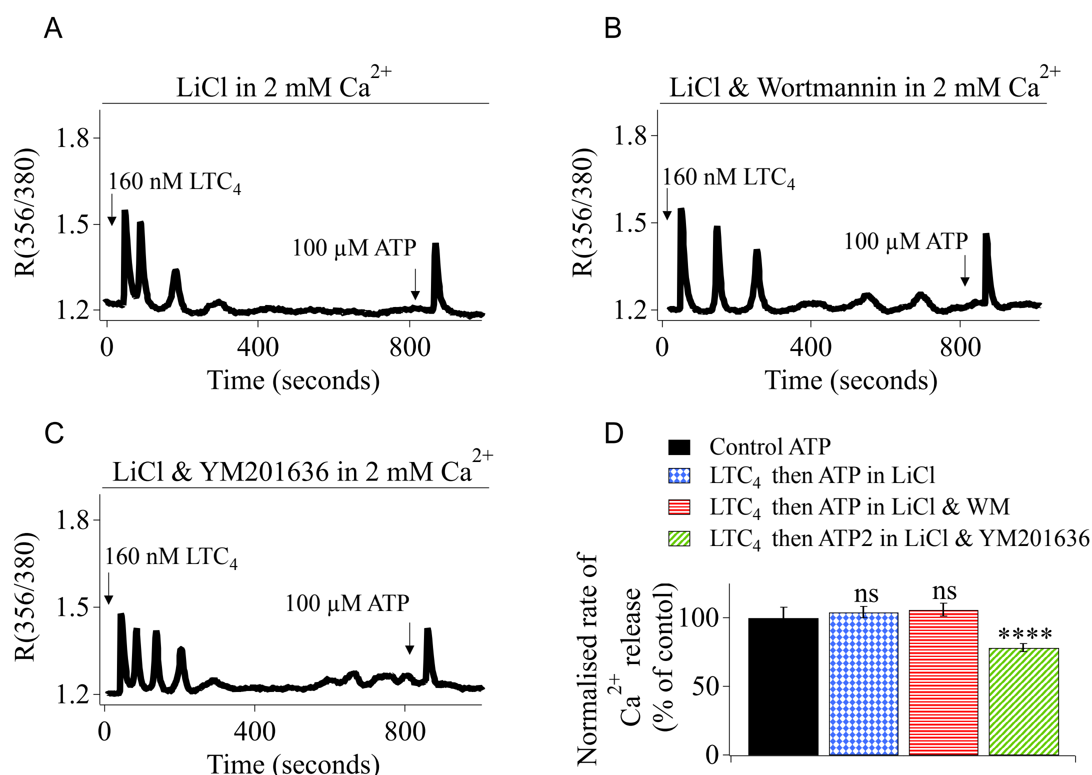

Figure 5.10. Access of PIP_2 pools by different receptors

(A) Representative graph showing the oscillatory calcium signal following the application of LTC_4 in cells bathed in the presence of external calcium and pre-treated with 15 mM of LiCl for 90 minutes; once the oscillations stopped, 100 µM ATP was applied. A similar experimental protocol was followed for both (B) and (C) with the additional application of 10 mM Wortmannin and 160 nM YM201636, respectively. (D) Collective data analysing the rate of ATP-induced calcium release from 30 cells from three independent experiments.

A reduction of 20% was observed in the rate of calcium release of the ATP-induced calcium signal, indicating that only part of the local pool around the vicinity of the P2Y receptor is provided by PI5P. Since PIP5K1 isoform distribution is not based on lipid rafts versus non-lipid rafts of the plasma membrane, the finding here supports the theory of PIP_2 compartmentalisation in the plasma membrane.

5.3 Key findings of this chapter

- LTC_4-mediated calcium signalling in RBL-2H3 cells is dependent on PI4P-specific pools.
- The conversion of PI4P into PIP_2 requires calcium entry via CRAC channels.
- RBL-2H3 cells express only two isoforms of PIP5K1: PIP5K1α and γ.
- Both isoforms of PIP5K1 support the oscillatory calcium signal induced by LTC_4 and thus provide an overlapping function in RBL-2H3 cells.
- The compartmentalisation of PIP_2 pools in the plasma membrane might not be dependent on PIP5K1 isoforms localisation in lipid and non-lipid rafts.

5.4 Discussion

To identify the role of PI4P and PI5P in supporting the oscillatory calcium signal by LTC_4, I have found that unlike PI4P, PI5P had no role (Fig 5.1). The amount of PI4P relative to PI5P is fiftyfold higher, and it has been suggested that most cellular PIP_2 comes from PI4P, so it was not unexpected that PI5P inhibition had no role in supporting the calcium signal induced by LTC_4 [482]. The observed inhibitory action of Wortmannin, but not the PIKfyve inhibitor YM201636, on the calcium signal (Fig 5.1C-F), indicated that the pathways involving phosphatidylinositol, PI4P and PIP_2, are the ones involved in supporting the calcium signal by LTC_4. The conversion of phosphatidylinositol into PI4P is mediated by the family of four PI4Ks with only two isoforms sensitive to Wort-

mannin [271, 273, 468, 469, 480]. Wortmannin was able to inhibit PIP_2 production in agonist-stimulated cells [483]. Accordingly, it was suggested that PI4KIIIα and PI4KIIIβ are the ones responsible for the plasma membrane PIP_2 pools [260][262]. Specifically blocking PI4KIIIβ shows no effect on agonist-mediated calcium signalling; therefore, PI4KIIIα was designated to be the main isoform in plasma membrane PIP_2 production [218, 260, 262]. my results showed no effect of Wortmannin pre-treatment on calcium release or entry (Fig 5.1E and F). The effect of Wortmannin and phosphoinositide levels on calcium entry seems to be inconsistent among multiple studies with many discrepancies. Korzeniowski et al. suggested that in COS cells, PI4P levels, (but not PIP_2), had an effect on calcium entry via Orai1 [245]. Walsh et al. provided evidence using a higher dose of Wortmannin in Hela cells that PIP_2 levels, but not PI4P, are responsible for regulating calcium entry by coupling STIM1 to Ora1, although overexpression of Orai1 eliminated the need for PIP_2 to regulate the coupling proces [246]. Finally, Calloway et al. suggested that it was not the levels of phosphoinositide, but the isoforms of the kinases, regulated STIM-Orai interaction by the formation of local PIP_2 pools, thus mutating the polyarginine sequence of Orai1 had no effect on calcium entry [244]. The differences between these studies could be attributed to the followed experimental protocol where the depletion of phosphoinositides occurred either prior to STIM1-Orai1 coupling or after. Moreover, pre-treatment times and Wortmannin dosages were not consistent among these studies. Furthermore, the expression levels of PI4Ks among the different cells types could be a contributing factor. It is clear that for the effect of PI4P or PIP_2 on calcium entry to be observed, a substantial depletion of polyphosphoinositides levels is needed. Supporting the notion that a substantial depletion is needed, in RBL-2H3 cells, following an agonist-mediated

depletion of polyphosphoinositides levels, the use of a Wortmannin dose that was tenfold higher and a period of pre-treatment three times longer than that suggested in Hela or COS cells were needed to achieve changes to the rate of calcium entry [243]. My data are in line with those published by Malth J et al. where the effect of PIP_2 on Orai1 was examined and it was concluded that it had no effect on Orai1-mediated calcium entry [484]. I showed that by using a dose of Wortmannin at the lower end of the suggested dose to affect PI4P, an effect on the calcium signal by LTC_4 was observed without affecting the rate of calcium entry or release.

PIP5KIβ was shown not be expressed in RBL-2H3 cells (Fig 5.4). This result was not anticipated on the basis that mast cells isolated from PIP5KIβ-deficient mice showed an augmented degranulation response following antigen stimulation [259]. As detailed in the results section, to confirm my findings, I used the same primer and followed the same experimental protocol to examine the levels of PIP5KIβ genomic expression. This was done to exclude any possible errors in the primer design or the experimental procedure. To my knowledge, no other reported work examining the role of endogenous PIP5KIβ in rat mast cells has been published, and the discrepancy between my results and those of Sasaki et al. is difficult to explain. One possibility is simply due to the variations in protein expression across species, especially as PIP5KIβ is not crucial for cell survival, as it was reported that bone marrow-derived macrophages isolated from mice do not express PIP5K1β with abundance in the two other isoforms [297]. Moreover, PIP5KIβ knockout mast cells showed only 15% reduction in the level of PIP_2 with an increase in IP_3 and $PI(3,4,5)P_3$ levels, and the observed effect was attributed to PIP5KIβ role in actin polymerisation [259]. Furthermore, it has been demonstrated that

PIP5KIβ-deficient mice were able to reproduce, and no anomalies or histological abnormalities were detected [259]. On the contrary, when PIP5K1γ was deleted, all animals died within 24 hours post-natally [394].

This project provided the functional profile of PIP5K1 isoforms role in mast cell signalling mediated by leukotriene receptors. Two previous studies have looked into PIP5K1 in mast cells; none of them examined the functional role of all the isoforms in the same cell line or primary cells. One study examined the role of PIP5K1γ in bone-marrow-derived mast cells from mice, and PIP5K1β was over-expressed and examined in an RBL-2H3 cell line [254]. The second study looked exclusively into the β isoform in bone-marrow-derived mast cells from mice [259]. In agreement with the results presented here, bone marrow-derived mast cells from mice lacking PIP5K1γ isoform showed a decreased antigen response [254]. Furthermore, it has been shown that the PIP_2 pool produced by PIP5K-Iγ87 is the one responsible for PLCβ-mediated calcium production when Hela cells are challenged with histamine, whereas the other isoforms have no role [485]. Moreover, platelets that lacked PIP5K1α showed a remarkable reduction in IP_3 levels following stimulation with thrombin [54]. Further supporting the role of PIP5K1γ in the agonist-mediated production of PIP_2 pools, it was suggested that ATP-mediated calcium production in cochlear non-sensory cells was dependent on PIP5K1γ [486].

Unlike PIP5K1γ, studies focusing on the PIP5KIα contribution to IP_3 -mediated calcium signalling are limited. Most studies investigating PIP5KIα look into its role in the phagocytic process [295, 296, 296, 487]. Consistent with the results presented in this chapter regarding PIP5KIα, it was suggested that PIP5KIα deficient platelets exhibited a reduction in PIP_2 and IP_3 levels following thrombin stimula-

tion [298]. Thus, the results here support the role of PIP5KIα in agonist-mediated PIP_2 production.

Taking into account the various functions mediated by PIP_2, it remains unclear whether or not there is a common pool of PIP_2 that mediates all the cellular activities or a specific pool for each cellular function. Assuming that a specific pool regulates PIP_2 function, how these pools are synthesised and distributed into the plasma membrane is not clearly established. Numerous studies have examined the hypothesis of the localisation of different isoforms of PIP5K1 to specific plasma membrane domains, as reviewed in detail by Kwiatkowska et al. [430]. It was shown that PIP_2 is confined to lipid rafts in multiple cell types [488–491]. At first glance, one might think that this hypothesis fits well for mast cells, especially since the class of 'immunoreceptors', such as B-cell receptors, some Fc-receptors and integrin receptors of T-cells, are believed to be dependent on lipid rafts for their activities [492–494]. In agreement with my results, more recent studies have critically analysed the local enrichment of PIP_2 to specific domains with the conclusion that the need for lipid rafts for the mobilisation and function of PIP_2 do not prove their association [495, 496]. Furthermore, studies using the FRET technique have provided evidence that no lipid raft-specific pools of PIP_2 were to be found [320, 495]. Despite the fact that the localisation of PIP_2 to certain domains is no longer supported, a global pool of PIP_2 accessible by all receptors is not the case here [495]. my data supports the concept of a decreased lateral mobility of PIP_2 observed in atrial cells where PIP_2 depletion is localised to GqPCRs [465, 497]. The notion of localised PIP_2 synthesis is supported by the concentration of PIP5K1s around the sites of actin polymerisation [295, 325, 498], or focal adhe-

sions [325, 326]. How different isoforms localise to certain cellular locations might be attributed to the lateral sequestration of phosphoinositides by certain plasma membrane proteins [430]. Another possibility is the recruitment of PIP5K1s to the plasma membrane by certain proteins such as talin and Rac, and this will be addressed in detail in the next chapter [430]. Finally, one possibility that needs to be addressed in the future is that different splice variants of PIP5K-Iγ might control the calcium signal induced by LTC_4, and thus the functional distinction of PIP5K1 in supporting the calcium signal might be at the level of splice variants and not the isoforms.

This chapter provided evidence that the calcium signal in RBL-2H3 is supported by PI4P and not PI5P-mediated PIP_2 production. Moreover, the conversion of PI4P into PIP_2 required calcium entry via CRAC channels. Furthermore, evidence is provided that implicated both PIP5K1α and PIP5K1γ in maintaining the PIP_2 pool needed to support the calcium signal induced by LTC_4. Finally, my results suggest that the observed calcium signal is dependent on a local rather than a global pool of PIP_2.

Chapter 6

The need to activate talin-1 to support the oscillatory calcium signal of LTC_4

6.1 Introduction

This chapter aims to extend the findings presented in the previous chapter concerning the conversion of PI4P into PIP_2 via PIP5KIα and PIP5K1γ regulated by calcium entry through Orai1. Given its reported role in mast cells' calcium signalling in addition to its clinical significance, this chapter investigates calcium's regulation of PIP5K1γ. Talin is a focal adhesion protein shown to activate PIP5K1γ and be regulated by calcium via multiple mechanisms. The role of talin in supporting the calcium signal mediated by LTC_4 in RBL-2h3 cells via its association with PIP5K1γ will be examined, as well as how calcium entry via CRAC channel

regulates talin.

PIP_2 functions as either a source of IP_3 due to PLC hydrolysis or a lipid messenger. PIP_2 's versatile functions have been attributed to numerous reasons and several hypotheses have been considered. One hypothesis involves the spatiotemporal pattern of PIP_2 production within cells [499], proposed to be directed by different PIP5K1 isoforms. Accordingly, if each isoform is localised to subcellular microdomains, then multiple PIP_2 pools will be produced that are distinct in their location and variable in their timing of PIP_2 production. The amino acid sequence of the catalytic domain of PIP5K1 shares an 80% identity among the three isoforms [292], the carboxyl and amino terminal variable regions of which are thought to target PIP5K1s to subcellular locations [292]. In support of this, the C-terminus of PIP5K1γ was found to interact with talin and to target the kinase towards focal adhesions [326]. PIP5KIα is furthermore thought to be targeted to the nucleus by its C-terminus [293], while Rac1 and ARF6 are suggested to recruit PIP5KIα to act in the plasma membrane [345]. In this context, RhoA has also been shown to interact with all PIP5K1 isoforms [345, 500, 501].

PIP5K1γ is a widely studied isoform recruited by talin to focal adhesions [326]. By extension, it was proposed that 26 amino acids in the C-terminal region of PIP5K1γ interact with talin, thereby leading to PIP5K1γ activation [326]. Further research has found that this interaction is regulated by the phosphorylation and dephosphorylation of PIP5K1γ at tyrosine 644 and serine 645, respectively [502], and that the phosphorylation at tyrosine 644 may be regulated by focal adhesion kinase (FAK) [503]. Interestingly, the phosphorylation at tyrosine 644 indirectly regulates the interaction of talin and PIP5K1γ, while the phosphory-

lation of tyrosine 644 by FAK prevents serine 645 phosphorylation by p35/Cdk5 [502]. Another protein found to activate PIP5K1γ is the adaptor protein complex AP-2 [348], which was shown to interact with PIP5K1γ in the same region as talin [348]. Contrary to what was observed for talin, the phosphorylation-mediated dephosphorylation of serine 645 is believed to depend on calcineurin [348].

Talin is a protein of 2,541 amino acids with an amino-terminal globular head and C-terminal rod [327]. Talin's globular head contains an amino acid sequence matching that of the ezrin, radixin, and moesin family of proteins and was accordingly named the FERM domain, which is composed of three subunits: F1, F2, and F3 [327]. The PTB domain of the F3 subdomain is thought to interact with PIP5K1γ [326], while FAK has also been shown to interact with talin in the FERM domain [504]. Talin's globular head is linked to the C-terminal rod via an 80-amino acid residue segment [328]. In its resting state, talin has been proposed to take an auto-inhibited conformation in which the C-terminal rod interacts with the FERM domain at F2 and F3 [330, 505]. This model of auto-inhibition is not unique to talin but observed in other proteins containing FERM domains [506].

Several studies have clarified calcium's effect on talin. It has been suggested, for example, that during inflammation, talin recruitment for adhesive contacts is mediated by calcium entry via CRAC channels [507]. More specifically, STIM1-mediated calcium signalling regulates the recruitment of FAK and talin for focal adhesions [508]. In microglial cells, talin was shown to be highly regulated by calcium entry via CRAC channels [509]. Moreover, believed to activate talin, calpain is also thought to depend on calcium entry [510, 511]. Calpain-mediated cleavage of talin is believed to be important for focal adhesion disassembly which

was defective in cells lacking STIM-1 [512] [510](Fig 6.1). Calpain activity was also revealed to depend on STIM1 and Orai1 levels in keratinocytes [513] and to become significantly reduced by the knockdown of STIM1 [514].

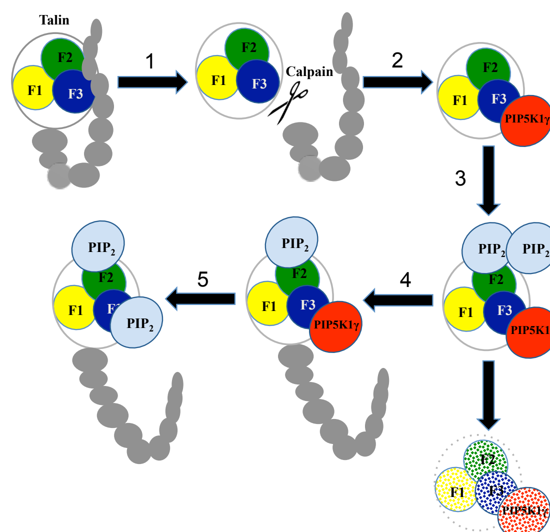

Figure 6.1. Calpain-mediated proteolysis of talin and PIP_2 production
In its resting state, talin is proposed to exist in an auto-inhibitory state in which the rod interacts with the F2 and F3 domains of the globular head. (1) Once calpain is activated, it cleaves talin at the linker region between the amino terminal globular head and the C-terminal rod, yielding a 47-kDa head and a 190-kDa C-terminal rod. The free head can function independently of the full-length protein. (2-3) PIP5K1 can now bind the free F3 subdomain, thus leading to the local enrichment of PIP_2 . (4) The binding of the surrounding un-cleaved talin to the newly produced PIP_2 helps to alleviate talins auto-inhibitory state and PIP5K1 can bind the F3 subdomain and further PIP_2 is produced. (5) Once higher levels of PIP_2 is produced PIP_2 bind both the F2 and F3 subdomains of talin and the free head eventually get ubiquitinated via Cyclin-Dependent Kinase 5 and Smurf1 mediated processes.

Findings that talin is highly regulated by calcium entry prompted me to propose that the mechanism by which calcium entry contributes to PIP_2 production involves the calcium-mediated regulation of talin. Talin-1 cleaved only given calcium entry via the CRAC channel. Furthermore, the oscillatory calcium signal in LTC_4 was examined in talin-1 knockdown cells, whose rundown of calcium oscillations was premature compared to that of control cells. Furthermore, adding

the PIP_2 substrate inositol and PI4P into the bath solutions failed to increase the oscillations compared to the control cells.

6.2 Results

6.2.1 Expression of talin isoforms in RBL-2H3 cells

Two talin genes that encode two isoforms are thought to occur in all vertebrates, though expression levels are believed to vary among different tissues [515]. Accordingly, the expression levels of the two isoforms were examined in RBL-2H3 cells using RT-PCR (Fig 6.2). Talin-1 was robustly expressed in RBL-2H3 cells, though talin-2 expression did not occur.

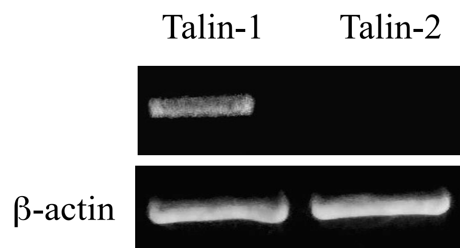

Figure 6.2. Talin isoforms in RBL-2H3 cells
RT-PCR shows the expression levels of talin-1 and talin-2 in RBL-2H3 cells. The lower panel shows the corresponding loading control using beta-actin.

6.2.2 The calcium-mediated cleavage of talin-1 depends on calcium entry via CRAC channels

Pull-down assays in leukocytes have revealed that kindlin-3 forms a complex with Orai1 and talin and that talin recruitment for adhesive contact sites is mediated

by CRAC channels [507]. This prompted me to examine if calcium entry in RBL-2H3 cells with endogenous talin-1 and Orai1 led to talin-1 cleavage. Accordingly, a western blot analysis was performed; resting cells revealed no proteolytic fragment of talin-1 (Fig 6.3). When cells were bathed in a calcium-free solution and calcium release from internal stores was activated by the addition of 2 µM thapsigargin, the talin-1 product was comparable to that observed in resting cells. Following the same protocol and adding calcium to the bath solution 4 minutes after applying 2 µM thapsigargin to activate CRAC channels resulted in two talin-1 cleavage products. Blocking CRAC channels with 10 µM of the specific CRAC channel blocker Synta for five minutes resulted in the abrogation of the talin-1 cleaved product.

To further situate the findings in a more physiological context, cells were stimulated with LTC_4 and talin-1 cleaved products observed. In cells stimulated with a submaximal dose of LTC_4 in the absence of calcium entry, in which cells were bathed in an external solution lacking calcium, no cleavage product of talin-1 was observed. On the contrary, when cells were bathed in a calcium-containing solution and stimulated with LTC_4, a proteolytic product of talin-1 appeared, while blocking CRAC channels with 10 µM Synta for five minutes attenuated the cleaved product. To localise the site of cleavage, a specific calpain inhibitor was used in cells stimulated with LTC_4 in the presence of external calcium. Ultimately, the cleaved product in cells pre-treated with 30 µM of calpain 2 inhibitor for 10 minuntes was significantly reduced. The talin-1 proteolytic product was quantified for each treatment and is shown in the histogram in Fig 6.3.

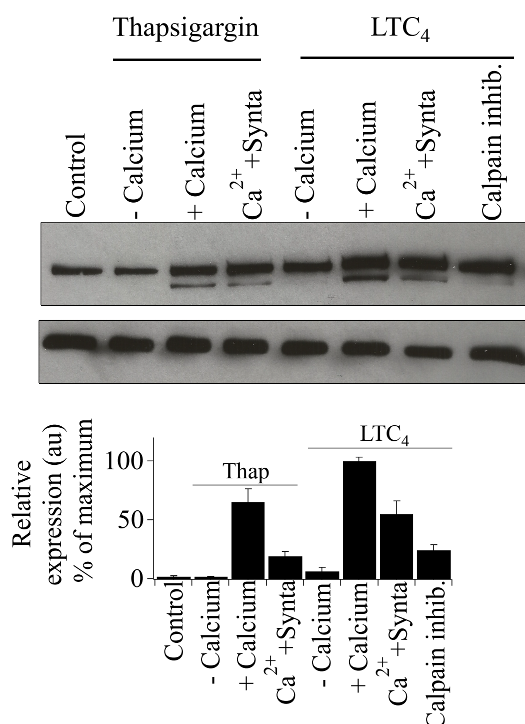

Figure 6.3. Talin-1 proteolysis requires calcium entry via CRAC channels
Representative western blot experiments show full-length talin and talin proteolysis in cells stimulated with either thapsigargin or LTC_4 in the presence or absence of calcium. Talin-1 proteolysis is clearly dependent on calcium entry via Orai1 in RBL-2H3 cells. The calpain 2 inhibitor was added to cells for 10 minutes prior to LTC_4 stimulation. The histogram shows data from two independent experiments aimed to quantify talin-1 proteolysis.

6.2.3 Knockdown of talin-1 in RBL-2H3 cells

To examine whether talin-1 is necessary to activate PIP5K1γ in mast cells by opening CRAC channels, I needed to decrease talin-1 expression in RBL-2H3 cells and to monitor the oscillatory calcium signal induced by LTC_4 . To decrease the expression of talin-1 in RBL-2H3 cells, small interfering RNA was introduced into the cells via electroporation. After 36 hours, protein expression levels were visualised using confocal microscopy in stained and fixed cells with immunohistochemical techniques. The expression levels in knockdown cells were reduced by approxi-

mately 52% compared to wild-type cells (Fig 6.4A). Data from three independent experiments were quantified and appear in the histogram in Fig 6.4B.

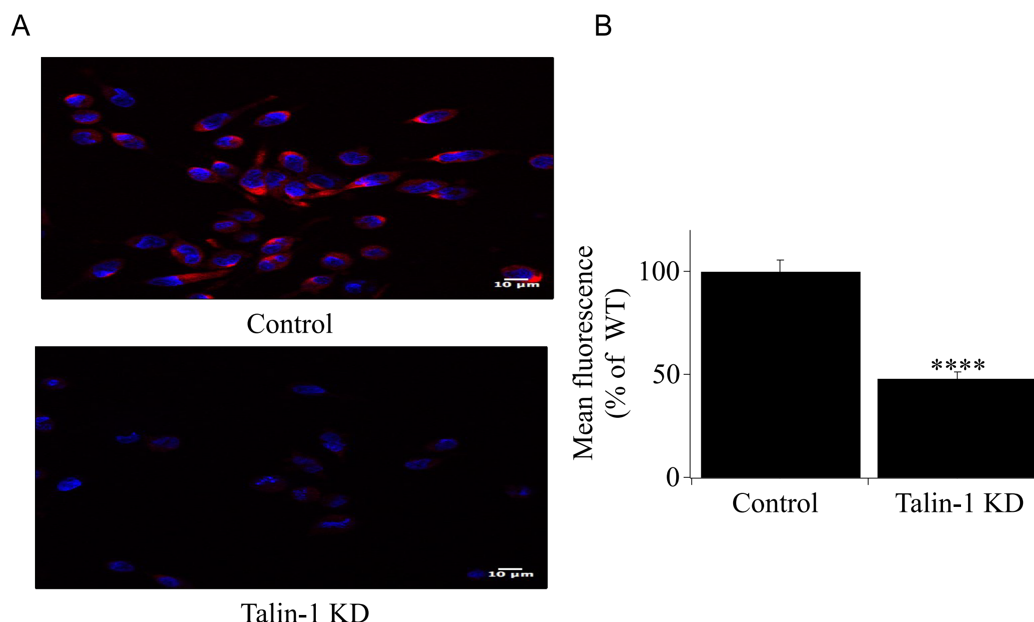

Figure 6.4. Knockdown of talin-1 in RBL-2H3 cells
The level of protein expression of Talin-1 in control cells and knock-down cells was obtained using confocal microscopy. (B) Collective data from three independent experiments appear in the histogram.

6.2.4 The role of talin-1 in LTC_4-mediated calcium signalling in RBL-2H3 cells

The previous chapter has shown that PIP5K1γ supports the calcium signal induced by LTC_4. Since talin is proposed to regulate PIP5K1γ [326], I sought to examine whether talin contributes to the calcium signal mediated by LTC_4 in RBL-2H3. To test how talin-1 knockdown cells affect the oscillatory calcium signal induced by LTC_4, cells were bathed in a solution containing external cal-

cium and stimulated with 160 nM LTC_4 . Since talin-2 was not expressed, siRNA against talin-2 was used as the control in this set of experiments. Talin-2 knock down cells produced an oscillatory signal that is similar to non-transfected cells (Fig 6.5C). Unlike in the control cells (Fig 6.5A)., in which a submaximal dose of LTC_4 effectively produced calcium oscillations on a low baseline lasting the duration of the recording period, talin-1 knockdown cells showed a significant reduction in the number of oscillations when stimulated with the same dose of LTC_4 (Fig 6.5B). The percentage reduction in calcium oscillations was 65.4% $\pm$ 4.1% when compared to the number of oscillations in the control cells. Depicted in the histogram are data from three independent experiments (Fig 6.5C).

The observed reduction in the number of oscillations was possibly attributable to the effect of talin-1 knockdown on calcium entry. To rule out this possibility, the cells were stimulated with 2 μM thapsigargin in the absence of external calcium, and calcium release from the internal stores was monitored. Calcium was then added to the bath solution, and the rate of calcium influx via CRAC channels in talin-1 knockdown cells and in the control cells was compared (Fig 6.6A). Both the rate of calcium release and entry in knockdown cells showed no significant differences compared to those of the control cells. Data from three independent experiments quantifying the rate of calcium entry appear in the histogram in (Fig 6.6B). To further confirm that talin-1 knockdown did not affect the calcium content in the internal store, cells were stimulated with a submaximal dose of LTC_4 in the absence of external calcium. The number of calcium oscillations in the control cells (Fig 6.6C) and in those in which talin-1 was knocked down (Fig 6.6D) were also statistically comparable, as no significant difference was observed. Collective

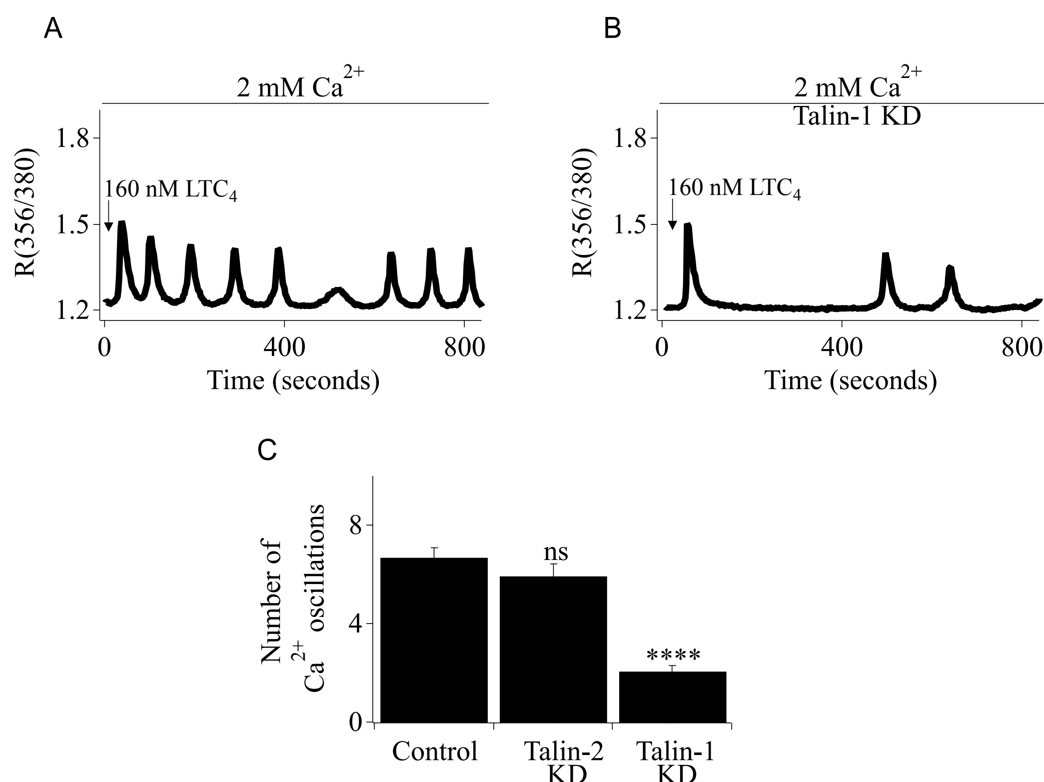

Figure 6.5. reduction in the number of calcium oscillations in talin-1 knock-down cells

(A) A representative cell shows LTC_4 -mediated calcium oscillation in cells stimulated in the presence of external calcium in (A) control cells and (B) talin1 knockdown cells. Collective data from three independent experiments appear in C.

data from two independent experiments are shown in the histogram in Fig 6.6E.

Lastly, to rule out that the observed effect did not stem from the reduction of PIP5K1s levels within the cells but were owing to talin, I examined the expression levels of PIP5K1α and PIP5K1γ in talin-1 knockdown cells. As shown in Fig 6.7A and B, the knockdown of talin-1 did not affect the expression levels of PIP5K1α. On the contrary, PIP5K1γ expression seemed to be slightly greater in talin-1 knockdown cells (Fig 6.7C and D).

In an attempt to link the observed rundown of the oscillatory calcium signal in

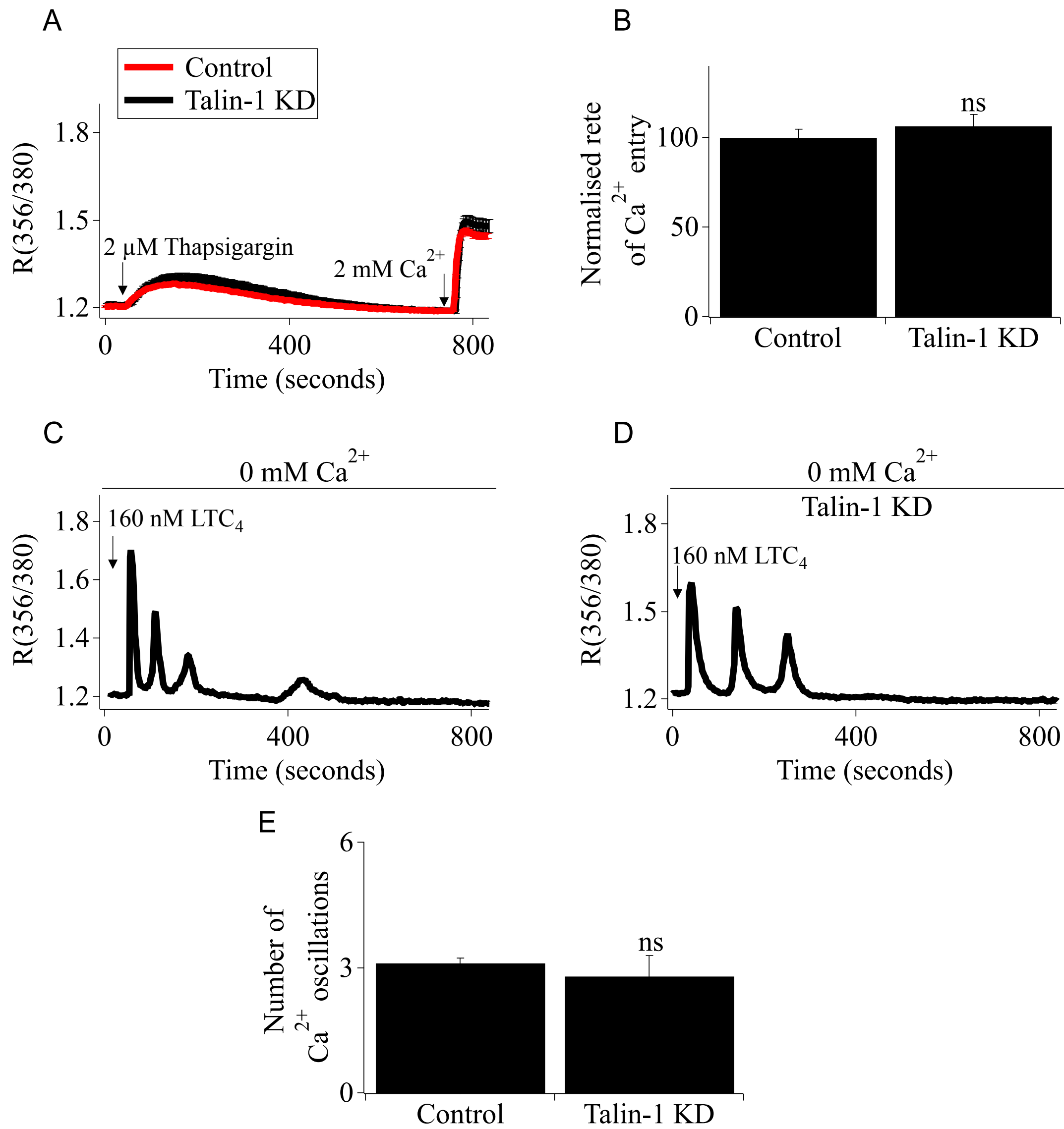

Figure 6.6. Calcium entry and release unaffected by talin-1 knockdown
(A) This representative experiment shows the average rate of calcium entry and release in talin-1 and control cells. Collective data from three independent experiments are shown in (B). (C) Representative cells show the oscillatory calcium response following LTC_4 stimulation in control cells and (D) talin-1 knock-down cells in the absence of external calcium. Collective data from two independent experiments is shown in D.

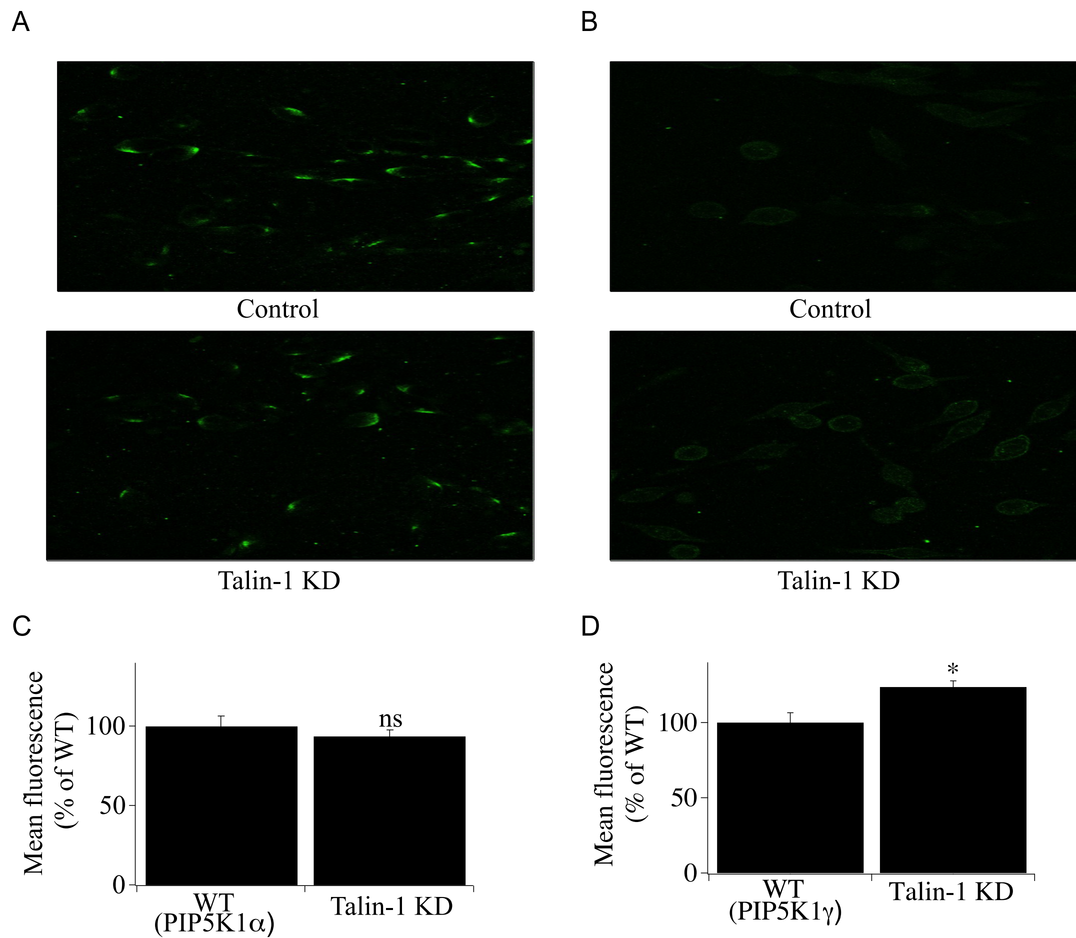

Figure 6.7. Expression levels of PIP5K1 isoforms in talin-1 knock-down cells
A representative image obtained using a confocal microscopy shows the expression of (A) PIP5K1α and (B) PIP5K1γ in control cells and cells in which talin-1 was knocked down. Levels of (C) PIP5K1α and (D) PIP5K1γ expression were quantified from a collection of cells from two independent experiments and are shown in the histogram.

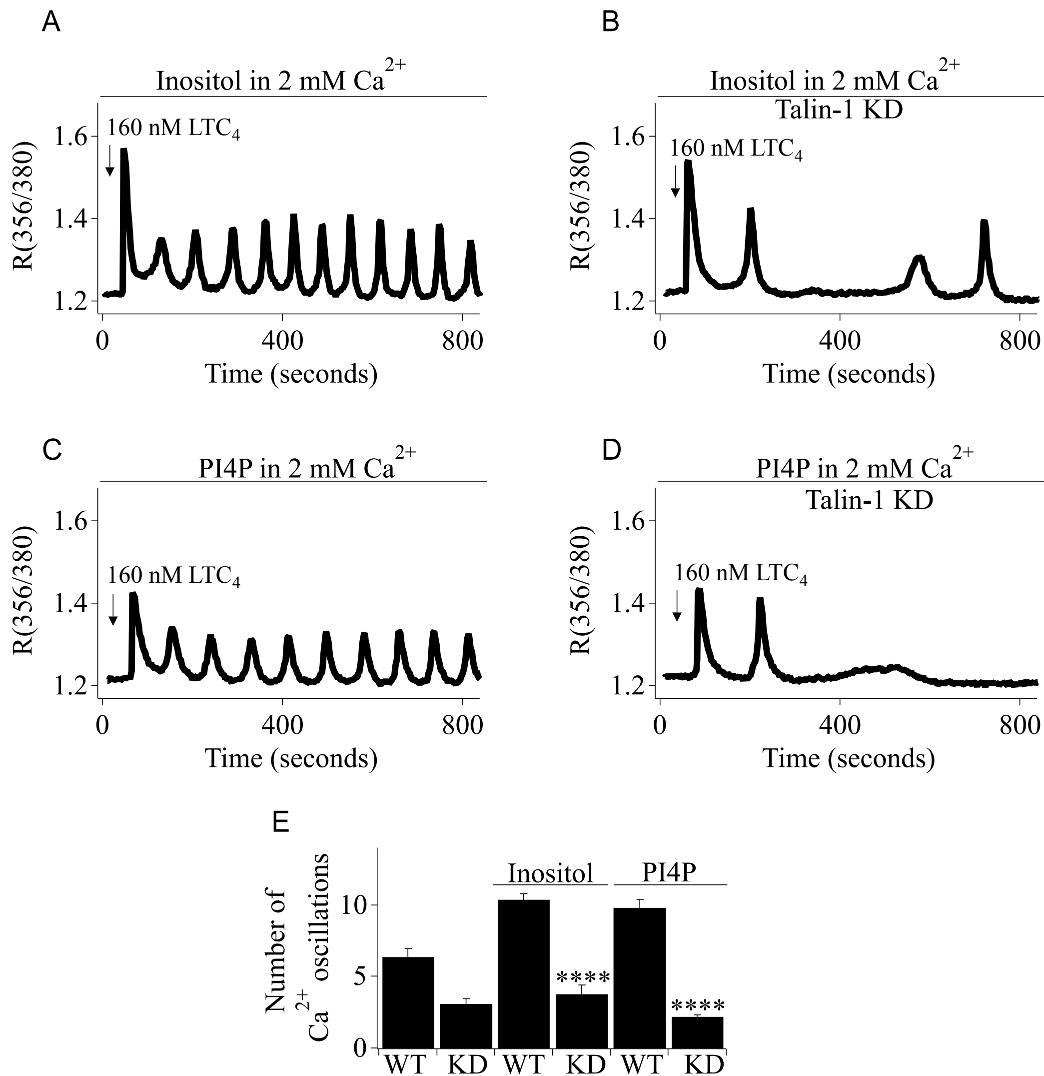

Figure 6.8. Phosphoinositide production is impaired in talin-1 knock-down cells
A representative graph showing the oscillations of (A) control cell and (B) talin-1 knock-down cell following the application of 160 nM LTC_4 in the presence of external calcium in cells pre-treated with 15 mM inositol. (C) Representative oscillations following the application of LTC_4 in a (C) control or (D) talin-1 knockdown cell, pre-treated cells with 70 µM PI4P. (E) This histogram shows the average number of calcium oscillations from collective data from two independent experiments.

talin-1 knockdown cells to talin-1's contribution to phosphoinositide production via PIP5K1γ, the conversion of exogenous inositol into PIP_2 was tested. Chapter 4 has discussed how the PIP_2 synthesis is a rate-limiting step and the addition of inositol to control cells led to $\approx$ 50% increase in the number of oscillations. Accordingly in an attempt to see if talin-1 knockdown cells were able to convert inositol to PIP_2 , 15 mM inositol was added to a calcium-containing bath solution. Unlike the control cells, pre-treatment with inositol in talin-1 knockdown cells led to a small increase in the number of oscillations following stimulations with a submaximal dose of LTC_4 , but this was not significant(Fig 6.8A and B). The number of oscillations was quantified from two independent experiments and shown in Fig 6.8E. This was further confirmed by the use of PI4P, like inositol, the application of PI4P to the control cells led a significant increase in the number of calcium oscillaions (Fig 6.8C). The application of PI4P to talin-1 knockdown cells failed to affect the number LTC_4-induced calcium signal (Fig 6.8D and E).

6.2.5 Calpain role in LTC_4 -mediated calcium signalling in RBL-2H3 cells

To further test the role of calpain which was shown to attenuated the cleaved product of talin-1 in Fig 6.3, I turned to Fura-2 calcium imaging. Following pre-treatment with 30 μM of calpain inhibitor for 10 minutes in cells bathed in a calcium containing solution, LTC_4 was applied and the number of calcium oscillations was quantified (Fig 6.9A). When compared to the control cells, the application of calpain 2 inhibitor resulted in 54.02% $\pm$ 3.5% reduction in the number of calcium oscillations when compared to control cells (Fig 6.9B). This reduction is most likely

due to the lost effect of calpain-mediated activation of talin-1, as the number of calcium oscillations in calpain inhibitor pre-treated cells when cells were bathed in calcium free solution is similar to the control cells (Fig 6.9C and D). Moreover, both the mechanism of calcium release and calcium entry were not affected when the cells were exposed to a calpain inhibitor for 10 minutes as shown in Fig 6.9E. Data from two independent experiments are shown in the histogram in Fig 6.9F.

6.3 Key Findings

- Talin-1 but not talin-2 is expressed in RBL-2H3 cells.
- The cleavage of talin-1 is dependent on calcium entry via Orai1.
- The oscillatory calcium signal mediated by LTC_4 was attenuated in talin-1 knockdown cells, possibly due to the reduction in PIP_2 levels.
- Inhibition of calpain 2 attenuated talin-1 cleaved product and led to an early rundown of the calcium signal induced by LTC_4 .

6.4 Discussion

The local enrichment of PIP_2 is mediated via PIP5K1 kinases, which have been proposed to be regulated via multiple partner proteins that yield spatiotemporally versatile PIP_2 pools [331]. An examination of many partner proteins in the literature to determine whether any are directly or indirectly regulated by calcium suggests that talin is a possible candidate for regulating PIP_2 production via the

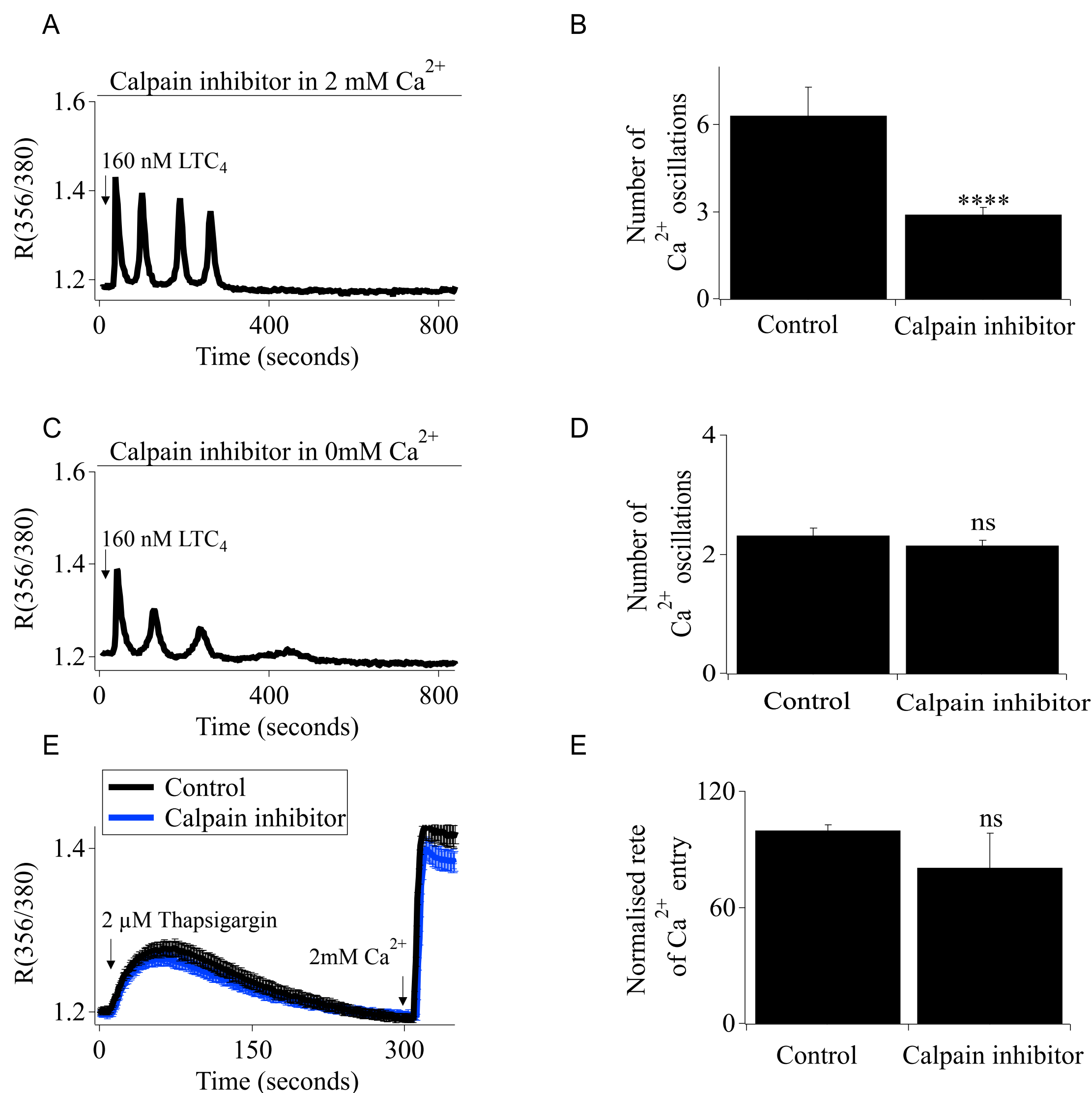

Figure 6.9. Calpain 2 inhibitor effect on LTC_4 - driven calcium oscillations
A representative graph showing the oscillations of (A) cell pre-exposed to 30 µM of calpain inhibitor for 10 minutes and stimulated with LTC_4 in the presence of external calcium. The average number of calcium oscillations obtained from three independent experiments is summarised and shown in (B). (C) Representative oscillatory calcium signal following LTC_4 stimulation in calpain 2 inhibitor pre-treated cells and in the absence of external calcium. (D) Histogram showing the average number of calcium oscillations from three independent experiments in calpain 2 inhibitor pre-treated cells and in the absence of external calcium. (E) The average rate of calcium entry and release in control cells and in those exposed to calpain 2 inhibitor for 10 minutes. Collective data from two independent experiments are shown in F.

activation of PIP5K1γ [326].

The talin protein is encoded by two different genes that yield two distinct isoforms with different functions [516]. The two isoforms share approximately 75% identity in their amino acid sequence [515], and are thought to mediate many functions [326, 510, 517, 518]. Talin-1 seems to play a chief role in embryogenesis, and mice lacking the gene failed to develop beyond gastrulation. This lethal phenotype was not rescued by talin-2 [519]. Expression levels of the two isoforms differ depending on the cell and tissue type, though talin-1 is typically more widely distributed [515]. This chapter's finding that talin-1 was expressed in RBL-2H3 but not talin-2 concurs with previous reports suggesting that talin-1 is expressed in nearly all tissues while talin-2 is predominantly seen in cardiac and skeletal muscles [515, 519].

Consistent with previous results, a global increase in calcium signal entry caused the cleavage of talin [507]. I showed specifically that calcium entry via CRAC channels in RBL-2H3 cells is needed for talin-1 cleavage. To further examine the role of talin-1 in the calcium signal mediated by LTC_4, I reduced the expression of talin-1 in RBL-2H3 cells. The calcium signal mediated by LTC_4 in talin-1 knock-down cells was reduced despite the presence of external calcium and of intact mechanisms of calcium release and entry. The observed reduction cannot be attributed to the levels of PIP5K1γ within cells, for when talin-1 was knocked down, the expression levels of PIP5K1γ within cells rose. This finding might support the fact that following PIP_2 production PIP5K1γ is ubiquitylated and degraded [520]. Since talin-1 is knocked down, the local production of PIP_2 becomes inhibited, and accordingly, PIP5K1γ begins accumulating in cells. This process is mediated by

the E3 ubiquitin ligase HECTD1 [520]. The failure of talin-1 knockdown cells to support the oscillatory calcium signal induced by LTC_4 is most likely due to the reduction of PIP_2 levels by PIP5K1γ. PIP_2 synthesis is impaired in talin-1 knockdown cells since adding inositol did not affect the number of calcium oscillations. PIP_2 levels are involved in a positive feedback mechanism for talin activation, and the local enrichment of PIP_2 allows talin to be unlocked for PIP5K1γ. It is believed that in its resting state, talin is restrained in an auto-inhibitory state in which the rod domain (talin R) interacts with the globular head (talin h) and prevents the head interaction with integrin β membrane-proximal cytoplasmic tails or PIP5K1α via the F3 subdomain [521]. The interaction of PIP_2 with talin has been shown to block the auto-inhibition of talin, and the local enrichment of PIP_2 caused its binding to the positively charged F2 and F3 subdomains of talin, thereby repelling the negatively charged talin R from binding to the head [522]. Since the F2 and F3 subunits of talin showed a stronger affinity for PIP_2 than other phospholipids [523], unlike PIP5K1γ, the binding of talin to PIP_2 helps to alleviate talin's auto-inhibitory state [329]. The PIP_2 -mediated unlocking of talin is believed to contribute to integrin β tails' binding to talin's head [524].

Talin activation has been shown to be mediated by the calcium-dependent calpain, which is believed to mediate the cleavage of talin between the amino terminal globular head and the C-terminal rod [525]. Calpain mediated the cleavage of talin release in the globular head from the C-terminal rod, yielding a 47-kDa head and a 190-kDa C-terminal rod [526]. Calpain belongs to the family of calcium-sensitive cysteine proteases and appears in two homologues μ-calpain (calpain 1) and m-calpain (calpain 2) whose primary difference lies in their calcium sensitivities, as

calpain 1 needs calcium concentration in the micromolar range for its activation, whereas the activation of calpain 2 needs a millimolar range of calcium [527, 528]. Calpain 2 is thought to be the isoform responsible for talin cleavage [510], and it is believed that the free head can function independently of the full-length protein following cleavage [529]. Integrin β tails compete with PIP5K1γ for the F3 subunit of talin, though once calpain is activated, it is thought that calpain cleaves the integrin β tails, thereby favouring the binding of PIP5K1γ to talin [530, 531]. In support of this, it has been suggested that increased intracellular calcium prompted focal adhesion disassembly [532, 533]. The activation of talin has been viewed according to its role in the assembly and disassembly of focal adhesions [508]. My results indicate that talin cleavage depends on calcium entry via CRAC channels, as the cleaved product size was reduced in cells in which CRAC channels were blocked despite the presence of external calcium, and were absent when there was no calcium entry. the findings further reveal that talin cleavage is mediated via calpain 2, since the pre-treatment with the calpain 2 inhibitor resulted in the inhibition of talin cleavage and an early rundown of LTC_4-mediated calcium oscillations was observed . Talin was observed to be cleaved by calpain in multiple cell types, including fibroblasts, platelets, and endothelial cells, in head and tail domains [534–536]. Calpain activity is believed to be regulated by SOCE [513, 514], and TRPC1 channels have also been shown to mediate the proteolysis of talin by calpain in neurons [537]. Lastly, the calcium ionophore ionomycin failed to cleave talin in T cells [538], suggesting that the calcium signal within specific cellular microdomains is responsible for talin cleavage.

The results here show that Orai1 is in continuous interaction with talin-1 levels

and that talin-1 proteolysis is dependent on calcium entry via CRAC channels. Furthermore, knockdown studies showed that talin-1 is needed to support the oscillatory calcium signal mediated by LTC_4, possibly through the activation of PIP5K1γ. These preliminary findings suggest the role of calcium entry in regulating PIP_2 production via the cleavage of talin.

Chapter 7

General discussion

The significance of understanding the mechanisms by which mast cells are activated cannot be overstated. This is because mast cells have been linked to multiple allergic and inflammatory conditions [154, 155]. Allergic conditions affect more than 60 million people in Europe and there are an estimated one billion cases around the world [539]. The prevalence of asthma in the United Kingdom is estimated at 15-20% with the majority of cases in children 5-14 years old [540, 541]. Moreover it has been found that allergic rhinitis is present in up to 26% of the United Kingdom population [542]. Prevalence of Nasal Polyposis, which is an allergic condition that shares many similarities with asthma and allergic rhinitis, is estimated to be between 1-4% [543, 544].

Despite many classes of medications that were introduced as controlling agents for asthma, 10% of asthmatic cases remain uncontrolled and constitute more than 75% of the total cost allocated for asthma management in health institutes and centers around the world [539]. Moreover, it has been found from a recent prospective

cohort study for 25 years, that the mortality rate among asthmatic patients was much higher when compared to the controlled group, mainly due to the consequent development of complications [545]. Similar to asthma, a percentage of allergic rhinitis patients remain symptomatic despite the presence of multiple anti-allergic medications available [546].

These data indicate that a new more specific treatment is required for the management of allergic conditions in general. Moreover, a better understanding of the underlying pathophysiological causes is needed in order to establish new therapeutic approaches.

Calcium entry was shown to be crucial for mast cell activation [28, 132]. This thesis expanded our understanding of the interplay between changes in intracellular calcium levels and phosphoinositide production in mast cells. Both calcium and PIP_2 have been linked to the regulation of multiple cellular signalling events [4, 231]. The temporal and spatial organisation of both was shown to be crucial for multiple downstream processes and effectors [4, 233]. In non-excitable cells, a significant fraction of calcium influx is via CRAC channels [28, 132]. One of the objectives of this thesis is to expand our knowledge of how CRAC channels are involved in regulating agonist-driven calcium response in mast cells. I have shown that in the rat mast cell line, RBL-2H3, calcium influx via CRAC channels was needed to maintain LTC_4 -driven calcium oscillations. No other means of calcium influx via other calcium permeable channels was able to sustain the oscillatory calcium signal to the extent achieved by CRAC channels. This result strengthens and is consistent with the proposed role of CRAC channels in calcium signalling as the main physiological route of calcium influx in non-excitable cells [367, 368].

Furthermore, using an siRNA approach directed towards STIM proteins, I have identified the need for STIM1 but not STIM2 to support LTC_4 -driven calcium oscillations. This finding is in line with other findings published earlier [395] and supports the designated housekeeping role of STIM2, given its higher sensitivity to detecting changes in calcium levels within the ER [42]. Given these findings, I was able to further investigate how precisely calcium entry via Orai1 is involved in LTC_4 -driven calcium oscillations.

Using cytoplasmic calcium ion imaging, I monitored the calcium signal generated by LTC_4 after pharmacological manipulation of phosphoinositide levels using LiCl, inositol, and PI4P . My data showed that similar to inositol, PI4P conversion to PIP_2 was observed only when there was calcium entry via CRAC channels. The kinases involved in the conversion of PI4P into PIP_2 are the family of the PIP5K1 kinases [290]. The results in this thesis showed that only PIP5K1α and PIP5K1γ are expressed in RBL-2H3 cells and are needed to support LTC_4 -driven calcium oscillations. I further investigated how calcium affected the activity of PIP5K1γ [254, 547]. Multiple PIP5K1-binding proteins were shown to be sensitive to changes in calcium levels, one of which is talin [321, 337, 548]. Talin, a focal adhesion protein, has been shown as being regulated by calcium and is required to interact with and activate PIP5K [326, 507]. Consistent with reports linking talin activity to calcium entry, I demonstrated that talin cleavage was dependent on calcium entry through CRAC channels [508, 509]. Moreover, I linked talin to the phosphoinositide pathway by showing that cells in which talin had been knocked down, were unable to support the oscillatory calcium signal generated by LTC_4 . To further strengthen this finding I were able to show that unlike control cells,

the addition of inositol or PI4P to talin knockdown cells had no major effect on the number of calcium oscillations. My data suggest that calpain might decode calcium oscillaions by cleaving talin and thereby activation of talin. However, a further work is needed to strengthen this [549].

The observed effect of calcium entry on PIP_2 production and thus the continuity of LTC_4 -mediated calcium signalling is most probably attributed to calcium microdomains near open CRAC channels and not through a global calcium rise. Several findings support this notion; first, both inositol and PI4P failed to rescue the calcium signal in LiCl pre-treated cells when bathed in a calcium-free solution despite a comparable rise in bulk calcium to that induced by CRAC channel opening. Second, blocking CRAC channels produced similar results to those obtained when cells were bathed in a calcium-free solution, and again inositol and PI4P failed to rescue the calcium signal in LiCl pre-treated cells. Third, the overexpression of alternative calcium permeable pathways such as TRPC3 channels failed to support the oscillatory calcium signal induced by LTC_4 .

Two general hypotheses are suggested for how the available PIP_2 at the plasma membrane is formed: the local synthesis and the local sequestration [290]. The local sequestration of PIP_2 relies on the strength of the electrostatic interaction between certain plasma membrane proteins and phosphoinositides thus restricting their diffusion [428]. The local synthesis hypothesis is based on the local synthesis of PIP_2 via PIP5K1s [430]. Although the local sequestration of PIP_2 cannot be ruled out, the findings in this thesis are in favour of the local synthesis of PIP_2 via PIP5K1 isoforms. I showed that both PIP5K1α and PIP5K1γ were needed to support the oscilatory calcium signal by generated by LTC_4 . I further showed

that the localisation of PIP5K1 isoforms in RBL-2H3 cells was not based on local enrichment of PIP_2 to specific domains. These results are consistent with previous reports [320, 495, 495, 496]. Finally, the results here are in agreement with the suggestion that localised PIP_2 enrichment is most probably due to PIP5K1-binding proteins such as talin [290, 326]. A recent study demonstrated that upon store depletion, the STIM1-Orai1 complex translocates between PIP_2 -rich and PIP_2 -poor domains [484]. In future studies, it would be interesting to know if the activation of certain PIP5K1 isoforms binding proteins via Oria1 is limited to a specific domain of the plasma membane.

Three splice variants of PIP5K1γ have been identified in and shared by all mammalians: PIP5K1γ87, PIP5K1γ90 and the neuronal specific PIP5K1γ93 [292, 302, 303]. The difference between PIP5K1γ87 and PIP5K1γ90 is the inclusion of exon 17 into PIP5K1γ90 , resulting in an extension in its carboxyl terminus. This additional extension is suggested to target PIP5K1γ90 in certain subcellular domains, and to regulate its phosphorylation by multiple proteins and to function as the interaction site with cell-regulating cytoskeleton proteins [292, 304]. It was suggested that PIP5K1γ87 is the isoform responsible for maintaining the PIP_2 pool when HeLa cells are challenged with histamine [550]. Mast cells PIP5K1γ87 and PIP5K1γ90 are shown to be responsible for antigen-mediated calcium signalling [254]. Accordingly, in future studies, one point that needs to be addressed is which PIP5K1γ isoform is involved in LTC_4 -driven calcium signalling. Moreover, further consideration should be given to examining the possibility of splice variants'-specific effect on the levels of downstream calcium-dependent targets, such as the calcium-dependent nuclear factor of activated T cells (NFAT)-driven

gene expression and the extracellular signal-regulated kinases (ERK) should be considered [203, 398].

The field of nuclear phosphoinositides is a growing one. PI4P, PIP_2 and PIP5K1α as well as the human-specific PIPKI-v4, were reported to be present within the nucleus [304, 551]. Changes in nuclear phosphoinositide levels have been linked to the initiation and regulation of multiple nuclear functions [551]. An increase has been reported in nuclear calcium levels following cell stimulation with a variety of stimuli including LTC_4 have been reported [552, 553]. It was shown that PIP5K1α targets nuclear speckles via interaction with a non-canonical poly(A) polymerase known as Star-PAP [293]. It would be interesting to test the possibility of calcium playing a role in modulating the activity of PIP5K1α or PIPKI-v4 via Star-PAP.

PI transfer proteins (PITP) consist of five members divided into two families based on the sequence homology [554]. The agonist used and cell type are responsible for determining which PITP will be used [555]. The only phosphoinositide in the inositol re-synthesis cycle that requires the activity of PI transfer proteins is PI. Therefore, the obtained data using PI4P in LiCl cells (Chapter five), suggest that the activity of PI transfer proteins per se is not needed to maintain LTC_4 -driven calcium oscillations. Although I showed that calcium entry is required for the conversion of PI4P to PIP_2 the effect of calcium entry on PI transfer proteins cannot be ruled out, especially as a recent report showed that the activity of the PI transfer protein, RdgBα, is modulated by calcium [556].

Given the time limitation of this doctoral project, I was not able to further investigate how calcium entry regulates PIP5K1α. PIP5K1α was shown to interact with and be regulated by Rac1 [331, 341]. The PIPKIα-Rac1 complex is shown to be nec-

essary for the complex spatial localisation of PIP5K1 and PIP_2 production [341]. The activity of Rac1 was shown to be regulated by calcium through its calmodulin-binding domain, thus making it an excellent candidate to be investigated as a potential calcium-sensnitive PIP5K1α-regulator in RBL-2H3 cells [338, 339]. Another possible mechanism by which calcium entry regulates PIP5K1α is via phospholipase D. It was shown that PLD interacts with and recruits PIP5K1α localisation in different cells [557, 558]. PLD is activated by the increase in intracellular calcium levels [559]. Specifically, it was shown that PLD is sensitive to calcium entry through CRAC channels [560].

Further understanding the precise mechanisms of the issues that I have addressed in this thesis and how they are linked to the activation of mast cells as well as their contribution in allergic and inflammatory reaction should help us to develop new, pathway-specific and promising therapeutic agents. This could be of a great clinical value, especially to patients with resistant allergic conditions where they will be no longer rely on multiple classes of general anti-inflammatory and immunomodulatory agents that are currently available.

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