JOHANNES KEPLER UNIVERSITÄT LINZ | JKU

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Faculty of Engineering

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Role of Calmodulin in Store-operated Calcium Entry

MASTER THESIS

to confer the academic degree of

Master of Science

in the Master Program

Biological Chemistry

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Submission:

Institute of Biophysics

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Linz, September 2014

University of South Bohemia in České Budějovice

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ACKNOWLEDGEMENTS

First and foremost, I would like to thank my supervisor, a. Univ.-Prof. Dr. Christoph Romanin, for giving me the chance to be involved in his research team. Furthermore I would like to express my sincere gratitude to my co-supervisor, Dr. Isaac Jardin-Polo, for his excellent guidance and insightful comments. Beside his scientific advice I enjoyed his unceasingly cheerful character and the hearty atmosphere he was always able to create. I greatly benefited from Dr.in Isabella Derler and Dr. Rainer Schindl introducing me to electrophysiological experiments and Dr. Martin Muik helping me with confocal FRET microscopy. Moreover, I thank all other members of the Ion Channel Group for the pleasant time I had at the Institute of Biophysics.

Special thanks go to Prof. RNDr.Libor Grubhoffer, CSc. and Univ.-Prof. Dr. Norbert Müller, coordinators of the Biological Chemistry program, for giving me the opportunity to carry out my master thesis in Linz.

Finally, I want to thank my family and Markus Gall for their constant support and encouragement throughout my whole study.

This master thesis was sponsored by a scholarship grant by the AKTION Österreich-Tschechien.

ABSTRACT

Store-operated calcium entry (SOCE) is a unique process by which depletion of intracellular calcium (Ca^{2+}) stores causes a Ca^{2+} influx through plasma membrane channels. In immune cells, SOCE is mediated through Ca^{2+} release-activated Ca^{2+} (CRAC) channels and represents the major pathway, which increases the intracellular Ca^{2+} level leading to lymphocyte activation and immune response. Two groups of proteins are required for the assembly of a functional CRAC channel. Those are the endoplasmic reticulum Ca^{2+} sensor stromal interaction molecule 1 (STIM1) and the plasma membrane protein Orai1. The focus of this study was to analyse the role of the small ubiquitous Ca^{2+}-binding protein calmodulin (CaM) in the STIM1-Orai1 association and its contribution to SOCE. In order to elucidate the interactions between those proteins, HEK293 cells were used as an expression system. Our results demonstrate that CaM is able to interact with both proteins of the CRAC channel. Using co-immunoprecipitation and confocal FRET microscopy, we determined that the most prominent interactions occur in a Ca^{2+}-dependent manner. By constructing various STIM1 fragments we were able to determine the location of the CaM binding site on STIM1 coiled-coil 3 (CC3) domain. Disruption of the CC3 to a more stretched structure enhanced this association. Furthermore, we found that the subsequent addition of STIM1 CRAC modulatory domain (CMD) to STIM1 CC3 induces CaM-STIM1 association under resting conditions and dissociation upon stimuli. The importance of CaM in the initiation of SOCE was further investigated by electrophysiological techniques. Although CaM can bind both STIM1 and Orai1 upon store depletion, the STIM1-Orai1 complex is entirely sufficient to induce SOCE. Our data along with recent publications suggest that CaM might act as a bridging protein between STIM1 and Orai1 triggering a conformational change which leads to fast Ca^{2+}-dependent inactivation of CRAC channels.

ABBREVIATIONS

aa	amino acid(s)
ab	antibody
a.u.	arbitrary unit
Ca^{2+}	calcium
$[Ca^{2+}]_i$	intracellular calcium concentration
$[Ca^{2+}]_{ER}$	concentration of calcium in endoplasmic reticulum
CAD	CRAC activation domain
CaM	calmodulin
CBD_{Orai1}	calmodulin binding domain of Orai1
CC	coiled-coil
Ccb9	coiled-coil domain containing region b9
cEF1	canonical calcium binding EF-hand
CFP	cyan fluorescent protein
CMD	CRAC modulatory domain
Co-IP	co-immunoprecipitation
CRAC	calcium release-activated calcium
CT	carboxy-terminus
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
E_{app}	apparent FRET efficiency
ECFP	enhanced cyan fluorescent protein
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid
ER	endoplasmic reticulum
ETON	extended transmembrane Orai1 N-terminal
EYFP	enhanced yellow fluorescent protein

FCDI	fast calcium-dependent inactivation
HC	heavy chain
hEF2	hidden EF-hand
HEK	human embryonic kidney
HEPES	4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
HRP	horse radish peroxidase
I_{CRAC}	calcium release-activated calcium current
IgG	immunoglobulin G
IO	ionomycin
IP_3	inositol 1,4,5-trisphosphate
IP_3R	inositol 1,4,5-trisphosphate receptor
LB	Luria Bertani
LC	light chain
lp	long pass
OASF	Orai1-activating small fragment
PBS	phosphate buffered saline
PBST	phosphate buffered saline-Tween solution
PI	protease inhibitor
PIP_2	phosphatidylinositol 4,5-bisphosphate
PLC	phospholipase C
PM	plasma membrane
PMCA	plasma membrane calcium ATPase
PVDF	polyvinylidene fluoride
RPM	revolutions per minute
S.E.	standard error
SAM	sterile-α motif
SCID	severe combined immunodeficiency
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SERCA	sarcoplasmic/endoplasmic reticulum calcium ATPase

SHD	STIM1 homomerization domain
SOAR	STIM-Orai activating region
SOC	store-operated calcium
SOCE	store-operated calcium entry
STIM1	stromal interaction molecule 1
Sulfo-NHS-LC-Biotin	sulfosuccinimidyl-6-(biotinamido)hexanoate
TEMED	*N,N,N',N'*-tetramethylethylenediamine
TM	transmembrane
Tris	2-Amino-2-(hydroxymethyl)-1,3-propanediol
WB	Western blot
YFP	yellow fluorescent protein

TABLE OF CONTENTS

1. INTRODUCTION

1.1. Calcium (Ca^{2+})

Ca^{2+} is a ubiquitous intracellular messenger engaged in diverse cellular processes involving gene transcription, muscle contraction and exocytosis. Deviation from the normal spatial and temporal Ca^{2+} boundaries can lead to apoptosis or necrosis [1]. Under resting conditions, intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is very low, approximately 100 nM. The concentration in the intracellular Ca^{2+} store (e.g. endoplasmic reticulum (ER), $[Ca^{2+}]_{ER}$) is more than three orders of magnitude higher, ranging from 0.4 to 1 mM. With extracellular $[Ca^{2+}]$ being approximately 1.2-2 mM a large concentration gradient between the extracellular milieu, cytoplasm and the intracellular Ca^{2+} store is generated. In order to maintain the $[Ca^{2+}]_i$ very low, cells employ various Ca^{2+} pumps and transporters such as sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA), which pumps Ca^{2+} into the ER, and plasma membrane Ca^{2+} ATPase (PMCA) that pumps Ca^{2+} out of the cell. In non-excitable cells, the main mechanism by which the $[Ca^{2+}]_i$ is increased involves a release of Ca^{2+} from intracellular stores (most remarkably ER) and Ca^{2+} influx from the extracellular space [2]. Electrically non-excitable cells rely on slower calcium signals which are accomplished by a mechanism in which plasma membrane (PM) receptors are activated in response to stimuli (e.g. binding of hormones) resulting in generation and release of inositol 1,4,5-trisphosphate (IP_3) to the cytosol. IP_3 binds to the IP_3 receptor (IP_3R) on the ER membrane and mediates the emptying of Ca^{2+} from the ER [3]. The store depletion leads to an influx of extracellular Ca^{2+} through PM channels by a process called store-operated Ca^{2+} entry (SOCE) [4, 5].

1.2. Store-operated calcium entry (SOCE)

SOCE requires members of two families of proteins: STIM molecules (STIM1 and STIM2) and Orai channels (Orai1, Orai2 and Orai3), STIM1 and Orai1 being the major contributors to SOCE. Under resting conditions when Ca^{2+} stores are full, STIM1 proteins are distributed throughout the ER membrane and act as ER Ca^{2+} sensors. Orai1 molecules are localized in the PM and function as pore-forming subunits of SOCE channels. SOCE is a fully

reversible process which is initiated when the ER Ca^{2+} stores are depleted and terminated in response to Ca^{2+} store refilling [4, 6].

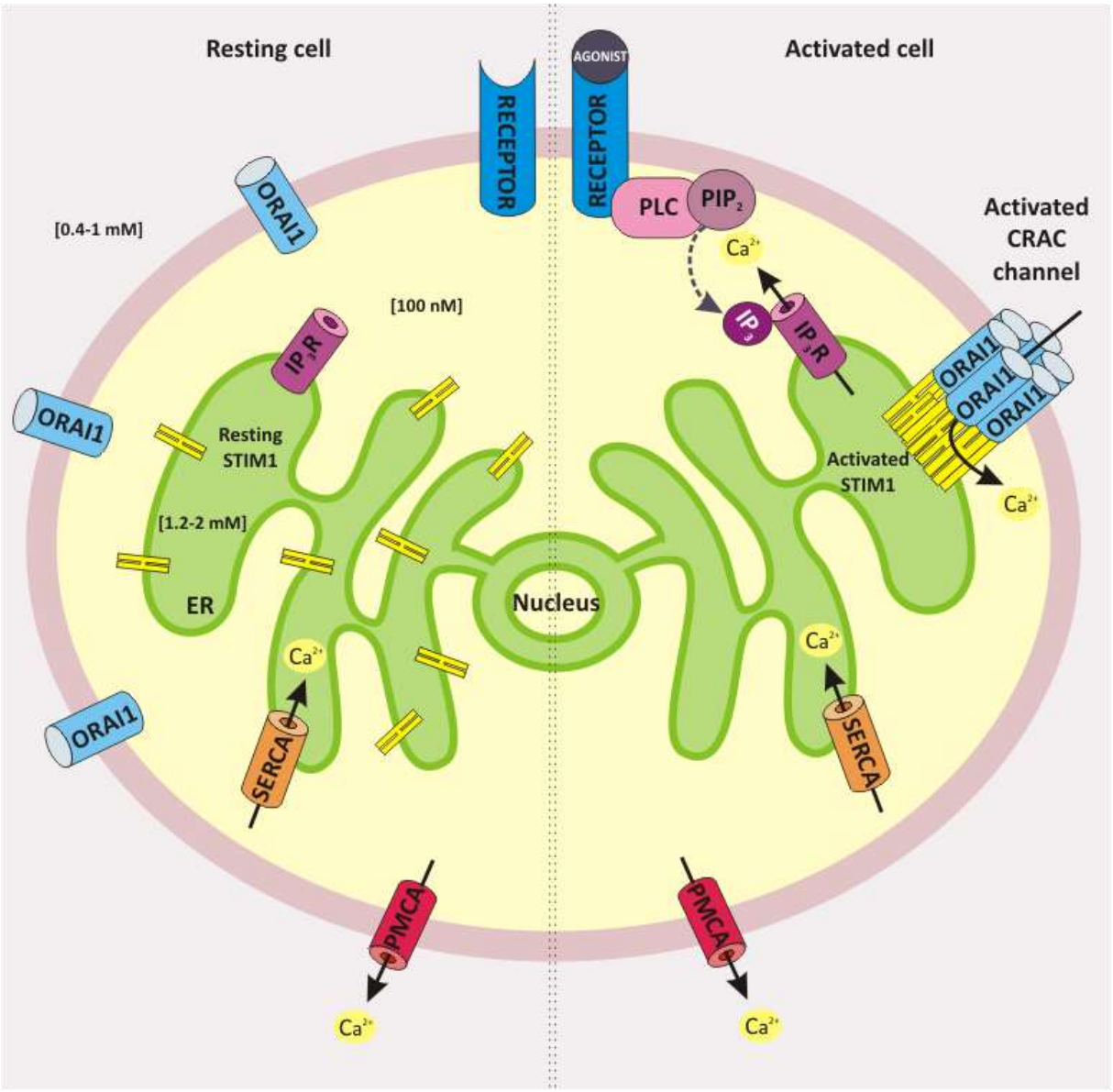

Figure 1: The Ca^{2+} signalling pathway highlighting the crucial steps of Ca^{2+} store depletion leading to SOCE initiation (modified from [7]). The left part of the scheme represents resting conditions and maintenance of Ca^{2+} level inside the cell by sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA) and plasma membrane Ca^{2+} ATPase (PMCA). The right part shows a cascade of events involving a ligand binding to a receptor, phospholipase C (PLC), phosphatidylinositol 4,5-bisphosphate (PIP_2), inositol 1,4,5-trisphosphate (IP_3) and its receptor (IP_3R) triggering the activation of Orai1 subunits of a Ca^{2+} release-activated Ca^{2+} (CRAC) channel by STIM1 oligomers and Ca^{2+} influx into the cytosol.

In non-excitable cells, the Ca^{2+} release from intracellular stores is induced by the activation of IP_3 receptors [8]. The IP_3/Ca^{2+} pathway is initiated when a ligand binds to phospholipase C (PLC) coupled receptors on the PM. The activated PLC hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP_2) and IP_3 and diacylglycerol are generated. The IP_3 molecule rapidly diffuses within the cytosol and directly binds to its receptor located on the ER. Such IP_3 binding causes a conformational change of IP_3R and the channel opens allowing Ca^{2+} stored at high concentrations in the ER to be released into the cytosol [9, 10] *(Figure 1)*. Depletion of ER Ca^{2+} stores causes dissociation of Ca^{2+} from the luminal EF hand binding domains of STIM1 which triggers activation and oligomerization of STIM1. The activated STIM proteins then translocate into ER-PM junctions where STIM1 oligomers form characteristic clusters called puncta. This relocalization allows the interaction with Orai proteins resulting in activation of Orai Ca^{2+} influx channels [11].

1.2.1. Ca^{2+} release-activated Ca^{2+} (CRAC) channel

CRAC channels belong to the class of store-operated calcium (SOC) channels. Being the best characterized SOC channels they are considered as a model for them. The CRAC current (I_{CRAC}) is mediated by functional CRAC channels made up of STIM1/Orai1 complexes [12]. STIM1 and Orai1 proteins are both necessary and entirely sufficient to mediate the process of store-operated channel function [13, 14]. The CRAC channels are highly selective to Ca^{2+}. Despite this high selectivity, they also possess the ability to conduct small monovalent cations (i.e. Na^+, Li^+, K^+) in divalent-free solution. Even μM Ca^{2+} concentrations in the solution inhibit the permeation of monovalent cations which is due to high affinity Ca^{2+} binding in the channel. Contrary to other Ca^{2+}-selective channels, unique features of CRAC channels are an extremely low single channel conductance and low permeability to large monovalent cations (i.e. Cs^+) [12].

Originally, it was assumed that the CRAC channel was a tetramer and required 2:1 stoichiometric ratio of STIM1:Orai1 to be maximally activated [15, 16]. However, the most recent evidence showed that Orai1 forms a hexameric assembly and six STIM1 are necessary to activate the channel [17, 18].

1.2.2. Functional role of SOCE

Though it was initially believed that SOCE is present only in non-excitable cells, nowadays it is evident that it occurs in virtually all cells including excitable cells like nerve and skeletal muscle cells [19]. In lymphocytes and other immune cells, SOCE represents the main Ca^{2+} influx pathway. SOCE through CRAC channels is required for the regulation of Ca^{2+}-dependent gene transcription and production of many cytokines. Mutations in Orai1 and STIM1 are mainly linked to immunodeficiency and autoimmunity. Immunodeficiency in CRAC deficient patients is characterized by repeated severe infections and the only functional therapy is hematopoietic stem cell transplantation [20]. In skeletal muscle, SOCE contributes to maintain long period muscle contractions. Whereas in non-excitable cells the full activation of SOCE requires tens of seconds, the activation in skeletal muscle is performed within a second. Malfunctions of SOCE and mutations in Orai1 and STIM1 can lead to muscular diseases [21]. SOCE plays a role in other biological processes as well, including endothelial proliferation, smooth muscle migration and proliferation, breast cancer cell migration and metastasis [22].

1.2.3. Stromal interaction molecule 1 (STIM1)

Only one kind of STIM protein is present in *Drosophila melanogaster*, while in vertebrates the STIM family comprises two closely related members, STIM1 and STIM2. Both STIM proteins are single-pass transmembrane proteins sharing a high sequence homology, however, there is a significant divergence within their C-terminal domain [23]. STIM1 is composed of 685 amino acids (aa) and is predominantly localized on the ER membrane with its N-terminus oriented into the ER lumen and C-terminus into cytosol [24]. Though around 25% of STIM1 can be found in the PM [25], PM STIM1 is not required for the CRAC channel activation [26]. Unlike STIM1, STIM2 is exclusively present in the ER [27]. Moreover, STIM2 is unable to produce a sufficient activation of Orai1 necessary for Ca^{2+} entry [28]. Its major role is the regulation of the Ca^{2+} influx and maintenance of Ca^{2+} homeostasis in cells [29].

When ER Ca^{2+} stores are filled (i.e. under resting conditions), STIM1 exists in the ER membrane as a dimer. Store depletion leads to the formation of STIM1 higher order oligomers [30, 31]. It can be assumed that ER STIM1 has five distinct functions in SOCE. Those are sensing of Ca^{2+} in the ER, signal initiation by oligomerization, translocation into ER-PM junctions,

organization of Orai1 subunits into clusters and activation of Ca^{2+} influx. In small cells, this entire intracellular process can occur within a minute [32].

The key component of the N-terminal domain of STIM1 is the EF-SAM domain. It contains an EF-hand pair which consists of a canonical Ca^{2+} binding EF-hand (cEF1) paired with a hidden EF-hand (hEF2). The hEF2 stabilizes the cEF1 via hydrogen bonding but does not bind Ca^{2+}. The paired EF-hands interact with the stability regulating sterile-α motif (SAM) mainly through hydrophobic interactions. Together this EF-SAM domain functions in Ca^{2+} sensing and its stability plays a major role in STIM1 function. When the luminal Ca^{2+} level decreases, Ca^{2+} dissociates from the EF-SAM domain causing its destabilization. The resulting conformational change leads to a partial unfolding and triggers the activation and oligomerization of STIM1 which represents the SOCE initiation mechanism. However, this process is entirely reversible when the luminal Ca^{2+} concentration is restored to its resting level [33]. Mutations in the EF hand domain resulting in loss of its Ca^{2+} binding ability mimic Ca^{2+} store depletion. When Ca^{2+} dissociates from the EF hand, translocation and oligomerization of STIM1 and subsequent activation of CRAC channels take place independently of store depletion [34].

While the N-terminal domain of STIM1 acts in regulation of the SOCE initiation, the cytosolic C-terminal domain is crucial for further STIM1 oligomerization, stabilization of oligomers, translocation of STIM1 oligomers to ER-PM junctions, interaction with and recruitment of Orai1 subunits to the same sites, gating of the CRAC channel pore and CRAC channel activation [35]. The C-terminal domain consists of three coiled-coil regions (CC1, CC2 and CC3), a STIM1 homomerization domain (SHD), a CRAC modulatory domain (CMD), a serine/proline-rich (S/P-rich) region and a lysine-rich region (K-rich) [36] *(Figure 2)*. It was found that a STIM1 fragment possessing only the cytosolic C-terminus (aa 233-685) is able to activate the CRAC channels independently of ER store depletion [37]. This finding lead to the identification of smaller STIM1 C-terminal fragments containing regions vital for the CRAC channel activation. These fragments are termed Orai1-activating small fragment (OASF, aa 233-450 or 233-474 in a longer version), coiled-coil domain containing region b9 (Ccb9, aa 339-444), CRAC activation domain (CAD, aa 342-448) and STIM-Orai activating region (SOAR, aa 344-422), SOAR being the smallest cytosolic fragment able to activate Orai1. All of them include the CC2 and CC3 domains and additional 39 residues [38].

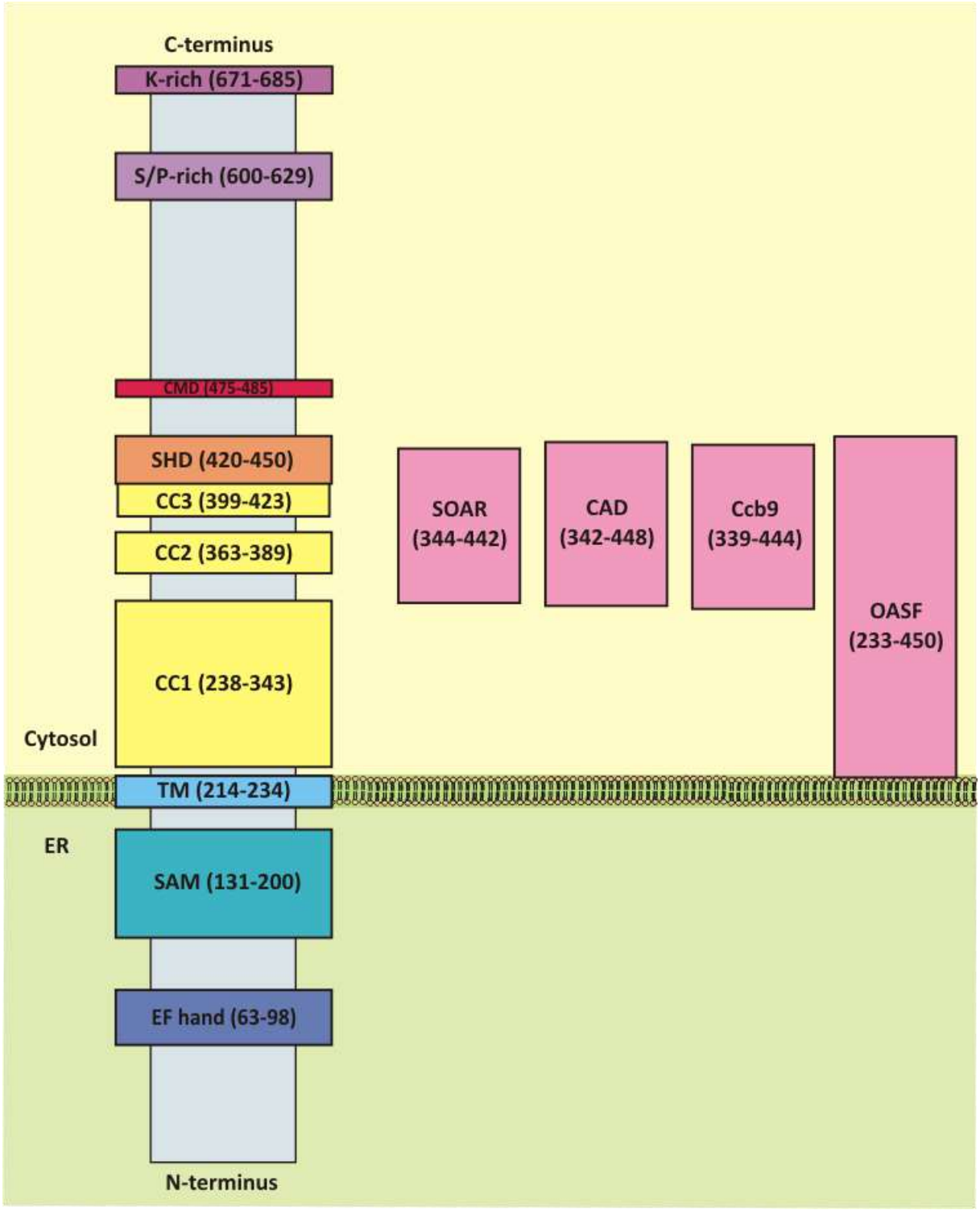

Figure 2: Schematic representation of STIM1 domain architecture. STIM1 contains an ER luminal N-terminal domain, a single transmembrane domain (TM) and a cytosolic C-terminal domain. The ER luminal N-terminal domain is composed of an EF-hand pair and a sterile-α motif (SAM). The cytosolic C-terminal domain consists of three coiled-coil regions (CC1, CC2 and CC3), a STIM1 homomerization domain (SHD), a CRAC modulatory domain (CMD), a

serine/proline-rich (S/P-rich) region and a lysine-rich (K-rich) region. Orai1-activating small fragment (OASF), coiled-coil domain containing region b9 (Ccb9), CRAC activation domain (CAD) and STIM-Orai activating region (SOAR) are smaller fragments of the cytosolic C-terminus involved in Orai1 channel activation.

Though, STIM1 lacking the C-terminal domain is able to oligomerize through EF-SAM interactions alone after store depletion [39], these oligomers are unstable. Therefore, cytosolic domains are necessary to stabilize store-dependent higher order oligomers. When stores are full, CC1 within STIM1 supports the formation of resting, inactive dimers. However, these dimers are not stable upon store depletion. Reinforcement by CAD is required to form stable, higher-order oligomers of STIM1 in response to store depletion [30, 31]. Deletion experiments in the OASF fragment further revealed a key component involved in the oligomerization. A homomerization domain (SHD, aa 420-450) is crucial for the formation of STIM1 dimers or larger assemblies which are required for the interaction with Orai1 and channel activation. C-terminal STIM1 fragments lacking this domain remain preferentially monomeric and are incapable of activating Orai1. Another identified domain within the STIM1 cytosolic part obtained by shortening of OASF is a modulatory domain controlling the extent of coupling to Orai1 [40]. This domain was called a CRAC modulatory domain (CMD, aa 475-485). CMD contains a cluster of seven negatively charged amino acids essential for the fast Ca^{2+}-dependent inactivation (FCDI) of Orai1. It was demonstrated that cytosolic C-terminal fragments both possessing or lacking CMD are able to conduct Orai1 Ca^{2+} currents, though fragments including CMD yield pronounced smaller currents. Fragments without CMD induced the maximal CRAC currents. Thus, CMD plays an inhibitory role and provides a negative feedback mechanism regulating Ca^{2+} entry [41]. Other domains within the C-terminus are the serine/proline-rich domain (aa 600-629) and the lysine-rich domain (also referred to as polybasic tail, aa 671-685). Though these domains are not required for the activation of Orai1 [42], they may assist in targeting and migration of STIM1 [43]. Particularly, the lysine-rich domain may help in localization of STIM1 oligomers into ER-PM junctions [44] by interactions with acidic phospholipids in the PM [45].

Although the major role of STIM proteins is to function as ER Ca^{2+} sensors, recent investigations revealed that they might even act as sensors of other stress conditions (such as oxidative stress, temperature variation and hypoxia) and mediate appropriate physiological responses in cells [46].

1.2.4. Orai1

Orai1 is the pore-forming subunit of CRAC channels [47]. It was the first identified member of the Orai protein family [48]. In addition to Orai1, mammalian cells express two other closely related homologues, Orai2 and Orai3 [49]. All three isoforms are able to generate SOCE when co-expressed with STIM1 in HEK 293 cells. However, Orai1 has the predominant role in mediating SOCE and efficiencies decrease in the order Orai1 > Orai2 > Orai3 [50, 51].

Human Orai1 is a 301 amino acid PM protein composed of four transmembrane (TM1-4) domains, cytosolic N and C termini, one intracellular loop connecting TM2 with TM3 and two extracellular loops *(Figure 3)*. The cytosolic N- and C-termini of Orai1 are essential for the functional activation of Orai1. The C-terminus of Orai1 (aa 265-301) includes a putative coiled-coil domain structure and plays a critical role in coupling to STIM1. Deletion of the Orai1 C-terminus eliminates the interaction of Orai1 with STIM1 and leads to a complete loss of Ca^{2+} entry [37]. The N-terminus (aa 1-90) of Orai1 is involved predominantly in the gating process [37]. In addition to its role in the gating process, the N-terminus provides a second binding site for STIM1. Though, the interaction is weaker than with the Orai1 C-terminus, the Orai1 N-terminus contributes to the overall stability of STIM1-Orai1 binding and effective and stable binding of CAD of STIM1 involves both surfaces [52]. The binding site is referred to as an extended transmembrane Orai1 N-terminal (ETON, aa 73-90) region and almost the whole ETON is required for STIM1-dependent Orai1 activation [53]. Proline/arginine-rich regions within the N-terminus are reported to contribute to the reactivation process of gating [54].

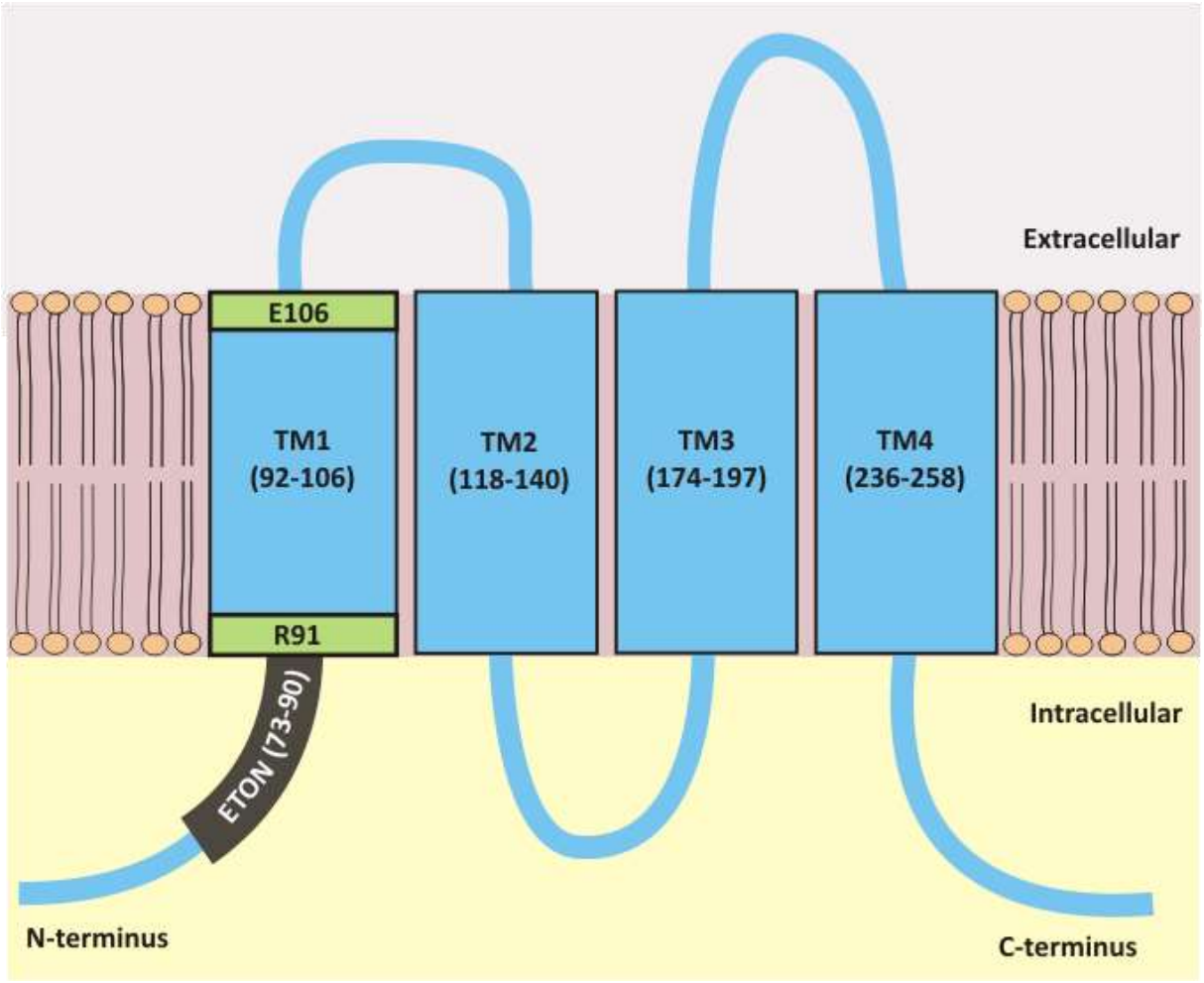

Figure 3: Schematic representation of Orai1 domain architecture. Orai1 includes C- and N-termini, transmembrane domains (TM1-4), an intracellular loop, extracellular loops, an extended transmembrane Orai1 N-terminal (ETON) region and some residues important for the Orai1 function.

The selectivity of the CRAC channel strongly depends on a glutamate residue located at position 106 (E106) in the TM1 domain of Orai1. E106 functions as a selectivity filter for Ca^{2+} ions. Substitution of glutamate to aspartate (E106D) significantly alters the ion permeability through the CRAC channel and the channel does not discriminate effectively between Ca^{2+} and monovalent cations [47].

Arginine at the site 91 (R91) is another important residue of Orai1. R91 forms a narrow part of the pore at the cytosolic side of the TM1 domain and has been proposed to be a part of the gate at the inner mouth of the CRAC channel [55]. Replacement of arginine by tryptophan (R91W) causes a hereditary severe combined immunodeficiency (SCID) [48]. R91W

mutation in the CRAC channel results in a complete loss of Ca^{2+} entry. Furthermore, a substitution of R91 by other highly hydrophobic amino acids (such as leucine, valine or phenylalanine) makes the channel non-functional as well [56].

The most recent crystallographic study of Orai from *Drosophila melanogaster* sharing 73% sequence identity with human Orai1 within its transmembrane region, reported a hexameric assembly of the Orai subunits forming the CRAC channel. The ion pore is located in the centre of the channel and is surrounded by TM domains in three concentric rings. TM1 forms the inner ring lining the ion pore, TM2 and TM3 domains constitute the middle ring and TM4 domain forms the outer ring [17] *(Figure 4).*

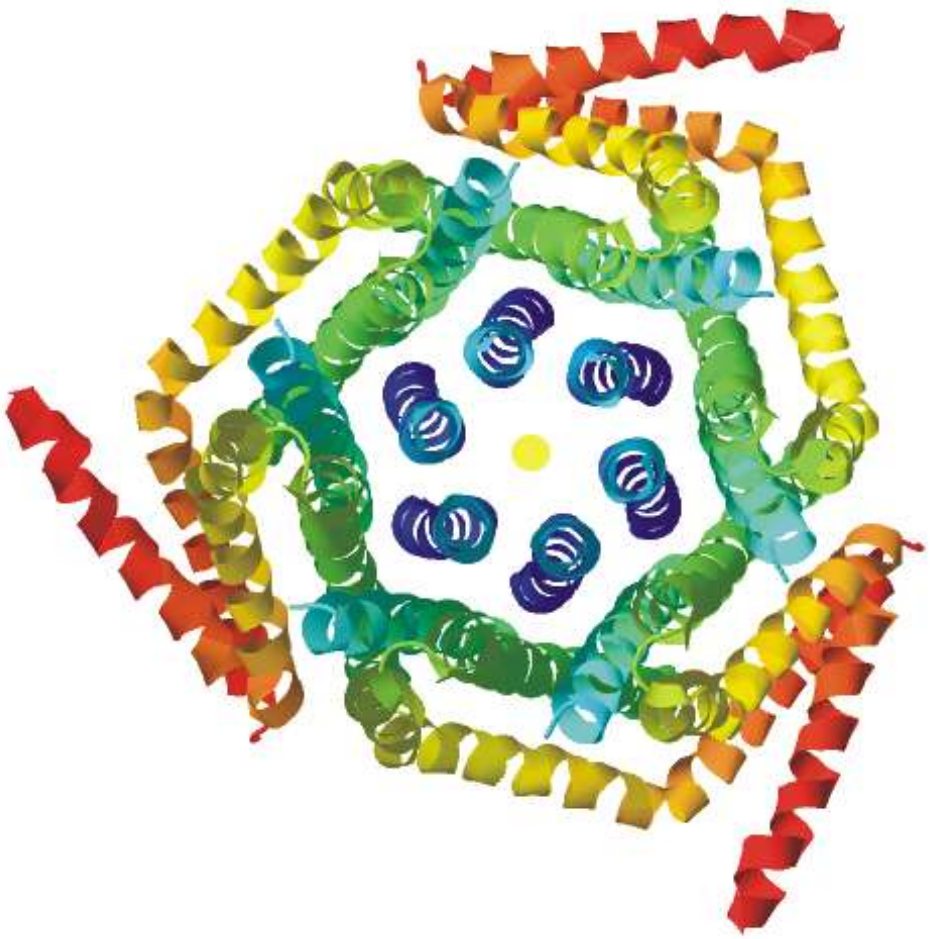

Figure 4: Cross-sectional view from the extracellular side on a hexameric assembly of Drosophila Orai subunits with a Ca^{2+} ion (yellow) depicted in its centre. Structural data are taken from the Protein Data Bank, accession code: 4HKR.

1.3. Fast calcium-dependent inactivation (FCDI)

Contrary to activation, much less is known about the mechanism of fast inactivation of CRAC channels. This process occurs in a Ca^{2+}-dependent manner [57] and is referred to as FCDI. The CRAC channel activity is individually regulated by local binding of cytosolic Ca^{2+} to the mouth of the channel [58]. FCDI is a complex process possibly influenced by the stoichiometry of binding between STIM1 and Orai1, STIM1 and Orai1 cytosolic domains and the selectivity centre within the Orai1 pore [18]. A proposed model for the underlying mechanism suggests a cooperative interplay of STIM1 and all cytosolic domains of Orai1, i.e. N- and C-termini and an intracellular loop [54]. A study using both Ca^{2+}-incompetent calmodulin (CaM) mutants and CaM inhibitor peptides revealed CaM to be involved in FCDI [59].

1.4. Calmodulin (CaM)

CaM is an intracellular Ca^{2+}-binding protein expressed in all eukaryotic cells. More than 0.1% of the total protein present in cells is CaM (10^{-6} - 10^{-5} M) and even higher levels are expressed in rapidly growing cells, especially those undergoing cell division and differentiation [60]. CaM binds to and regulates a high number of diverse target proteins involved in a variety of different biological processes. Many of those proteins are incapable of binding Ca^{2+}. Therefore, they require CaM to sense changes of $[Ca^{2+}]_i$ and mediate the initial Ca^{2+} signal resulting in altering their activity. CaM can function as an activator or inhibitor. CaM is a relatively small protein consisting of 148 amino acids. CaM contains four EF-hands each of which can bind one Ca^{2+} ion. The N-terminal lobe is formed by the first EF-hand pair and the C-terminal lobe by the second pair *(Figure 5)*. Each EF-hand motif has about 30 residues in length. Thus, only a short part of the protein sequence including a linker located in the central region connecting both lobes does not participate in Ca^{2+} binding. Furthermore, Ca^{2+}-binding affinities of the lobes are not identical. The C-terminal lobe Ca^{2+}-binding affinity is much higher. Ca^{2+} binding to EF hands leads to a conformational change forming hydrophobic cavities. The conformational change enables Ca^{2+}-CaM to recognize the specific CaM-binding domain of a target protein and bind to it via hydrophobic interactions between surface cavities of CaM and anchor residues of the CaM-binding peptide [61].

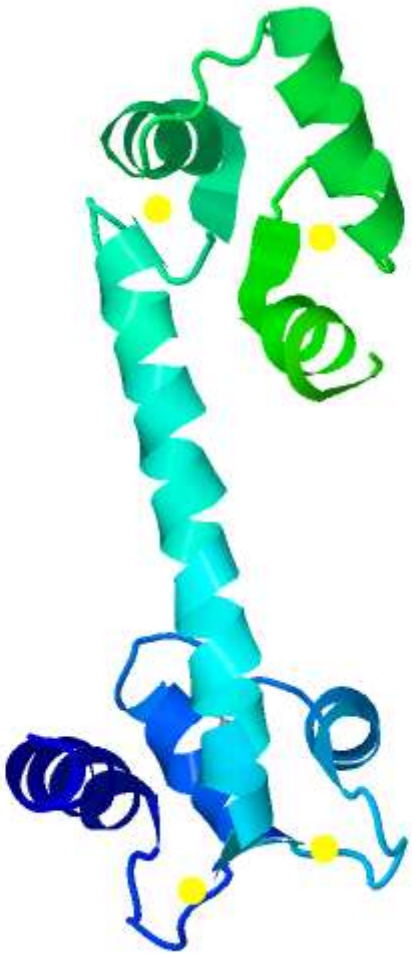

Figure 5: Crystal structure of Ca^{2+}-CaM. Structural data are taken from the Protein Data Bank, accession code: 1CLL.

It was shown that CaM binds in a Ca^{2+}-dependent manner to a membrane-proximal CaM binding domain of Orai1 (CBD_{Orai1}, aa 68-91) localized within the Orai1 N-terminal domain. Ca^{2+}-CaM binding to this domain is required for I_{CRAC} inactivation. Mutations within this region disrupting this binding block the inactivation of I_{CRAC} [62]. A crystal structure of CBD_{Orai1} in complex with Ca^{2+}-CaM revealed an interaction only via the C-lobe of CaM. However, experimental studies in solution showed C- and N-lobes of CaM both binding to CBD_{Orai1}, CaM N-lobe having a four times weaker affinity than CaM C-lobe. The interaction is performed through a tryptophan at the position 79 (W79) of CBD_{Orai1} and the binding mode of CaM and CBD_{Orai1} was suggested to be at a 1:2 ratio. The binding is proposed in a sequential manner. At first the C-lobe of CaM interacts with CBD_{Orai1} and then the next step is the N-lobe interaction with another CBD_{Orai1} [63].

This evidence displayed that the C-terminal region of STIM1, intracellular loop of Orai1 and CaM bound to the N-terminus of Orai1 all contribute to the process of FCDI of CRAC channels [41, 62, 64]. However, the mechanism underlying the interactions among them is not yet fully understood.

The aim of this work is to study the role of CaM in the formation, maintenance and/or dissociation of the STIM1/Orai1 complex and its contribution to SOCE using different molecular biology, electrophysiology, confocal microscopy and biochemistry approaches.

2. MATERIALS AND METHODS

2.1. Preparation and transfection of plasmid DNA

2.1.1. Construction of plasmids

Table 1 shows constructs cloned into vectors with a corresponding antibiotic resistance. All C-terminal fragments of STIM1 were cloned either into pEYFP-C1 expression vectors for EYFP-labelling or pECFP-C1 (both Clontech) for ECFP-labelling. Calmodulin was cloned into pEYFP-C2 and pECFP-C1 (both Clontech) vectors and pcDNA 3.1/V5-His TOPO® (Invitrogen) vectors were used for untagged constructs.

Table 1: Plasmid construction

Construct	Vector	Resistance
STIM1	pcDNA 3.1/V5-His TOPO® (Invitrogen)	ampicillin
Orai1		
STIM1	pECFP-C1 (Clontech)	kanamycin
STIM1 CT		
CaM		
STIM1	pEYFP-C1 (Clontech)	kanamycin
STIM1 CT		
STIM1 233-474		
STIM1 233-343		
STIM1 344-399		
STIM1 400-474		
STIM1 400-474 L416S L423S		
STIM1 400-485		
CaM	pEYFP-C2 (Clontech)	kanamycin

2.1.2. Bacterial transformation

A 150 µL aliquot of *E. coli XL1-Blue competent cells* (Stratagene) stored at -80 °C was thawed on ice (10 min). 1 µL of desired DNA was added and the mixture was kept on ice (30 min). Transformation was induced by a heat shock method (2 min, 42 °C). 150 µL of tempered LB medium (42 °C) (Fischer BioReagents) were added and the suspension was kept under constant shaking in an incubator (1 h, 37 °C). The LB-bacterial mixture was plated according to its bacterial resistance on an agar Petri dish with the antibiotics kanamycin or ampicillin (50 µg/mL) (Sigma-Aldrich) and incubated overnight (37 °C). A single colony was selected and used to inoculate 100 mL of LB medium containing 50 µg/mL kanamycin or ampicillin (Sigma-Aldrich). The inoculated medium was cultured under constant agitation in a water bath incubator (10-15 hours, 37 °C).

2.1.3. Isolation of plasmid DNA

Plasmid DNA was isolated and purified by PureYield™ Plasmid Midiprep System (Promega) and its concentration was measured by NanoDrop™ 2000 Spectrophotometer (Peqlab). This plasmid DNA was further used for cell transfection.

2.1.4. Cell transfection

HEK (human embryonic kidney) 293 is a permanently adenovirus type 5 transformed human cell line [65] and was used as an expression system to obtain the proteins of interest. HEK293 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM High Glucose with Sodium Pyruvate, with L-Glutamine, PAA) supplemented with 10% fetal bovine serum (Gibco) and 1% Penicillin/Streptomycin (PAA). The transient transfection was performed using a lipid transfection reagent (TransFectin™ Lipid Reagent, Bio-Rad) and plasmid DNA of various STIM1 constructs and/or Orai1 and/or calmodulin, STIM1 and Orai1. After the incubation with the transfection reagent (20 min), the mixture was added dropwise to the HEK293 cells. MOCK cells (untreated with plasmid DNA) were used as a negative control. The cells were grown in a humidity controlled incubator (5% CO_2, 37 °C).

2.2. Protein analysis

2.2.1. Co-immunoprecipitation (Co-IP)

20-24 hours after transfection cells were harvested and culture medium was discarded. The reagents used for Co-IP are listed in *Table 2*. The adherent cells were scratched off the Petri dish using a plastic cell scraper and Ca^{2+}-free extracellular medium (pH 7.4) and gently centrifuged (1 min, 4,600 RPM). The remaining DMEM-based culture medium was removed by washing the cells twice in Ca^{2+}-free extracellular medium (pH 7.4). The suspension was divided into two fresh Eppendorf tubes, one was used as a control and the second one for further stimulation, and the cells remained resting (10 min). 100 µM EDTA, a calcium chelating agent, was added to the control samples to quench all the traces of Ca^{2+} in the extracellular space. The control samples were lysed in ice-cold lysis buffer (pH 8.0) supplemented with 20 µL/mL protease inhibitor (PI) which prevents proteolytic cleavage of proteins. Contrary to control samples, 2 mM Ca^{2+} was provided to the treated cells to allow the activation of cytosolic CaM. Afterwards, the cells were stimulated with 10 µM ionomycin (3 min) (Sigma-Aldrich), which is a calcium ionophore used to deplete intracellular calcium stores [66]. The stimulation was stopped by addition of ice-cold lysis buffer (pH 8.0) supplemented with 20 µL/mL PI and additional 2 mM Ca^{2+}. The lysed cell suspensions were subjected to sonication (5 s, 40% amplitude) using Sonopuls GM70 (Bandelin) sonifier and kept on ice (1 h). Then the suspension was centrifuged (10 min, 14,000 RPM) to remove membranes and other organelles debris. 1 µg/mL Co-IP antibody was coupled to 25 µL of agarose beads with covalently-linked Protein A (Protein A Agarose, Millipore) and the supernatant containing proteins was transferred to the antibody-coupled beads. The mixture was incubated overnight on a rotating platform (4 °C).

2.2.2. SDS-PAGE and Western Blot

Table 3 represents all reagents used for SDS-PAGE and blotting. The lysate-bead mixture was centrifuged (1 min, 10,000 RPM) and washed twice in ice-cold PBS (pH 7.2). After these washing steps, the antibody-coupled beads with the target protein and all proteins bound to it were isolated and other proteins from the cells were discarded. The antibody-protein complex was eluted from the beads by heating the samples (10 min, 75 °C) in 18 µL of

Laemmli's buffer (pH 7.4). The eluted mixture was separated by 10% SDS-PAGE (150 V). After separation the proteins bands were transferred (100 V, 90 min) from the gel onto a blotting membrane (Immun-Blot® PVDF Membrane for Protein Blotting, pore size: 0.2 µm, Bio-Rad) to be readily accessible to specific antibodies.

Table 2: Reagents used for Co-IP

Ca^{2+}-free extracellular medium (pH 7.4)	145 mM NaCl 10 mM glucose 10 mM HEPES 5 mM KCl 1 mM $MgCl_2$
Ionomycin	Ionomycin from *Streptomyces conglobatus* (Sigma-Aldrich), stock solution prepared in DMSO
Lysis buffer (pH 8.0)	100 mM NaCl 20 mM Tris 2 mM EDTA 10% glycerol 0.5% Nonidet P40 (Roche)
Protease inhibitor (PI)	cOmplete EDTA-free Protease Inhibitor Cocktail Tablets (Roche), 1 tablet dissolved in 1 mL H_2O
Co-IP antibody	Anti-Calmodulin Antibody (Millipore)
	Anti-GFP (Roche)

Table 3: Reagents used for SDS-PAGE and blotting

PBS (pH 7.2)		137 mM NaCl 2.7 mM KCl 11.3 mM Na_2HPO_4 1.1 mM NaH_2PO_4 1.5 mM KH_2PO_4
Laemmli's buffer (pH 7.4)		70 mM Tris 10% glycerol 0.002% bromophenol blue 2% SDS 0.5% β-mercaptoethanol (Sigma-Aldrich)
Polyacrylamide gel	Resolving gel (10%)	1.5 M Tris (pH 8.8) 33% Acrylamide/bis-acrylamide 30% solution (Sigma-Aldrich) 10% SDS 2.2 mM ammonium persulfate 0.05% TEMED (Sigma-Aldrich)
	Stacking gel (4%)	0.5 M Tris (pH 6.8) 13% Acrylamide/bis-acrylamide 30% solution (Sigma-Aldrich) 10% SDS 4.4 mM ammonium persulfate 0.1% TEMED (Sigma-Aldrich)
Marker		Precision Plus Protein™ All Blue Standards (Bio-Rad)
Electrophoretic buffer		25 mM Tris 205 mM glycine 0.1% SDS
Blotting buffer (pH 8.3)		31 mM Tris 142 mM glycine 20% isopropanol

2.2.3. Immunodetection

The immunodetecting steps following the blotting were carried out under constant agitation. All reagents used in the immunodetection are listed in *Table 4*. The membrane was placed into blocking buffer (1 h) to prevent non-specific binding and then incubated with the primary antibody (50 min). Unbound antibody was removed by washing with PBST (6 x 5 min). Incubation with the secondary antibody conjugated with horse radish peroxidase (HRP) (40 min) and washing with PBST (6 x 5 min) were performed. ECL (enhanced chemiluminescence) reagents (2 min) were used to evoke the chemiluminescence reaction. The protein complexes were visualized in a dark chamber. A highly sensitive chemiluminesce film (Amersham Hyperfilm™ ECL, GE Healthcare) was placed over the membrane and closed into a cassette (Amersham Hypercassette Autoradiography Cassette, GE Healthcare). Afterwards the film was put into a bath with developer solution and fixed in a fixative solution bath.

2.2.4. Data analysis

Western blot images were processed and analysed using *ImageJ* program (National Institutes of Health). An area of each band was obtained from an intensity histogram. The relative density of each protein band on a blot was calculated using control samples as a reference.

Table 4: Reagents used for immunodetection

Blocking buffer	5% solution of non-fat dry milk in PBST
Primary antibody (dilution 1:1,000 in blocking buffer)	Anti-GFP, mouse $IgG_1\kappa$ monoclonal antibody (clones 7.1 and 13.1.) (Roche)
	Anti-Orail1 antibody produced in rabbit (Sigma-Aldrich)
PBST	PBS (pH 7.2) + 0.1% Tween® 20 (Sigma-Aldrich)
Secondary antibody (dilution 1:10,000 in PBST)	Mouse IgG, horse radish peroxidase linked whole antibody (from sheep) (GE Healthcare)
	Monoclonal Anti-Rabbit IgG (γ-chain specific)-Peroxidase Clone RG-96 (Sigma-Aldrich)
ECL reagents	Clarity™ Western ECL substrate (peroxide solution) Clarity™ Western ECL substrate (luminol/enhancer solution) (both Bio-Rad)
Developer solution	ILFORD MULTIGRADE (Harman), 1:9 dilution
Fixative solution	ILFORD RAPID FIXER (Harman), 1:4 dilution

2.2.5. Biotinylation

All reagents used in the biotinylation method are shown in *Tables 2-5.* Transfected cells followed the same harvesting and washing steps as described above. After washing the cells were gently resuspended in biotinylation reagent and incubated at room temperature on a rotating platform (30 min). Biotinylation reagent was used for specific cell surface protein labelling. It is negatively charged reagent which is unable of cell membrane permeation. Therefore, only surface membrane proteins of whole cells can be biotinylated. It reacts with primary amines in proteins, such as lysine side chains and N-termini of polypeptides and forms stable amide bonds [67, 68]. After centrifugation (1 min, 4,600 RPM) cells were washed twice in PBS (pH 8.0) with 100 µM Tris to remove excess non-reacted biotinylation reagent. Lysis in ice-cold lysis buffer (pH 8.0) supplemented with 20 µL/mL PI and sonication (5 s, 40% amplitude) by Sonopuls GM70 (Bandelin) were performed and the suspension was kept on ice (1 h). The lysed soluble fraction was centrifuged (10 min, 14,000 RPM) and incubated overnight with 25 µL of agarose beads with covalently immobilized streptavidin (High Capacity Streptavidin Agarose Resin, Pierce) on a rotating platform (4 °C). Afterwards the lysate-bead mixture was centrifuged (1 min, 10,000 RPM) and washed twice with ice-cold PBS (pH 7.2). 18 µL of Laemmli's buffer (pH 7.4) were added. After heating (10 min, 75 °C) biotinylated cell surface membrane proteins were resolved by 10 % SDS-PAGE (150 V) and blotted (100 V, 90 min) on a PVDF membrane (Immun-Blot® PVDF Membrane for Protein Blotting, pore size: 0.2 µm, Bio-Rad). The proteins were detected as described above using anti-GFP (Roche) as a primary antibody and HRP-linked mouse IgG (GE Healthcare) as a secondary antibody and analysed.

Table 5: Reagents used in cell surface biotinylation

PBS (pH 8.0)	137 mM NaCl 2.7 mM KCl 11.3 mM Na_2HPO_4 1.1 mM NaH_2PO_4 1.5 mM KH_2PO_4
Biotinylation reagent	EZ-Link® Sulfo-NHS-LC-Biotin (Pierce), 0.5 mg/1 mL PBS (pH 8.0)

2.3. Confocal Förster resonance energy transfer (FRET) microscopy

FRET experiments were performed at room temperature according to [40]. The reagents are listed in *Table 6*. CFP (donor) and YFP (acceptor) were used as a FRET pair. The fluorescence images were recorded by QLC100 Real-Time Confocal System (VisiTech International) with two Photometrics CoolSNAPHQ monochrome cameras (Roper Scientific), one for CFP- and another for YFP-imaging. The system was coupled to an Axiovert 200M microscope (Zeiss). Two diode lasers (445 and 515 nm) (Visitron Systems) were used as a light source for the excitation of fluorophores. The appropriate excitation wavelengths were selected by Acousto Optical Tuneable Filter (VisiTech International). The dual port adapter (Chroma Technology) contained 505lp (long pass) dichroic filter, 485/30 cyan emission filter and 535/50 yellow emission filter. 24-48 hours after the transfection, HEK293 cells grown on a 12 mm glass coverslip were washed twice with Ca^{2+}-free extracellular medium (pH 7.4) and transferred into a measurement chamber. FRET of transfected HEK293 cells co-expressing both CFP- and YFP-labelled proteins was investigated. 2 mM Ca^{2+}-containing extracellular solution (pH 7.4) was added through a perfusion system. The FRET experiments were carried out in the absence and after the addition of 10 µM ionomycin (Sigma-Aldrich). The sample was illuminated with 445 nm and fluorescence of both CFP and YFP was simultaneously recorded using two camera systems. The two recorded images taken at one time point were merged and the same procedure was performed at the illumination with 515 nm. Therefore, one image illustrating the fluorescence under CFP excitation and one image depicting the fluorescence under YFP excitation were obtained. The images were recorded consecutively with a minimum delay and the illumination times for CFP and YFP were 900 ms per image. Prior to the calculation the images had to be corrected due to cross-talk and cross-excitation. This was done by the determination of appropriate calibration factors for each construct on the day of the FRET experiments. In order to perform the calibration, HEK293 cells expressing only CFP-labelled proteins and only YFP- labelled proteins were used. The calibration factors were then subtracted from FRET signal detected during the experiments.

Table 6: Reagents used in confocal FRET microscopy

Ca^{2+}-free extracellular medium (pH 7.4)	145 mM NaCl 10 mM glucose 10 mM HEPES 5 mM KCl 1 mM $MgCl_2$
2 mM Ca^{2+}-containing extracellular solution (pH 7.4)	145 mM NaCl 10 mM glucose 10 mM HEPES 2 mM $CaCl_2$ 5 mM CsCl 1 mM $MgCl_2$
Ionomycin	Ionomycin from *Streptomyces conglobatus* (Sigma-Aldrich), stock solution prepared in DMSO

2.3.1. Image acquisition and data analysis

Visiview 2.1.1 software (Visitron Systems) was used for image acquisition and to control the setup during measurement. FRET images were analysed with a custom-made program [69] integrated in *MatLab 7.0.4* (MathWorks) using a method proposed by [70].

2.4. Electrophysiology

Electrophysiological experiments were carried out 12-48 h after transfection, using the patch clamp technique in whole-cell recording configuration at room temperature *(Figure 6)*. The current measurements were performed according to [71]. Reagents used in patch clamp experiments are listed in *Table 7*. Transfected HEK293 cells grown on a 12 mm glass coverslip were washed twice with Ca^{2+}-free extracellular medium (pH 7.4). The coverslip and bath electrode were placed into a recording chamber and the chamber was filled with 10 mM Ca^{2+}-containing extracellular solution (pH 7.4). A glass pipette with a tip diameter of 1 µm was filled with internal pipette solution (pH 7.2) containing 20 mM EGTA chelator to buffer cytosolic Ca^{2+} and induce passive store depletion and thus I_{CRAC} activation when in contact with cell

interior [57]. An Ag/AgCl electrode used for voltage recording and current transduction was placed inside the glass pipette. A small pressure was applied to the pipette to keep the tip free of contamination. The tip was pressed against the plasma membrane and a GΩ seal (gigaseal) between the tip and the membrane patch was formed by a gentle suction. Whole-cell recording was achieved by applying more suction, which raptured the membrane patch and thus allowed contact of the cell interior with the internal pipette solution (pH 7.2). Voltage ramps were applied in 5 s intervals from a holding potential of 0 mV, covering a range of -90 to 90 mV over 1 s. Liquid junction potential arising from the differences in concentration and mobility of ions between the extracellular solution (pH 7.4) and internal pipette solution (pH 7.2) prior the seal formation was corrected by applying +12 mV. At the end of the experiment the extracellular solution (pH 7.4) was exchanged through a perfusion system by 10 µM La^{3+} to perform leak correction by subtracting the remaining currents.

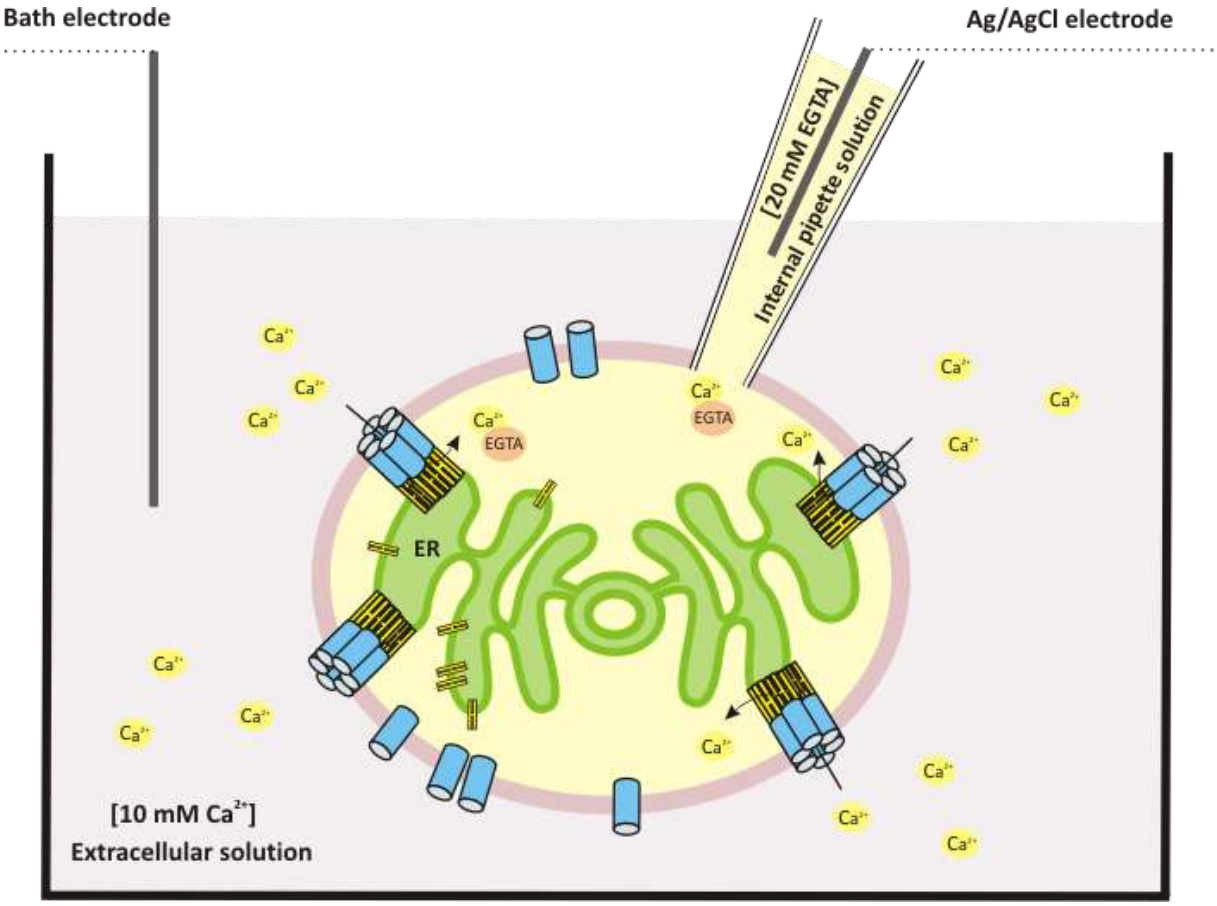

Figure 6: Whole-cell patch clamp configuration

Table 7: Reagents used in patch clamp experiments

Ca^{2+}-free extracellular medium (pH 7.4)	145 mM NaCl 10 mM glucose 10 mM HEPES 5 mM KCl 1 mM $MgCl_2$
10 mM Ca^{2+}-containing extracellular solution (pH 7.4) (bath solution)	145 mM NaCl 10 mM glucose 10 mM HEPES 10 mM $CaCl_2$ 5 mM CsCl 1 mM $MgCl_2$
Internal pipette solution (pH 7.2)	145 mM cesium methanesulfonate 20 mM EGTA 10 mM HEPES 8 mM NaCl 5 mM $MgCl_2$

2.4.1. Image acquisition and data analysis

After amplification of the currents by amplifier, *Clampex 8.2* (Molecular Devices) was used to acquire and process the electrophysiological data. Data were further analysed by *Clampfit 8.2* (Molecular Devices) and *OriginPro 8.1* (OriginLab).

3. RESULTS AND DISCUSSION

3.1. Verification of expression of various vectors

In order to obtain optimal results in our experiments, the prepared plasmid DNA of various constructs, transfection efficiency and rate of protein expression were verified. HEK293 cells, used as an expression system, were transiently transfected with a plasmid DNA of interest using a lipid-based transfection reagent. Those cells which were not treated with the plasmid DNA (Mock) were used as a negative control. After incubation in a humidity controlled incubator and removal of traces of the culture medium, cells were lysed in a lysis buffer and subjected to sonication. The proteins were resolved using SDS-PAGE and blotted onto a membrane. Since STIM1 and all the protein fragments were YFP-labelled, anti-GFP antibody and HRP-conjugated mouse IgG antibody were used for the detection.

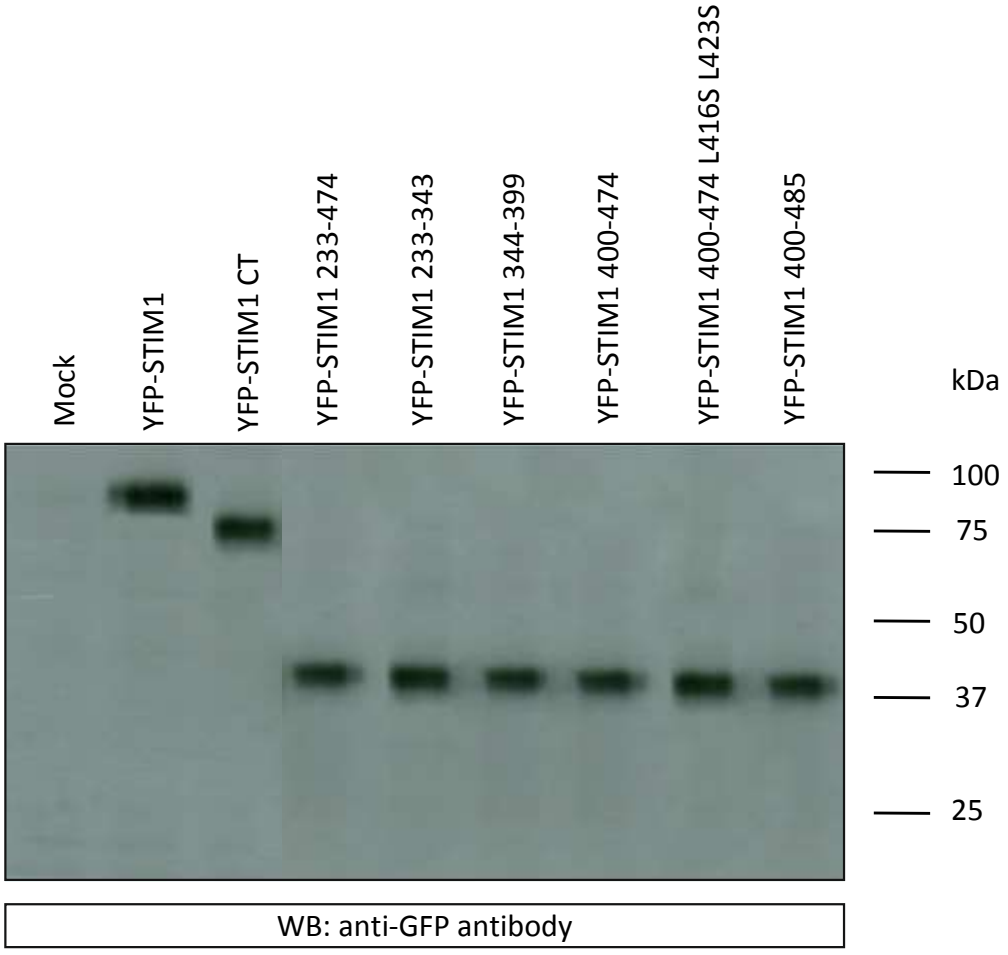

Figure 7: Expression of various vectors in HEK293 cell line.

As expected, no YFP-labelled proteins were expressed in Mock *(Figure 7)*. The immunoblot further represents from left to right STIM1 wild type, STIM1 C-terminus (CT), STIM1 233-474 fragment which was described by our group as the fragment able to fully activate Orai1 [40], STIM1 233-343 containing STIM1 CC1, STIM1 344-399, STIM1 400-474 including STIM1 CC3 and STIM1 homomerization domain (SHD), STIM1 400-474 L416S L423S comprising mutations in SHD that enhance STIM1 activation of Orai1 [71] and STIM1 400-485 including CC3, SHD and the CRAC modulatory domain (CMD). All the fragments showed the expected molecular weight increased by approximately 27 kDa due to the YFP-label and were expressed at similar levels in our cell type. These constructs were further used to elucidate possible region(s) where calmodulin may interact with STIM1.

3.2. Interaction of calmodulin with STIM1

Calmodulin being a small intracellular protein with a molecular weight of 16.7 kDa [72] is regarded as a transducer of Ca^{2+} signals in eukaryotic cells. CaM is activated upon Ca^{2+} binding. Such Ca^{2+}-loaded CaM is able to recognize a large number of diverse proteins and regulate their activities in a calcium dependent manner [61, 73]. Within its vast scope of activity, we attempted to examine whether it interacts also with the Ca^{2+} sensor STIM1, a 77 kDa single spanning ER membrane protein with its N-terminus oriented into ER lumen and C-terminus into cytosol [24]. The methods employed in this section to detect the protein-protein interactions were co-immunoprecipitation and confocal FRET microscopy. In order to carry out the FRET measurements, CFP-labelled proteins were used as donors and YFP-labelled proteins as acceptors.

3.2.1. Interaction of calmodulin with full-length STIM1

At first we investigated, whether there might be an interaction of full-length STIM1 with CaM. For Co-IP experiments, HEK293 cells were transfected with plasmid DNA of YFP-labelled STIM1 wild type (WT) and unlabelled CaM. 100 µM calcium chelating agent EDTA, was added to the control samples to establish a Ca^{2+}-free environment surrounding the cells. 2 mM Ca^{2+} was provided to those cells which were further stimulated with 10 µM ionomycin (3 min). Due to its lipophilic nature, ionomycin does not translocate Ca^{2+} only across the plasma membrane but it also reaches calcium stores where it causes a release of Ca^{2+} and thus an

increase of intracellular Ca^{2+} levels [57, 74]. The cells were lysed by sonication in lysis buffer. Free CaM and CaM in a potential complex with STIM1 WT were isolated using anti-CaM antibody and protein A Agarose beads. After separation by SDS-PAGE and blotting, the co-immunoprecipitated protein complexes were detected using anti-GFP antibody and HRP-linked mouse IgG secondary antibody.

Despite the bands corresponding to the denatured heavy chain and light chain of the antibody used for Co-IP *(Figure 8A)*, no clear band of the STIM1 size is visible in the control. These bands arose due to the fact that the antibodies used for Co-IP and Western blot (WB) detection were from the same species (mouse). Anti-GFP antibody employed in Co-IP formed an immune complex with CaM and the complex was captured on agarose beads carrying immobilized Protein A. After elution, all the components of the immune complex were denatured and analysed by SDS-PAGE, followed by WB analysis. The heavy chain and the light chain were recognized as well by the HRP-conjugated secondary antibody and depicted on the immunoblot [75]. Contrary to the control, samples stimulated with a calcium ionophore ionomycin revealed an appearance of a strong STIM1 band above 100 kDa (including an YFP-tag of approximately 27 kDa) indicating that STIM1 interacts with CaM. The association upon depletion of intracellular stores greatly increased from 2104 ± 305 to 15976 ± 1000 a.u. (arbitrary unit) representing the mean ± S.E. (standard error) of the number of experiments equal to 5. Based on the results of Co-IP experiments, we can assume that STIM1 interacts with CaM and this interaction is Ca^{2+}-dependent.

HEK293 cells co-expressing both CFP-labelled STIM1 WT and YFP-tagged CaM were used in the confocal FRET microscopy. Control experiments were carried out in the presence of 2 mM Ca^{2+} extracellular solution *(Figure 8B)*. The red and green fluorescent images displayed a homogenous cellular distribution of both proteins. Compared to the resting conditions in the control, addition of 10 µM ionomycin apparently changed the distribution of STIM1 *(Figure 8C)*. The depletion of intracellular Ca^{2+} stores caused an accumulation of STIM1 into clusters (or so called puncta formation) [76].

Although we were able to detect the interaction between full-length STIM1 and CaM using biochemical experiments, the interaction was not observed from the FRET data. One explanation for the absent interaction in the FRET experiment could be the relatively bulky fluorescent labels which might hinder the interacting sites and thus avoid the association of STIM1 with CaM.

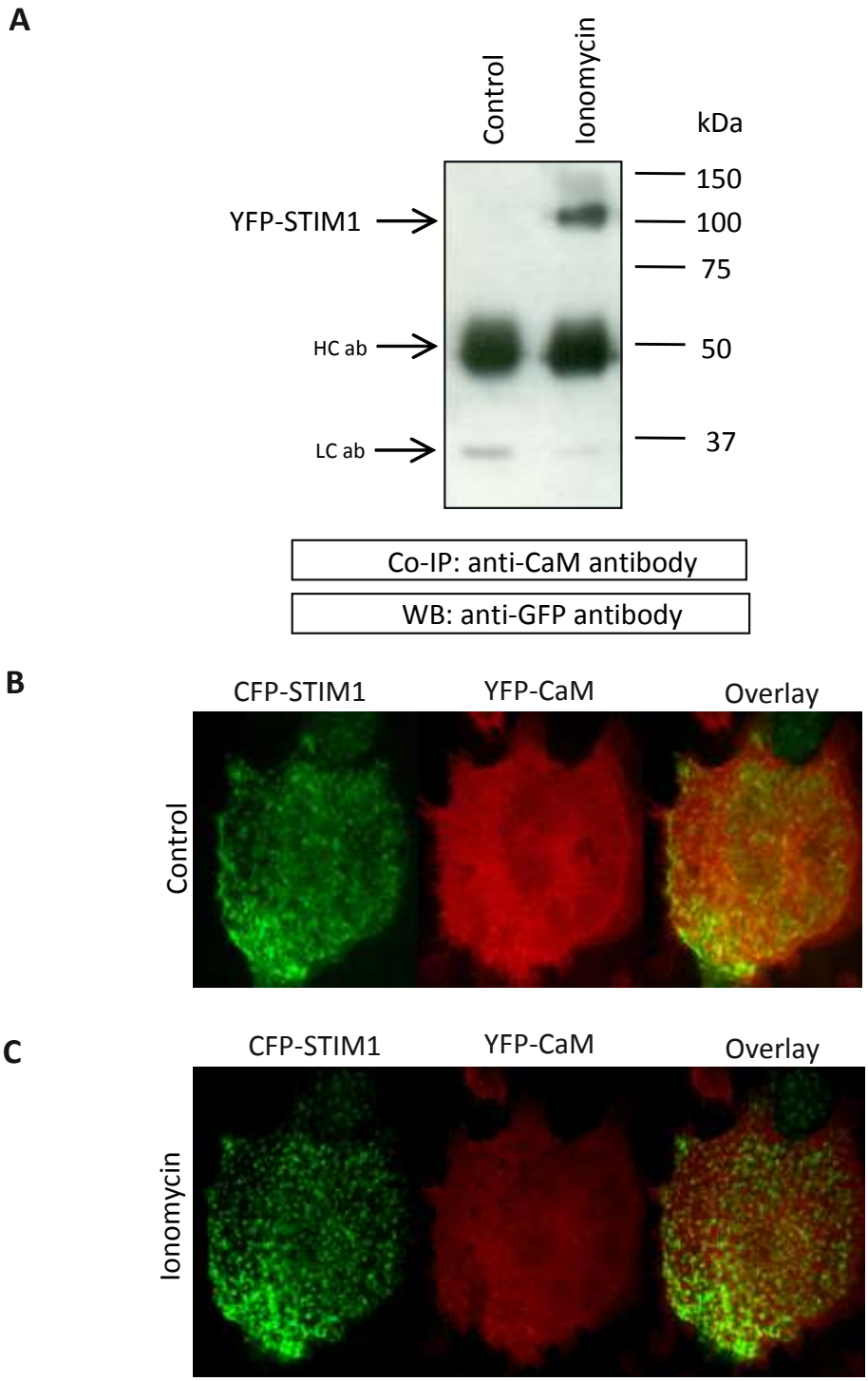

Figure 8: Interaction between CaM and STIM1. (A) Co-IP experiments with Western blot analysis of HEK293 cells expressing unlabelled CaM and YFP-STIM1 treated without (control) or with 2 mM Ca^{2+} and 10 µM ionomycin, HC and LC ab indicate heavy chain and light chain of Co-IP antibody, respectively. (B) and (C) confocal FRET microscopy images of a HEK293 cell expressing CFP-STIM1 and YFP-CaM showing localization and red-green overlay before (B) and after store depletion (C) induced by 10 µM ionomycin.

3.2.2. Interaction of calmodulin with cytosolic C-terminus of STIM1

Due to the fact that CaM is a cytosolic protein, in our next step we focused on an interaction of CaM with the cytosolic part of STIM1, i.e. C-terminal region (aa 233-685). The cytosolic portion of STIM1 lacking the luminal Ca^{2+} sensing function contains domains which are crucial for coupling to Orai1 and CRAC channel activation [37]. The domains within the cytosolic part are namely CC1-CC3 domains, SHD, CMD, Ser/Pro-rich and Lys-rich domains. Those domains encompassing regions important for the CRAC channel activation are Orai1-activating small fragment (OASF), coiled-coil domain containing region b9 (Ccb9), CRAC activation domain (CAD) and STIM-Orai activating region (SOAR) [36].

In order to investigate, whether the cytosolic part of STIM is responsible for the interaction with CaM, plasmid DNA of YFP-labelled STIM1 CT (aa 233-685) and unlabelled CaM were used for the HEK293 cell transfection. The same conditions of Co-IP procedure as stated above were established for the control and 2 mM Ca^{2+} and 10 µM ionomycin were provided to stimulated samples. The antibody used for Co-IP was anti-CaM and the detection was performed using anti-GFP and HRP-conjugated mouse IgG antibodies.

As can be seen from the immunoblot *(Figure 9A)*, there is strong association of CaM with the cytosolic STIM1 CT in the samples treated with ionomycin which raised the cytosolic Ca^{2+} levels. The values of the association under the resting state and upon the store depletion correspond to 3151 ± 394 (n=5) and 14997 ± 1205 (n=5), respectively.

CFP-CaM and YFP-STIM1 CT were co-expressed in HEK293 cells and analysed by confocal FRET microscopy. As expected CaM is uniformly distributed throughout the cytosol and is found also in the nucleus [60] *(Figure 9B and C).* On the other hand, when only the STIM1 CT fragment was used instead of the full-length STIM1, a clear cytosolic distribution was displayed. Furthermore, no clustering is visible. This is due to the fact that it is lacking the luminal N-terminal region which anchors STIM1 within the ER membrane and ER localization is as well a prerequisite for the formation of STIM1 clusters [37].

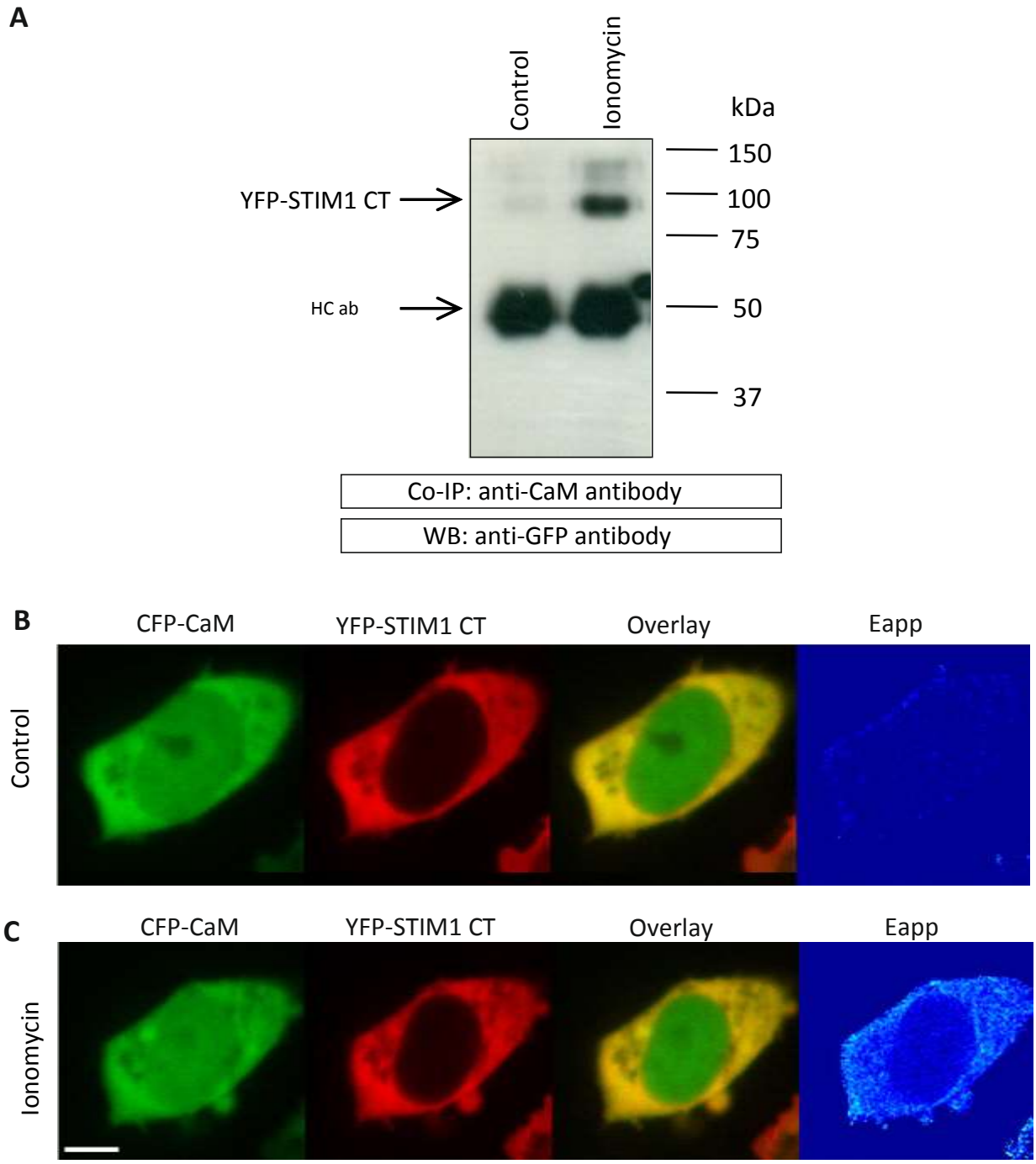

Figure 9: Interaction between CaM and STIM1 C-terminus. (A) Co-IP experiments with Western blot analysis of HEK293 cells expressing unlabelled CaM and YFP-STIM1 CT treated without (control) or with 2 mM Ca^{2+} and 10 µM ionomycin, HC ab indicates a heavy chain of Co-IP antibody. (B) and (C) confocal FRET microscopy images a HEK293 cell expressing CFP-STIM1 CT and YFP-CaM showing localization, red-green overlay and pseudocolor image obtained from calculated apparent FRET efficiency (Eapp) before (B) and after store depletion (C) induced by 10 µM ionomycin.

Pseudo-colour FRET image displayed a very low FRET signal in the control containing 2 mM extracellular Ca^{2+} solution *(Figure 9B)*. Very weak interactions were detected under the resting conditions in the Co-IP experiments as well but only little importance was attached to them due to the fact that they could represent a consequence of improper washing followed by an activation of some cells. Nevertheless, the interactions detected in the FRET experiments under the resting conditions might indicate that CaM weakly associates with STIM1 CT already when the level of cytosolic Ca^{2+} is low. The FRET signal in the cytosolic region strongly increased after the addition of ionomycin *(Figure 9C).* Therefore, the interactions are significantly reinforced when the cytosolic Ca^{2+} concentration is raised by the depletion of the internal Ca^{2+} stores.

The Co-IP and FRET experiments demonstrated that the interaction between CaM and STIM1 takes place within the cytosol and it is Ca^{2+}-dependent. Furthermore we can conclude that the CaM-interacting STIM1 domain has to be present within the cytosolic C-terminal region of STIM1.

3.2.3. Interaction of calmodulin with cytosolic fragments of STIM1

Considering the finding that cytosolic CT of STIM1 associates with CaM, we further aimed at closer characterization of the regions of the cytosolic portion of STIM1 underlying the interaction with CaM. For this purpose, various YFP-labelled fragments of STIM1 were constructed *(Figure 10)* and overexpressed together with CFP/unlabelled CaM in HEK293 cell line. Co-IP experiments were carried out under Ca^{2+}-free conditions as well as under addition of 2 mM Ca^{2+} and stimulation with 10 µM ionomycin. STIM1 fragments in a protein complex with CaM were isolated using anti-CaM antibody and detected by anti-GFP and HRP-linked mouse IgG antibodies. FRET analysis was carried out in 2 mM Ca^{2+} containing extracellular solution and depletion of intracellular stores was induced by addition of 10 µM ionomycin. CFP-CaM and YFP protein were additionally co-expressed as a control to exclude any influence of the label.

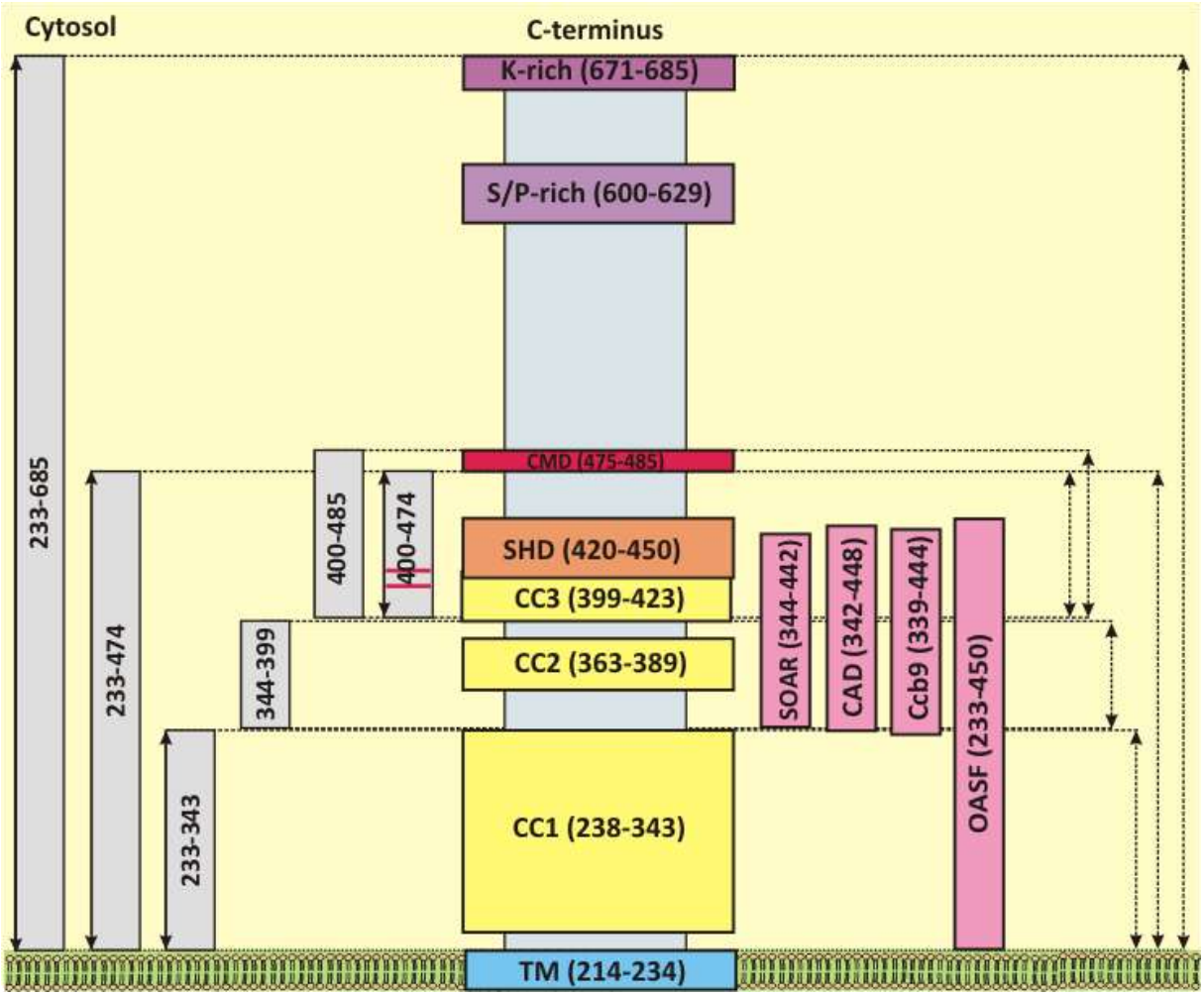

Figure 10: Cytosolic fragments of STIM1. Red bars in the fragment 400-474 indicate mutations L416S and L423S.

The largest STIM1 fragment 233-474 (extended OASF) comprised all three coiled-coil domains essential for interactions with Orai1 and SHD domain involved in coupling and activation of Orai1 as well [40]. Co-IP experiments revealed a clear interaction with CaM when ionomycin was involved *(Figure 11A, Table 8)*. Nevertheless, the interaction was not as strong as it was obtained when the whole C-terminal portion of STIM1 was used. Similar associating activity upon store depletion was observed while using FRET confocal microscopy *(Figure 11C)*.

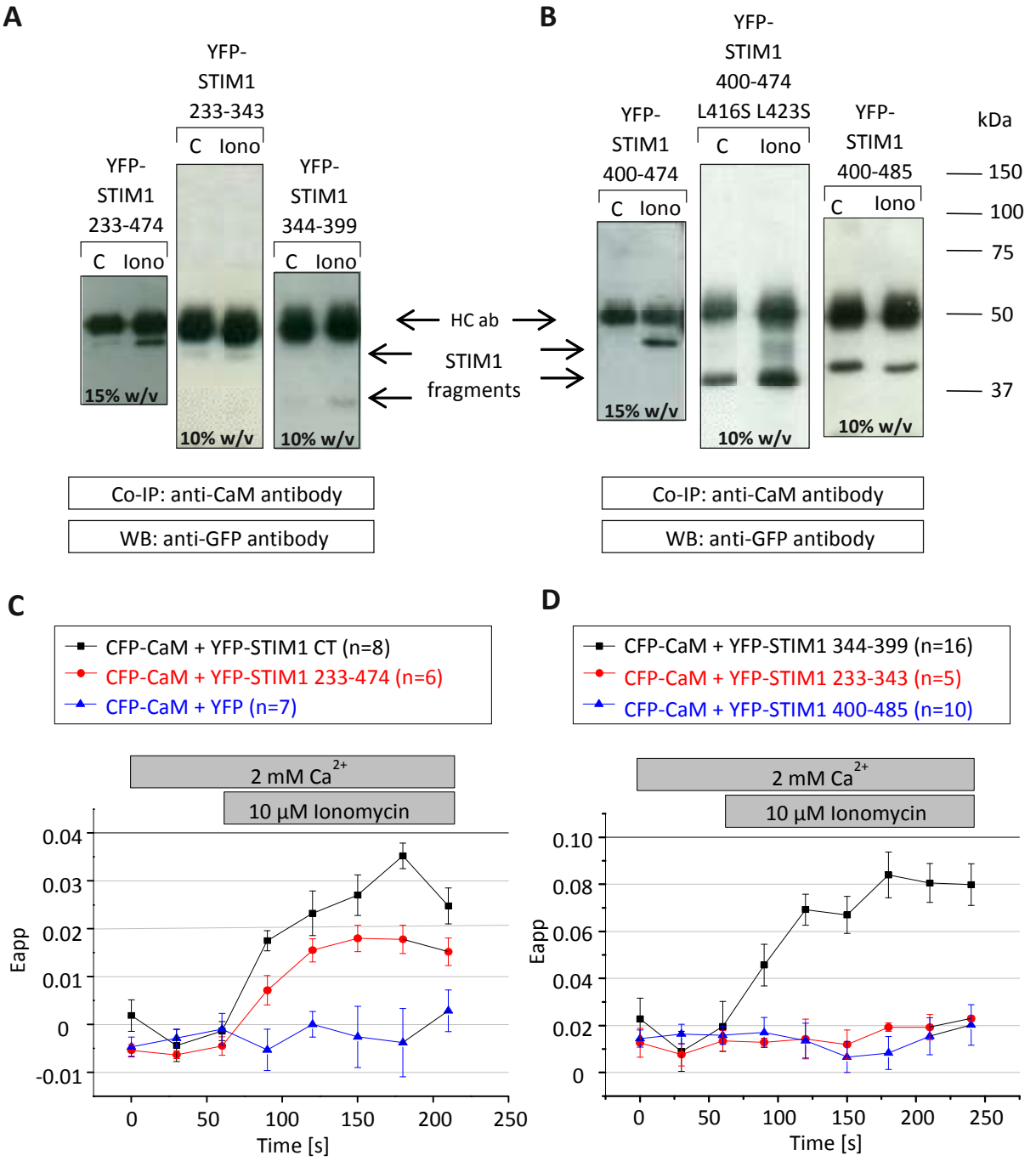

Figure 11: Interaction between CaM and cytosolic STIM1 fragments. (A) and (B) Co-IP experiments and Western blot analysis of HEK293 cells expressing unlabelled CaM and individual YFP-tagged STIM1 fragments treated without (control) or with 2 mM Ca^{2+} and 10 µM ionomycin, w/v represents weight/volume percentage concentration of SDS-PAGE, C means control, Iono stands for ionomycin and HC ab indicates heavy chain of Co-IP antibody. (C) and (D) FRET experiments of HEK293 cells expressing CFP-CaM and individual YFP-STIM1 fragments showing interactions between CFP-CaM and some YFP-TIM1 fragments before and after store depletion induced by 10 µM ionomycin.

Table 8: Association of CaM with cytosolic STIM1 fragments. The values are shown as the mean ± S.E. of the number of experiments n depicted in brackets

Fragment	Control [a.u.]	Ionomycin [a.u.]
STIM1 233-474	1147 ± 1404 (n=6)	12224 ± 2491 (n=6)
STIM1 233-343	1374 ±419 (n=5)	1499 ± 267 (n=5)
STIM1 344-399	907 ± 218 (n=4)	1394 ± 175 (n=4)
STIM1 400-474	954 ± 201 (n=6)	10840 ± 2501 (n=6)
STIM1 400-474 L416S L423S	13140 ± 3708 (n=4)	21623 ± 1277 (n=4)
STIM1 400-485	12564 ± 3086 (n=4)	9937 ± 2426 (n=4)

The next analysed fragment STIM1 233-343 contained only the CC1 domain. This domain, being involved in the formation of resting dimers of STIM1 [31] is situated in close proximity to the TM domain. Although Co-IP results showed slight association of this fragment with CaM upon ionomycin stimulation *(Figure 11A, Table 8)*, FRET analysis could not detect any interaction with CaM *(Figure 11D)*. A reason for that may lie in the size of CaM or the fragment which are both smaller than the size of the attached respective fluorescent tag.

The STIM1 344-399 fragment included only the CC2 domain which plays an important role in STIM1-Orai1 coupling [77]. Both Co-IP and FRET experiments demonstrated presence of the interaction between this fragment and CaM when stimulated with ionomycin *(Figure 11A and D, Table 8)*.

The STIM1 400-474 fragment contained only the CC3 domain and SHD. As can be seen from Co-IP experiments *(Figure 11B, Table 8),* treatment with ionomycin lead to a strong association with CaM upon the depletion of internal Ca^{2+} stores.

Additionally, the STIM1 400-474 fragment encompassing point mutations within the third coiled-coil domain causing a destabilization of the CC3 structure was used. In this fragment, leucines at the positions 416 and 423 were mutated to serines (L416S and L423S). These specific point mutations have been shown to enhance co-clustering with Orai1. Their introduction into the full-length STIM1 locks STIM1 in a constantly active state which results in coupling to Orai1 and activation of CRAC currents without store depletion [71]. Using Co-IP

experiments *(Figure 11B, Table 8)*, the mutated form showed a very strong association with CaM independent of store depletion.

The last examined STIM1 400-485 fragment comprised CC3, SHD and CMD domains. Although FRET analysis detected no signal *(Figure 11D)* which could be caused again due to the small size of CaM or the fragment with regard to the anchored fluorescent tags, Co-IP method demonstrated a clear interaction with CaM *(Figure 11B, Table 8)*. Remarkably, this was the only non-mutated fragment showing a strong interaction with CaM in a Ca^{2+}-free environment. The association with CaM was even slightly reduced when the stores were depleted. CMD (aa 475-485) represents a domain which has an inhibitory impact on the STIM1/Orai1 communication and contributes to the process of fast Ca^{2+}-dependent inactivation of a CRAC channel [40, 41, 62]. Thus, CaM may play a role in the inactivation of CRAC channels or in regulating STIM1 oligomerization.

Comparison of the values of association between CaM and individual STIM1 fragments *(Table 8)* enabled us to determine the CaM binding domain. The largest fragment, STIM1 233-474 fragment showed a strong association with CaM upon store depletion. When the shorter fragments STIM1 233-343 and STIM1 344-399 including CC1 and CC2 domain, respectively, were used only a weak interaction with CaM was observed. However, a strong interaction upon store depletion was observed at the STIM1 400-474 and STIM1 400-485 fragments. Both of these fragments contain SHD as well as CMD. By introducing mutations into STIM1 400-474 leading to destabilization of the CC3 structure, we obtained a fragment which showed the strongest association with CaM of all our experiments. Therefore, our data provide a clear evidence that the CaM binding domain exists within the CC3 domain (aa 399-423) of STIM1.

All cytosolic fragments demonstrated an intensified interaction upon store depletion evoked by ionomycin. Interestingly, the STIM1 400-485 fragment was the only fragment showing a different behaviour. In this case, the low cytosolic Ca^{2+} levels in the control represented the strongest association with CaM and even a slight reduction was noticed upon the depletion of internal stores. This association under resting conditions was about ten times higher than detected at all other non-mutated fragments. This indicates that at the resting levels, CaM could be already pre-bound to the cytosolic part of STIM1. *Swindells et al.* [78] demonstrated that CaM, whose N- and C-terminal domains comprise two EF-hands per each domain, can take up three different conformations depending on the availability of Ca^{2+}. Upon Ca^{2+} binding, both Ca^{2+}-loaded domains of CaM adopt an open conformation in which

hydrophobic surfaces of each domain are exposed to the solvent to enable binding to its target proteins. In the absence of Ca^{2+}, the N-terminal domain of apo-CaM (Ca^{2+}-free calmodulin) adopts a closed conformation with a tight packing arrangement incapable of binding peptides. Contrary to the N-terminal domain, the C-terminal domain of apo-CaM in the absence of Ca^{2+} adopts a semi-open state by a partial exposure of some hydrophobic residues to be accessible to the solvent which leads to increased conformational freedom so that interaction with some proteins is possible. Therefore, it can be assumed that this cytosolic STIM1 fragment is capable of interacting with the semi-open form of CaM, i.e. with the C-terminal domain of CaM. Concerning all the other non-mutated fragments, store depletion and increase of the cytosolic Ca^{2+} concentration leads to binding of Ca^{2+} to EF-hand pairs of CaM which evokes a conformational change and CaM adapts the open form [78].

In comparison to the previous experiment, the whole C-terminus showed much stronger interaction after the store depletion than the individual non-mutated cytosolic STIM1 fragments. It indicates that there could be another interaction site for CaM. Taking into consideration that the end region of STIM1 was the only part of cytosolic STIM1 which was not subjected to our investigation, the second binding site of CaM should be located between the residues 486-685. This suggestion would be in agreement with the study by *Bauer et al.* [79] who tested the polybasic tail of STIM1 (aa 667-685) on the presence of interaction with CaM. The authors presented that the polybasic tail rich in hydrophobic residues binds to hydrophobic sites of CaM and stated this interaction to be Ca^{2+}-dependent. Therefore, it indicates that the polybasic region of the STIM1 could represent the second binding site for CaM. Additionally, *Mullins et al.* [62] later determined that the deletion of the end region of STIM1 CT (aa 491-685) does not affect fast Ca^{2+}-dependent inactivation of a CRAC channel.

3.3. Interaction of calmodulin with Orai1

In the next set of experiments, we checked an interaction of CaM with a 33 kDa plasma membrane protein, Orai1. For this purpose HEK293 cells overexpressing together unlabelled Orai1 and YFP-CaM were used. 100 µM EDTA was added to the control samples for setting the extracellular Ca^{2+}-free environment and additional 2 mM Ca^{2+} was provided to cells treated with 10 µM ionomycin. The cells were lysed and anti-GFP antibody and Protein A agarose beads allowed an isolation of the YFP-labelled protein complex. The detection of the co-immunoprecipitated Orai1 with YFP-CaM was achieved using anti-Orai1 antibody and HRP-conjugated anti-rabbit IgG antibody.

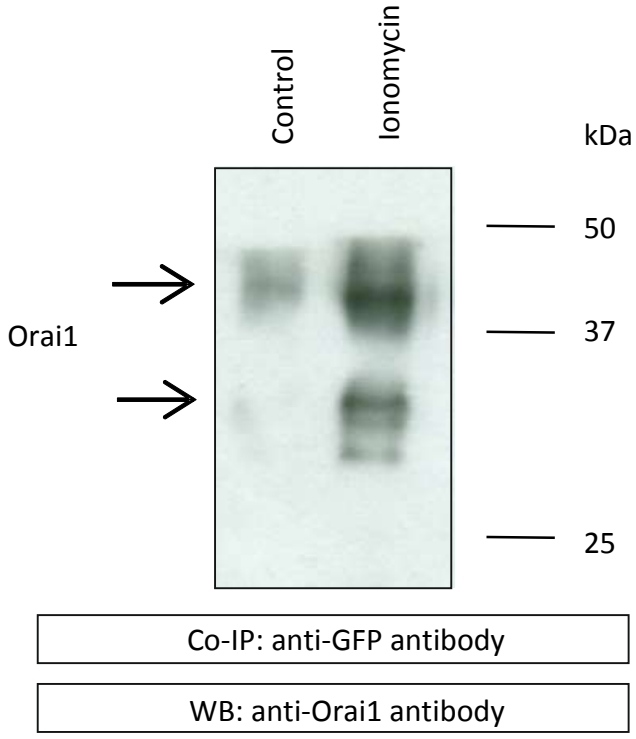

Figure 12: Interaction between Orai1 and CaM. Co-IP experiments with Western blot analysis of HEK293 cells expressing YFP-CaM and Orai1 treated without (control) or with 2 mM Ca^{2+} and 10 µM ionomycin.

The immunoblot *(Figure 12)* shows a characteristic glycosylation pattern of Orai1 when expressed in HEK293 cells. This is probably due to the presence of the *N*-glycosylation site, asparagine at the position 223 (N223), within the second extracellular loop of Orai1 connecting transmembrane domain 3 and 4. Nevertheless, the glycosylation leading to an

increase of the typical Orai1 molecular mass was shown to be not required for the function of Orai1 [47, 51]. The smear visible in the control was caused most likely due to an activation of some cells during washing steps and does not interpret any significant band. Therefore, the normalized values of the association of Orai1 with CaM after the stimulation with ionomycin give a 2.97 ± 0.11 increase (n=5) compared to resting levels.

Using Co-IP technique, we could proof that Orai1 interacts with CaM upon depletion of internal Ca^{2+} stores and Ca^{2+} entry. Our results fully correspond to the study by *Mullins et al.* [62], who demonstrated that CaM binds to Orai1 in a Ca^{2+}-dependent manner. The authors additionally determined the CaM binding domain to be in the N-terminus of Orai1 (CBD_{Orai1}, aa 68-91) and assessed this binding as a requirement for the occurrence of the fast Ca^{2+}-dependent inactivation of CRAC channels. *Liu et al.* [63] later found out that each domain of CaM can bind one CBD_{Orai1} and a 1:2 binding mode is implemented. In the same study they showed that the C-terminal domain of CaM has approximately four times higher affinity for Ca^{2+} than the N-terminal domain. Hence, they proposed that at first C-domain interacts with CBD_{Orai1} and afterwards the interaction of N-domain with another CBD_{Orai1} can occur.

3.4. Effect of calmodulin on store-operated calcium entry

Electrophysiological experiments using patch clamp in the whole-cell recording mode were performed to examine the role of CaM on the initiation of the store-operated calcium entry. HEK293 cells exhibit very small and nearly undetectable endogenous I_{CRAC}. Large inward currents can be recorded when STIM1 and Orai1 are co-expressed together [80]. Therefore, the currents in HEK293 cells transfected with CFP-STIM1 and unlabelled Orai1 as well as cells transfected with CFP-STIM1, unlabelled Orai1 and YFP-CaM were monitored. Activation of I_{CRAC} was achieved by the passive depletion of calcium stores by 20 mM EGTA diffusing from the pipette solution. EGTA causes a gradual Ca^{2+} leakage out of the internal stores by buffering the cytosolic Ca^{2+} and keeping its concentration at low levels [57]. The measured currents of individual cells were normalized by the respective cell capacitances, averaged and plotted as a function of time (± S.E.).

The STIM1/Orai1-expressing HEK293 cells produced a current with the typical features of I_{CRAC} [14]. The time course of current development *(Figure 13A)* shows an inward current induced by the passive depletion of Ca^{2+} stores. The currents in STIM1/Orai1/CaM-expressing HEK293 cells exhibited a similar behaviour in response to activation with EGTA as those cells co-expressing only STIM1/Orai1 and no remarkable change in current was monitored. Therefore, we can conclude from the electrophysiological data that CaM does not affect the CRAC channel activation or alter the initiation of SOCE.

Based on the electrophysiological results, a biotinylation method was employed to investigate, whether the presence of CaM influences an expression of Orai1 in the plasma membrane. Those transfected HEK293 cells overexpressing both unlabelled STIM1 and YFP-Orai1 were used as a control and those cells overexpressing together unlabelled STIM1, YFP-Orai1 and unlabelled CaM were used for the determination of the level of Orai1 expression when CaM is present. Mock cells were used as a negative control. Cells were incubated with a biotinylation reagent which enabled labelling of the cell surface proteins. Non-reacted reagent was removed by 100 µM Tris. After cell lysis by sonication in lysis buffer, biotinylated proteins were extracted using agarose beads with a covalently immobilized streptavidin. YFP labelled Orai1 was detected by anti-GFP antibody and HRP-linked mouse IgG antibody.

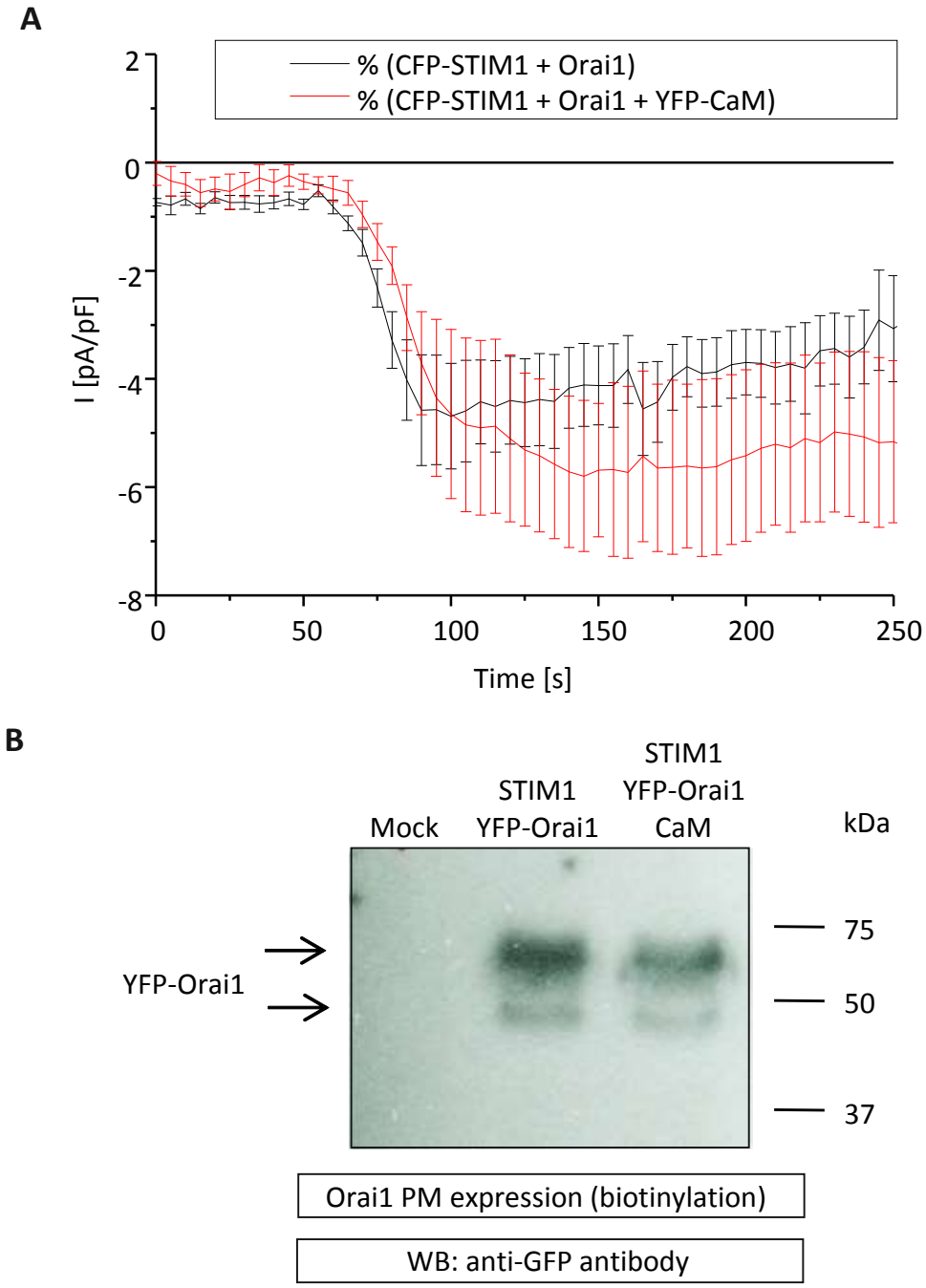

Figure 13: Role of CaM in store-operated calcium entry in HEK293 cells.

(A) Time course of inward currents activated by EGTA-induced passive store depletion in cells co-expressing CFP-STIM1, unlabelled Orai1 and YFP-CaM in comparison with cells co-expressing only CFP-STIM1 and unlabelled Orai1. Error bars illustrate mean ± S.E. (B) Biotinylation experiments with Western blot analysis of HEK293 cells expressing only STIM1 and YFP-Orai1 or STIM1, YFP-Orai1 and CaM demonstrating Orai1 expression in the plasma membrane in the absence or presence of CaM.

YFP-Orai1 appeared as a double band *(Figure 13B)* of expected size. The dominant upper band of the higher molecular mass is most likely a result of a post-translational modification (glycosylation) of Orai1 [51, 81]. Experiments in the absence and presence of CaM revealed a similar results regarding to the expression of Orai1 in the plasma membrane. The values of the Orai1 expression, 22104 ± 3578 (n=5) and 21967 ± 2947 (n=5) correspond to STIM1/Orai1 and STIM1/Orai1/CaM, respectively. Therefore, cell surface biotinylation demonstrated that CaM does not affect abundance of Orai1 in the plasma membrane.

The electrophysiological and biotinylation experiments revealed that CRAC channel is assembled and SOCE is initiated without an affection of CaM. Taking into consideration our results and the fact that CaM is involved in the fast Ca^{2+}-dependent inactivation of CRAC channels [59], it seems that the function of CaM related to the CRAC channel activity lies only in the mediation and regulation of the inactivation. This process occurs in a Ca^{2+}-dependent manner but the molecular mechanism underlying Ca^{2+}-dependent gating of the CRAC channel has not yet been understood [18]. To date, a proposed model suggests an interplay of STIM1 and cytosolic regions of Orai1 comprising the N- and C-termini and intracellular loop [54].

The most recent study by *Kar et al.* [82] focused on interactions between Orai1 and CaM supports the possibility of another bridging protein to contribute to the association between CaM and Orai1. Based on our results we can confirm that indeed another protein is involved and that is STIM1. A hypothesis consistent with existing data describing the mechanism of interaction between CaM and CRAC channel proteins could encompass the following steps. When cytosolic Ca^{2+} levels in resting cells are low, apo-CaM is apparently pre-bound with its C-terminal domain to a binding site located in the cytosolic region of STIM1. Depletion of internal Ca^{2+} stores leads to an oligomerization of STIM1 [11] and conformational change of CaM from the semi-open state into the open-state [78]. This rearrangement in which more hydrophobic surfaces are exposed, according to our results strongly enhances the interaction with the cytosolic portion of STIM1. Due to the finding that Orai1 associates with CaM only upon store depletion at the increased concentration of cytosolic Ca^{2+}, translocated STIM1 most likely brings the attached CaM into puncta to be in close proximity to Orai1. Thus, CaM can bind to the CaM binding domain of Orai1 in a certain point of Ca^{2+} entry and participate in the conformational change inactivating the CRAC channel and preventing further Ca^{2+} entry. Our finding that CaM is anchored to STIM1 is also in accordance with a study by *Litjens et al.* [59]. The authors proposed that in the case of involvement of CaM in

FCDI, it is unlikely for CaM to be freely mobile in the cytosol. Neither can it be excluded that Orai1 binds also some molecules of Ca^{2+}-loaded CaM and not only those brought by STIM1.

CaM as well as other proteins, like the scaffold family protein Homer [83], SARAF [84] or Septin [85] may indeed have a supportive/regulatory/modulatory role in endogenous systems, but they are not required for the full activation of the STIM1-Orai1 complex in our expression system.

4. CONCLUSION

Our study on HEK293 cell line as an expression system shows that CaM is able to bind both proteins of the CRAC channel. Using Co-IP method and confocal FRET microscopy, we determined that the strongest association of CaM with full-length STIM1 and Orai1, respectively, occurs upon depletion of intracellular Ca^{2+} stores evoked by the stimulation with ionomycin. Therefore, it is store depletion and Ca^{2+}-dependent. Deletion of the luminal portion and retaining only the cytosolic part of STIM1 revealed that the interaction of STIM1 with CaM takes place within the cytosol. By constructing cytosolic STIM1 fragments of various lengths and introducing mutations, we demonstrated that the CaM binding domain is present within the CC3 (aa 399-423) of STIM1. The interaction of all fragments except one was more pronounced upon store depletion. The STIM1 400-485 fragment showed approximately ten times higher association under resting conditions when the level of intracellular Ca^{2+} is low, compared to the other non-mutated fragments. It seems that CMD (aa 475-485) might stand behind the occurrence of this phenomenon. Furthermore this observation indicates that in resting cells, apo-CaM, particularly the semi-open form could be already pre-bound with its C-terminal domain [78] to the cytosolic part of STIM1.

Increase of cytosolic Ca^{2+} concentration causes a conformational change and CaM adapts the open form [78]. Nevertheless, the association between CaM and individual non-mutated STIM1 fragments upon store depletion was not as pronounced as in the case of the whole C-terminal STIM1. This might suggest that there is another binding site for CaM located between the residues 486-685.

Despite our previous finding that CaM associates with both STIM1 and Orai1, cell surface biotinylation and electrophysiological experiments demonstrated that CaM neither affects the abundance of Orai1 in the plasma membrane nor the activation of SOCE. Thus, the functional CRAC channel is assembled and SOCE is induced without any contribution of CaM. Based on our results and recent publications related to CaM and fast Ca^{2+}-dependent inactivation of CRAC channels [54, 59, 62-64], it appears that the function of CaM lies solely in Ca^{2+} sensing and triggering a conformational change which inactivates the CRAC channel and prevents further Ca^{2+} entry. CaM could shield the STIM1 CMD domain and upon Ca^{2+} influx

through Orai1, the activated CaM dissociates from STIM1 and uncovers the CMD domain leading to inactivation of Orai1. On the other hand, CaM may regulate STIM1 oligomerization mediated by CC3. Upon increases in the cytosolic Ca^{2+} level the activated CaM could associate to STIM1 CC3 disrupting STIM1 oligomerization and stopping or down-regulating Orai1 activity. These results open new possibilities into the regulation of CRAC channels and further investigations should be performed to finally elucidate the role of CaM in the regulation of SOCE.

LIST OF FIGURES

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