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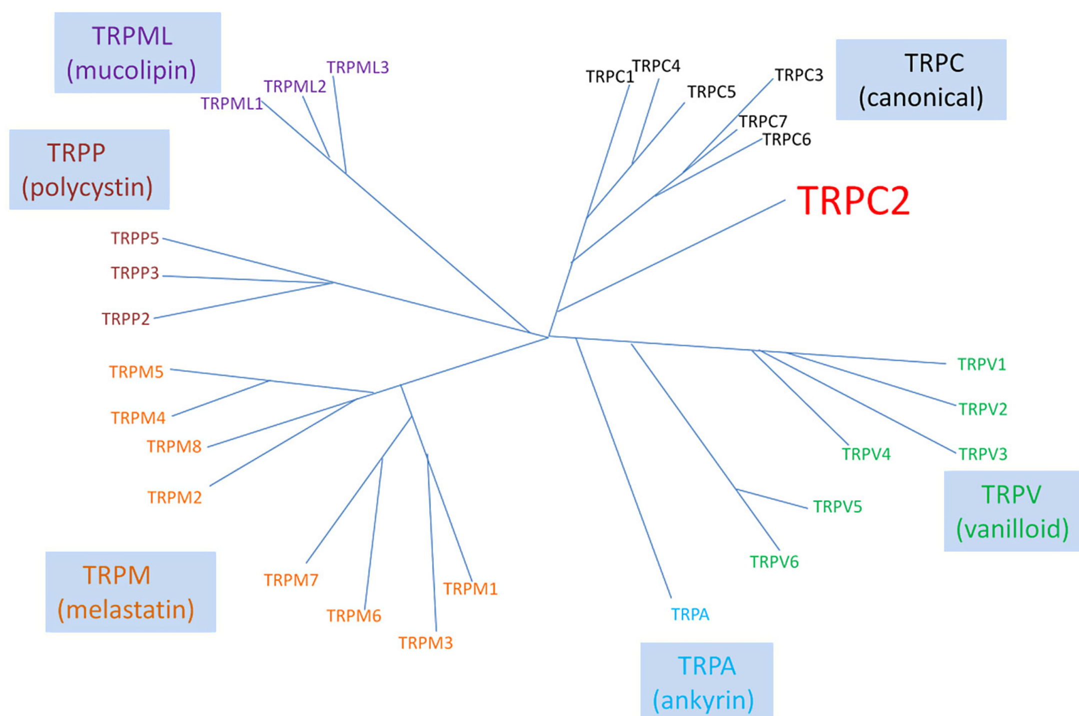
Role of the canonical transient receptor potential channel 2 (TRPC2) in thyroid cell function



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Role of the canonical transient receptor potential channel 2 (TRPC2) in thyroid cell function

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2013

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To my Father

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ABSTRACT

Transient receptor potential canonical (TRPC) channels are widely expressed in mammals and function in many physiological important processes. Among the seven TRPC channels, little is known about to the physiological significance of TRPC2 and its regulation.

For the first time, we report the presence of TRPC2 channel in rat thyroid FRTL-5 (Fischer rat thyroid low-serum 5%) cells and primary rat thyroid cells. In rodents, TRPC2 was thought to be exclusively expressed in the vomeronasal organ. To investigate the physiological importance of the channel, we have developed stable TRPC2 knock-down cells (shTRPC2) using short hairpin RNA. Knockdown of TRPC2 have resulted in profound differences in cellular functions and was found to have important downstream effects on thyroid cells. Knock-down of TRPC2 have resulted in decreased ATP-evoked calcium entry and inward current but enhanced the basal calcium entry. The expression of PKCβ1 and PKCδ, the activity of SERCA and the calcium content in the endoplasmic reticulum was decreased in shTRPC2 cells. Moreover, via TRPC2, communication between the calcium and cAMP signaling pathways existed and that regulated the expression of the TSH receptor. We also studied the significance of the TRPC2 channel in the regulation of FRTL-5 cell proliferation, migration, adhesion and invasion, all of which were attenuated in shTRPC2 cells. Taken together, these results presented a novel insight into how TRPC2 channels function in rat thyroid FRTL-5 cells.

LIST OF ORIGINAL PUBLICATIONS

This Thesis is based on the following original publication and manuscripts, which are referred to in the text by their Roman numerals:

I. Löf C, **Sukumaran P**, Viitanen T, Vainio M, Näsman J and Törnquist K. Communication between the calcium and cAMP pathways regulate the expression of the thyroid-stimulating hormone receptor: TRPC2 in the center of action. Mol. Endocrinol (2012). 12, 2046-57.

II. **Sukumaran P**, Löf C, Kemppainen K, Kankaanpää P, Näsman J, Viitanen T and Törnquist K. Canonical transient receptor potential channel 2 (TRPC2) regulates basal calcium homeostasis in rat thyroid FRTL-5 cells: Importance of PKC and STIM2. J. Biol. Chem (2012) 287, 4435-60.

III. **Sukumaran P**, Löf C, Viitanen T, Pulli, L and Törnquist K. Significance of transient receptor potential canonical 2 (TRPC2) channel in the regulation of rat thyroid FRTL-5 cell proliferation, migration, adhesion and invasion. Manuscript submitted.

ABBREVIATIONS

$[Ca^{2+}]_i$	Intracellular free calcium concentration or cytoplasmic free calcium concentration
2APB	2-aminoethoxydiphenylborane
Caly A	calyculin A
cAMP	Cyclic adenosine monophosphate
CDKs	Cyclin-dependent kinases
CREB	Cyclic AMP response element-binding protein
DAG	Diacylgycerol
ER	Endoplasmic reticulum
ERK	Extracellular signal regulated kinase
FRTL-5	Fisher rat thyroid low-serum 5% cell
GPCR	G protein coupled receptor
G_s	G stimulatory alpha subunit
HBSS	Hank's balanced salt solution
IP_3	Inositol-1, 4, 5-trisphosphate
IP_3R	Inositol trisphosphate receptor
MAPK	Mitogen-activated protein kinase
MMP	Matrix metalloproteinase
NCX	Sodium calcium exchanger
NFAT	Nuclear factor of activated T-cells
OAG	1-oleoyl-2-acetyl-sn-glycerol
ORAI	Calcium release-activated calcium channel protein
PBS	Phosphate-buffered saline
PIP2	Phosphatidylinositol 4, 5-bisphosphate
PIP3	Phosphatidylinositol 3, 4, 5-Trisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PM	Plasma membrane
PMCA	Plasma membrane Ca^{2+}-ATPase
ROCE	Receptor-operated calcium entry
RT-PCR	Reverse transcriptase-polymerase chain reaction
S1P	Sphingosine-1-phosphate
SERCA	Sacro/Endoplasmic Recticulum Ca^{2+}-ATPase
SOCE	Store-operated calcium entry
STIM	Stromal interaction molecule
Tg	Thyroglobulin
TRPC	Transient receptor potential canonical channel
TSH	Thyroid stimulating hormone
TSHR	Thyroid stimulating hormone receptor

1. INTRODUCTION

Calcium is one of the prominent regulators for a variety of cellular processes such as gene transcription, proliferation, motility and apoptosis (Berridge et al., 2003). To make this a possibility, the cells have evolved various mechanisms to regulate cellular calcium levels, where calcium channels play a key role.

The transient receptor potential (TRP) channels have been studied extensively since the discovery of the first member of the family by Wes et al (1995) and Zhu et al., (1995) and the TRP channels have been shown to play a prominent role in regulating cellular functions. The canonical transient receptor potential (TRPC) channel subfamily consists of seven members (TRPC1-7) with diverse modes of regulation and physiological function. Among the seven different members of the TRPC, the TRPC2 channel, being a pseudogene in human, is perhaps the least investigated one. Physiological roles of TRPC2, have been studied in mature sperm and the vomeronasal sensory system of rodents (Yildirim and Birnbaumer, 2007; Kiemnec-Tyburczy et al., 2011). In the vomeronasal sensory organ, TRPC2 was found to constitute the transduction channel activated through a signaling cascade initiated by the interaction of pheromones (Kiselyov et al., 2010). In sperm, TRPC2 is activated by the sperm's interaction with the oocyte (Jungnickel et al., 2001).

Due to the availability of FRTL-5 cells of rat thyroid epithelium, these cells have been studied *in vitro* to a greater extent than that of any other endocrine gland (Kimura et al., 2001). In FRTL-5 cells, of the all TRPC channels, only TRPC2 was expressed. TRPCs commonly forms heterodimers with other TRPCs (Abramowitz and Birnbaumer, 2009; Nilius and Owsianik, 2011). Comparatively little is known in regard to TRPC2 regulation and interaction with other calcium regulating signaling molecules and its physiological significance (Abramowitz and Birnbaumer, 2009). Taken together, these explain the reason for using FRTL-5 cells as our study model.

This Thesis deals with the presence and significance of TRPC2 channels in FRTL-5 cells. It investigates how TRPC2 regulates various cellular processes, such as proliferation, migration and invasion. The role of TRPC2 channels as a regulator of calcium homeostasis in thyroid cells is also studied with an emphasis on Stromal interaction molecule-2 (STIM2) and PKCs. The Thesis also investigates the role of TRPC2 in regulating the expression of thyroid-stimulating hormone receptor via cAMP and extracellular signal-regulated kinase 1/2 (ERK1/2) pathways. Taken together, the results presented in this Thesis gives novel insight into how TRPC2 channels function in rat thyroid FRTL-5 cells.

2. REVIEW OF THE LITERATURE

2.1 Calcium signaling

Even though it was serendipity by Sidney Ringer in 1910 to discover the importance of calcium in cellular function, calcium is an important ubiquitous intracellular signaling molecule in all cells. In the last 102 years, after Ringer's discovery, remarkable progress has been made to understand the molecular and physical mechanisms that are affected by calcium. All cells via various calcium channels, pumps and calcium binding proteins, strictly control the intracellular free calcium concentration $[Ca^{2+}]_i$, at low nanomolar levels because a small increase in intracellular free calcium concentrations will result in various cellular processes, ranging from short-term responses, such as muscle contraction, secretion, neuronal transmission, to long term modulation of cell growth and gene transcription. Changes in the cytosolic calcium concentration are also resisted by resident, high affinity calcium binding proteins. The concept of calcium as a universal second messenger started to evolve after the discovery of various calcium binding proteins such as troponin C by Ebashi and Kodama, (1966) and calmodulin by Kakiuchi et al., (1981) and Cheung, (1982).

Calcium sensitive photoproteins and fluorescent calcium indicators were the key factor for such rapid and ardent development of this field. Hence today calcium research is mainly dependent on the imaging and patch clamp techniques. A milestone achievement came with the development of the calcium indicator dyes Quin2 by Tsien et al. in 1982 and Fura-2 by Grynkiewicz et al. in 1985. Fura-2 can be used to estimate the actual concentration of calcium in the cytosolic compartments. Fura-2 exhibits a progressive wavelength shift in its excitation spectra, as calcium is bound. The ratio of the fluorescence emission (340/380nm) can be then calibrated and used for quantitative measurement of intracellular free calcium concentrations. These techniques along with the modern development of molecular and genetic techniques have added new aspects to the field of calcium research. Now specialized indicators are available which can measure calcium in particular organelles such as the mitochondria, ER and the nucleus (Titushin et al., 2011; Tian et al., 2012).

The cells have access to two source of signal calcium, an infinite supply of external calcium mediated by the channels and more finite internal stores such as the ER (Berridge, 1997). In general, when a stimulus activates the 7-transmembrane G-protein coupled receptors, it results in the dissociation of the G protein complex into βγ and α subunits. The βγ subunit activates the phospholipase-C (PLC). PLC acts on

phosphatidylinositol 4, 5-bisphosphate (PIP2) and catalyses its dissociation into diacylglycerol (DAG) and inositol 1, 4, 5-trisphophate (IP3). The released IP3 diffuses through the cytosol and binds to the IP3R in the ER, resulting in the opening of IP3-gated calcium release channels, releasing the stored calcium into the cytosol. DAG may activate receptor-operated calcium channels. Once the stored calcium in the ER is depleted, it is refilled by the activation of store-operated calcium channels in the plasma membrane and by the action of sacro/endoplasmic reticulum calcium-ATPase (SERCA) in the ER. Excess cytosolic calcium is harmful for the cells. Hence, the excess calcium is pumped out to the exterior of the cell, mainly by plasma membrane calcium-ATPase (PMCA) and sodium-calcium exchangers (NCX). The following chapters will give a broad detail in the different components and regulators of the above said signaling pathway with emphasis on the non- selective calcium channel TRPC2 (transient receptor potential canonical channel-2).

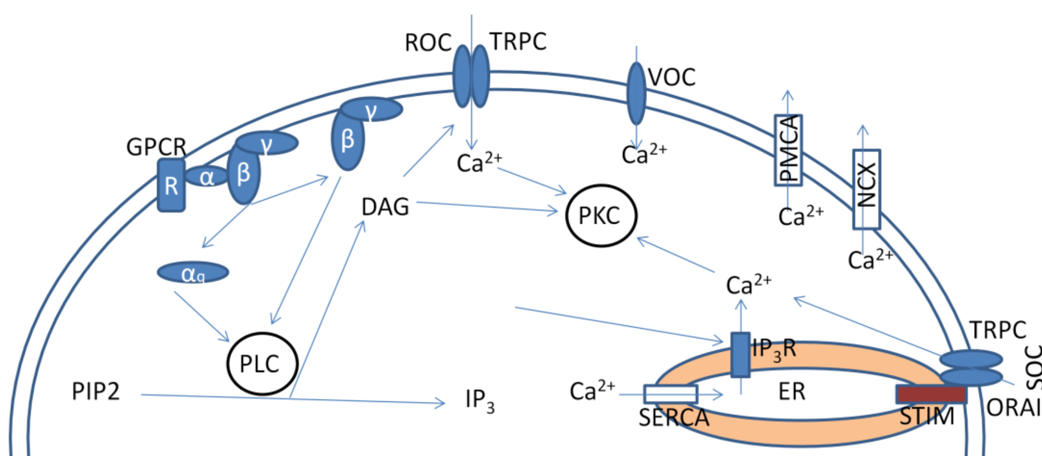

Figure 1. Schematic picture of the common calcium signaling pathways.

All cells have several mechanisms for regulating cytosolic calcium concentration, but not necessarily all of the mechanism shown here are present in one single cell type. The activation of G coupled protein receptor complex dissociates and activates the enzyme PLC which catalyses the dissociation of PIP2 to form DAG and IP_3. DAG activates receptor-operated channels (ROC). IP_3 binds to its receptors in the ER (IP_3R) resulting in the release of the stored calcium from the ER. Emptying of the ER calcium activates the STIM protein to translocate to the PM and binds to calcium release-activated calcium channel protein (ORAI), thereby

activating store-operated channels (SOC). TRPCs can act as a ROC and SOC. Increased calcium in the cytosol is pumped out by PMCA and NCX. SERCA refills the stores. DAG and calcium activates PKC which results in various downstream effects. For abbreviation and more information see the text.

2.2 Calcium channels

Normally the cytoplasmic free calcium concentration $[Ca^{2+}]_i$ is kept very low (10^{-7} M) compared to the extracellular fluid (10^{-3} M) (Yamakage and Namiki, 2002). Neuronal, hormonal or other stimuli cause an influx of calcium into the cells, specifically through the calcium channels in the plasma membrane or by the sequential release from the ER, thereby increasing the $[Ca^{2+}]_i$.

In electrically excitable cells, such as neuronal and muscle cells, a rise in calcium is initiated by the opening of voltage-dependent calcium channels in the plasma membrane. Here the rise in calcium is very rapid and achieves a high concentration of calcium immediately.

The calcium channels in the plasma membrane are largely divided into three main categories on the basis of their regulatory mechanism (Berridge, 1997)

- Voltage-operated calcium channels
- Receptor-operated calcium channels and
- Store-operated calcium channels

2.2.1. Voltage-Operated calcium channels

In muscles cells, glial cells and neurons cells, mainly voltage-operated calcium channels regulate the fast increase of calcium influx. They were first identified by Paul Fatt and Bernard Katz in crustacean muscles (Fatt and Katz, 1953). Voltage-operated channels are also present at low level, even though the function is not yet understood, especially in cells not traditionally considered as excitable, such as cells in the immune system (Cahalan et al., 2001). Carbone and Lux in 1975 first divided the voltage-operated channels into two major groups on the basis of biophysical and

pharmacological properties. They are high voltage activated channels and low voltage activated channels (Dolphin, 2006). These are further subdivided into L, N, P/Q and R type as high voltage activated channels and T-type as low voltage activated channels. R type channels is occasionally classified as inter-mediate voltage-activated channels (Yamakage and Namiki, 2002).

L-type channels are responsible for excitation-contraction coupling of skeletal, smooth and cardiac muscles and also for hormonal secretion in endocrine cells (Dolphin, 2006). Nimodipine and nifedipine inhibits the L-type channels (Yamakage and Namiki, 2002).

N-type channels are widely distributed in neuronal cells (Fox et al., 1987). They are very important in the presynaptic nerve terminal with an inactivation rate of 100 $msec^{-1}$ (Borle, 1981). They are blocked by ω-conotoxin (Yamakage and Namiki, 2002).

P/Q-type channels were named so because they were first identified in Purkinje cells (Somlyo and Somlyo, 1994). These channels have monovalent ion selectivity in the absence of divalent cations. In 1995 Randall and Tsien designated the Q-type as a separate channel because the Q-type rapidly gets inactivated compare to P-type channels. They are also inhibited by ω-conotoxin (Yamakage and Namiki, 2002).

R-type channels current remains even after all the other channels are blocked by the selective channels blockers (Pearson et al., 1995). The biophysical properties of the R channels are different from N and Q channels, thereby classified as inter mediate-voltage-activated channels (Yamakage and Namiki, 2002).

T-type channels were first discovered in neurons of the guinea pig inferior olivary nucleus (Llinás and Yarom, 1981). T-type channels open with weak depolarization. The T-type channels are activated at more negative voltage compared with other voltage-operated channels, thereby classified as low voltage gated channels (Yamakage and Namiki, 2002). Channel opening is inhibited at a holding potential of more negative than -60mV. These channels are inhibited by ω-agatoxin and mibefradil.

Molecular cloning has revealed the presence of at least seven different genes encoding the high voltage gated channels (Somlyo and Somlyo, 1994). The channel pores and voltage sensing domains form the α subunits and there are various splice variants, making the structural morphology very complex (Dolphin, 2006).

2.2.2. Receptor-operated calcium channels

The receptor-operated calcium entry (ROCE) pathway is activated by second messengers following G-protein evoked activation or tyrosine kinase receptor activation of PLC (Salmon and Ahluwalia, 2011). In many non-excitable cells such as hepatocytes, ATP, acetylcholine, ADP, S1P, TSH, vasopressin and histamine activates receptor-operated calcium influx (Sho et al., 1991; Salmon and Ahluwalia, 2011). The mammalian family of TRP channels, has recently emerged as molecular candidates involved in receptor-operated calcium entry (Plant and Schaefer, 2005). Among the TRP channels, the transient receptor canonical (TRPC) channels are studied more exclusively. Even though TRPC1 can couple to STIM and Orai protein and stimulate store-operated calcium entry (SOCE) (Liu et al., 2000), TRPC4 and TRPC5 has been shown to be a strong candidate to be a receptor-operated calcium channels (Plant and Schaefer, 2005). Receptor-operated channels have an inevitable role in the functioning of human neutrophils, where it regulates the motility and migration of neutrophils. (Salmon and Ahluwalia, 2011). Gd^{3+} does not inhibits receptor-operated calcium entrybut it inhibits SOCE and the ROCE (Salmon and Ahluwalia, 2011). Thapsigargin, an inhibitor of SERCA, separates SOCE from ROCE-activity. Thapsigargin induces depletion of the internal stores in a second messenger independent manner (McFadzean and Gibson, 2002).

2.2.3. Store-operated calcium channels

Store-operated calcium channels are activated following the depletion of the intracellular calcium stores within the endo/sacroplasmic reticulum (Parekh and Putney, 2005). The concept of store-operated entry (SOCE) was first proposed in 1986 by Putney and was originally called capacitative calcium entry and later as calcium release-activated calcium entry. Intracellular calcium stores are often referred as a “life-giving” signal since intracellular calcium stores are involved in sperm motility, acrosome reaction and in creating calcium oscillations in fertilization of the egg (Parekh and Putney, 2005). At present a good perceptive knowledge of organelles that function as calcium stores and how calcium is released from the stores is present (Berridge et al., 2003). Since a fall in the ER calcium content is necessary for SOCE stimulation, a major regulator of the calcium entry pathway must be present in the maintenance of the ER calcium levels (Parekh and Putney, 2005).

2.3. STIM and ORAI interaction

STIM, the calcium censor present in the ER (Liou et al., 2005; Roos et al., 2005), and Orai, the channel pore forming subunit in PM (Prakriya et al., 2006; Vig et al., 2006) have been identified as the most essential components enabling the reconstitution of the calcium release-activated channels or CRAC that mediates the SOCE (Cahalan, 2009; Deng et al., 2009; Braun et al., 2012). STIM, a single pass membrane protein in the ER, detects the change in ER calcium concentration through a conserved calcium binding domain. (Dziadek and Johnstone, 2007; Zheng et al., 2011). When ER-stored calcium depletes, it leads to oligomerization and relocalization of STIM to the PM (Liou et al., 2005; Dziadek and Johnstone, 2007; Cahalan, 2009; Feske, 2010). STIM then binds to the four transmembrane Orai protein (Prakriya et al., 2006; Frischauf et al., 2008; Feske, 2010). Binding of STIM to Orai results in the opening of the channel, allowing the calcium to enter into the cell and thereby increasing the cytosolic calcium concentration (Liou et al., 2005; Prakriya et al., 2006; Barr et al., 2009; Cahalan, 2009; Deng et al., 2009; Braun et al., 2012).

STIM1 was first identified as an important protein that is required for SOCE, when high-throughput RNAi screens for searching the molecules involved in SOC of Drosophila S2 and HeLa cells were performed (Liou et al., 2005; Spassova et al., 2006b). STIM has two homologous proteins, STIM1 and STIM2 (Cahalan, 2009). STIM1 and STIM2 share moderate sequence similarity and maintain similar structural domains (Hogan et al., 2010). STIM1 and STIM2 function as calcium sensors with different sensitivities for the ER calcium (Collins and Meyer, 2011). STIM1 and STIM2 have distinct roles in regulating calcium homeostasis in the cells (Braun et al., 2012). STIM2 plays a central role in regulating and maintaining the basal cytosolic calcium levels (Brandman et al., 2007). STIM2 regulates the ER calcium concentration by acting as a feedback regulator that senses small change in ER calcium and translates them into the gating of Orai1 pores (Brandman et al., 2007; Collins and Meyer, 2011).

2.4. Transient receptor potential channels

The transient receptor potential (TRP) channels superfamily is one of the largest families of cation channels (Nilius and Owsianik, 2011). The canonical transient receptor potential (TRPC) channels is a seven-member subfamily of the TRP family of ion channels and are present in a wide variety of cells (Graham et al., 2012). The other TRP family subfamilies are TRPM (melastatin), TRPP (polycystin), TRPV (vanilloid), TRPML (mucolipin), TRPA (ankyrin) and TRPN (NOMPC-like); the latter is found

only in invertebrates and fish (Nilius and Owsianik, 2011). Phylogenetic tree of the mammalian (human) TRP channel superfamily is shown below.

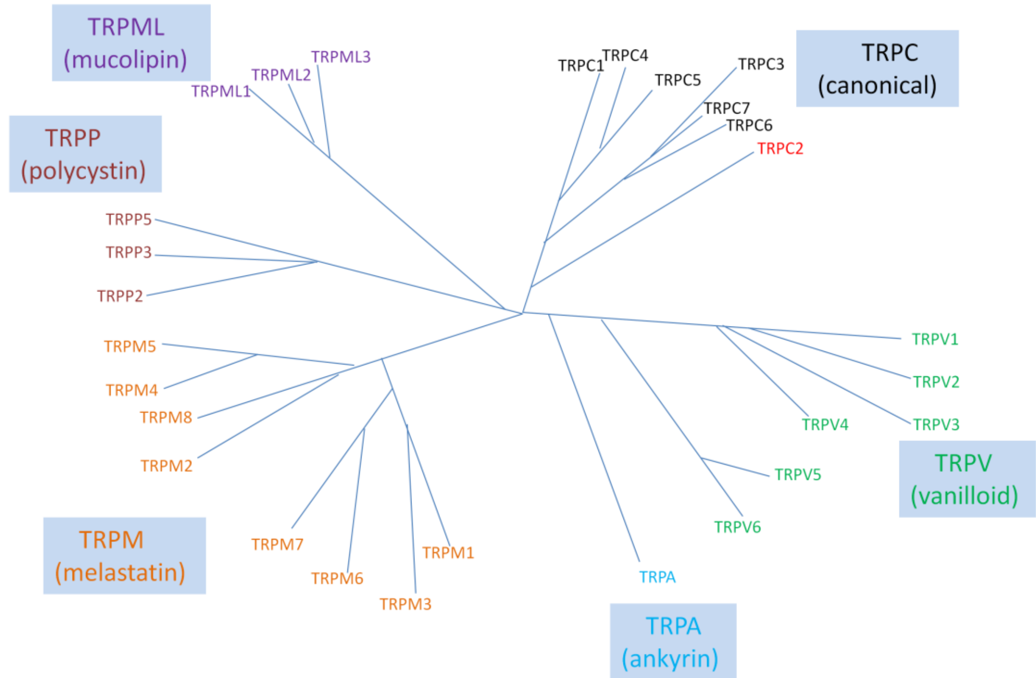

Figure. 2. Phylogenetic tree of the mammalian (human) TRP channel superfamily. TRPML (mucolipin), TRPP (polycystion), TRPM (melastatin), TRPA (ankyrin), TRPV (vanilloid) and TRPC (canonical). TRPC2 is a pseudogene in human. Modified figure from Nilius, 2007.

The TRP channels are involved in regulating various cellular functions, ranging from pure sensory function to molecular regulation. Hence they serve as gatekeepers for transcellular transport of sodium and calcium ions (Nilius and Owsianik, 2011). Currently there are more than 100 TRP genes identified in various animals, making the channels more complex and diverse (Nilius and Owsianik, 2011). Most of the TRPs, especially TRPCs function as homotetramers, even though the formation of heteromultimeric channels have also been reported (Nilius and Owsianik, 2011). The

TRP protein in general is a six putative transmembrane protein with a pore forming reentrant loop between S5 and S6 (Owsianik et al., 2006; Gaudet, 2008).

2.5. Canonical Transient receptor potential channels

The canonical transient receptor potential channels (TRPC) is a subfamily of transient receptor potential channels that have the highest degree of homology to the Drosophila photoreceptors' TRP (Kiselyov and Patterson, 2009). TRPCs are non-selective cationic channels with more selectivity over calcium than sodium and potassium (Song and Yuan, 2010). TRPC channels have been related to several calcium-dependent physiological processes in different cell types, ranging from proliferation to exudation or contractility, under both physiological and pathological conditions (Flockerzi, 2007). All the TRPCs are activated through downstream pathways of PLC stimulation (DeHaven et al., 2009). The family comprises of seven members (TRPC1-7) and is widely expressed (Abramowitz and Birnbaumer, 2009). Of all the seven different members of TRPC, the TRPC2 (a pseudogene in human) is perhaps the least investigated one (Yildirim and Birnbaumer, 2007). TRPCs assemble both as homomeric and heteromeric complexes (Schaefer, 2005). The role and function of the TRPCs varies in contest with its presence in various cells (Abramowitz and Birnbaumer, 2009).

2.5.1. TRPC1

TRPC1 was the first mammalian TRPC channel to be identified (Wes et al., 1995; Abramowitz and Birnbaumer, 2009). TRPC1 forms homo and heterodimers with other TRPCs, mainly TRPC4 and TRPC5 (Hofmann et al., 2002; Strübing et al., 2003). TRPC1 is strongly suggested to be involved in SOCE and has been shown to have interaction with STIM1 and ORAI protein (Ambudkar et al., 2007; Cheng et al., 2011). TRPC1 is present in a wide variety of tissues, mainly in the brain and neuronal cells (Hofmann et al., 2002). They are also present in the prostate gland, liver, salivary gland and many other epithelial tissues (Ambudkar et al., 2007; Abramowitz and Birnbaumer, 2009). The first physiological role of TRPC1 was identified by Ambudkar and colleagues in salivary gland (Singh et al., 2001). Disruption of TRPC1 expression in the salivary gland resulted in an increased salivary sodium, potassium and chloride concentration (Liu et al., 2007). TRPC1 is associated with the regulation of cell volume in liver (Chen and Barritt, 2003). TRPC1 regulates the endothelial permeability in human pulmonary artery endothelial cells (HPAEC) and NF-kappaB-

regulates the TRPC1 expression which is an essential mechanism of vascular inflammation (Paria et al., 2004, 2006). TRPC1 expression in vascular smooth muscle is mediated by hypoxia-inducible factor-1(HIF-1) (Wang et al., 2006). TRPC1 is also an important regulator of cardiac hypertrophy in rodents (Abramowitz and Birnbaumer, 2009) and in neuronal function and development in rodents (Maric et al., 2000).

2.5.2. TRPC2

Being a pseudogene in human, the TRPC2 is considered to be unique and perhaps the least investigated TRPC channel. Relatively little is known about its physiological significance and interaction with other calcium regulating signaling molecules (Abramowitz and Birnbaumer, 2009). TRPC2 exist as a pseudogene in the genome of old world monkey and human (Liman, 2003; Zhang and Webb, 2003). In rodents, loss of TRPC2 expression results in reduction in the ability to detect pheromones leading to gender specific behaviors (Yildirim and Birnbaumer, 2007; Abramowitz and Birnbaumer, 2009). TRPC2 is present in the vomeronasal organs (VNO) which are responsible for detecting water soluble pheromones. Liman et al., (1999) showed for the first time the role of TRPC2 in vomeronasal sensory neurons, and ultrastructural analysis revealed that VNO sensory receptor cells express TRPC2. TRPC2 knockout mice had a phenotype in which the sensory response to pheromones in urine was abolished and the male failed to recognize the difference between male and female (Liman et al., 1999; Leypold et al., 2002; Stowers et al., 2002). TRPC2 knockout male mice started to show sexual behavior towards other male mice (Stowers et al., 2002). The female TRPC2 knockout mice started showing male characteristic behaviors such as mounting, pelvic thrust and ultra-sonic vocalizations (Kimchi et al., 2007; Abramowitz and Birnbaumer, 2009).

Detail study of the TRPC2 gene by Vannier et al., (1999), found two splice variants (TRPC2a and TRPC2b), which increased SOCE and ROCE when heterologously expressed in COS-M6 cells. Hofmann et al., (2000) showed another two novel variants of TRPC2, mTRPC2α and mTRPC2β, which when expressed in HEK-293 cells did not enhanced SOCE and ROCE, which may be because they failed to reach the PM and were retained in intracellular membranes (Hofmann et al., 2000). Studies in fibroblast cells, CHO cells by Gailly and Colson-Van Schoor in 2001 showed involvement of TRPC2 protein in store-operated calcium influx. In HEK-293 cells, the inward current after stimulation of purinergic receptor with ATP was increased after transiently expressing TRPC2 (Jungnickel et al., 2001). Lucas *et al.*, compared DAG-induced inward currents in vomeronasal sensory neurons of TRPC2

knockout mice and wild type mice and found that the inward current in TRPC2 knockout mice was severely reduced (Lucas et al., 2003).

STIM1 binds to TRPC2 via its ezrin/radixin/moesin (ERM) domain (Huang et al., 2006; Yildirim and Birnbaumer, 2007) and enkurin and ankyrin binds to its long N terminal (Tang et al., 2001; Sutton et al., 2004). Calmodulin (CaM) binds to both the N and C-terminal region (Tang et al., 2001).The adaptor protein Homer 1 co-immunoprecipitates with TRPC2 (Yuan et al., 2003; Mast et al., 2010) and a complex between TRPC2 and IP3R3 has also been shown in the VNO (Brann et al., 2002).

TRPC2 plays a major role in regulating calcium homeostasis in sperm cells in rodents (Jungnickel et al., 2001; Yildirim and Birnbaumer, 2007). In sperm, it seems like TRPC2 functions as a SOC channel, since an antibody against TRPC2 decreased both thapsigargin-induced and zona pellucida- (ZP3) induced calcium entry (Jungnickel et al. 2001). TRPC2 regulates the entry of calcium into mouse sperm which is triggered by egg ZP3 (Jungnickel et al., 2001), but the mechanism of activation is not known since TRPC2 deficient mice are fertile (Abramowitz and Birnbaumer, 2009). Studies by Sutton et al., (2004) showed that interaction of other adaptor proteins such as enkurin, in egg ZP3 evoked an increase in the calcium levels.

TRPC2 participates in the erythropoietin-evoked calcium increase in murine haematopoietic (HCD-57) cells (Chu et al., 2002). Studies also showed that the erythropoietin-modulated calcium influx through TRPC2 is mediated by IP3R and phospholipase C gamma, which interacts to form a signaling complex (Tong et al., 2004).

2.5.3. TRPC3

TRPC3 is involved with the control of neuronal function and development both in rat (Li et al., 1999) and in human (Riccio et al., 2002). TRPC3 interacts with other TRPCs and forms heterodimers, especially TRPC1 in cerebellar Purkinje cells and skeletal myogenesis (Lemmon, 2005; Hartmann et al., 2008; Cheung et al., 2011) and with TRPC6 in sensory neuronal cells (Quick et al., 2012). TRPC3 mediate activation of a slow, mixed-cation excitatory postsynaptic conductance in cerebellar Purkinje cells (Kim et al., 2003; Hartmann et al., 2008). TRPC3 is also expressed in the kidney collective duct, skeletal muscles, cardiovascular system and uterine myometrium and has a significant role in their function and regulation (Abramowitz and Birnbaumer, 2009). The multifunctional transcription factor TFII-I has an associated role for TRPC3 in cognition (Caraveo et al., 2006). TRPC3 is activated by DAG (Birnbaumer, 2009).

In HEK-293 cells, TRPC3 translocation depends on its interaction with PLCγ through an intermolecular pleckstrin homology (PH) domain (van Rossum et al., 2005).

2.5.4. TRPC4

The physiological role of TRPC4 is perhaps best studied in the regulation of endothelial cell function (Abramowitz and Birnbaumer, 2009). In vascular endothelial cells, TRPC4 is a required component of SOCE and is part of regulating the vascular tone by regulating the calcium entry signal transduction (Freichel et al., 2001). TRPC4 is also required for cystic fibrosis transmembrane conductance regulator (CFTR) activation in vascular endothelial cells (Wei et al., 2001). TRPC4 is involved in response to neural injury and regulation of neurite outgrowth (Abramowitz and Birnbaumer, 2009). In gastrointestinal pacemaker cells and in mouse visceral smooth muscle cells TRPC4 are important for the control of muscarinic stimulation (Lee et al., 2005). ATP induces an increased expression of TRPC4 which requires cAMP response element-binding protein (CREB) phosphorylation (Zhang et al., 2004). TRPC4 in human is also involved in proliferation of corneal epithelial cells (Yang et al., 2005).

2.5.5. TRPC5

TRPC5 plays an important role in CNS and is important in neuronal function. TRPC5 is mainly involved in the regulation of hippocampal neurite length and growth cone morphology in young rat hippocampal neurons (Greka et al., 2003; Abramowitz and Birnbaumer, 2009). TRPC5 is also regulating the neurite outgrowth (Hui et al., 2006). It is also expressed in epithelia cells (mainly in gastrointestinal mucosa) and in the cardiovascular system (Abramowitz and Birnbaumer, 2009). TRPC5 mediates S1P-induced smooth muscle migration (Xu et al., 2006). Like TRPC1 and TRPC3, TRPC5 also interacts with STIM1 and Orai1 (Ma et al., 2008).

2.5.6. TRPC6

TRPC6 also plays an important role in the CNS (neuroblastoma cells and cerebellar granule cells), cardiovascular system (vascular smooth muscle, heart and endothelium) and in epithelia cells (lung and kidney gromerulus) (Abramowitz and Birnbaumer, 2009). In vascular smooth muscle (VSM) TRPC6 acts as a stretch-activated ion channel (Spassova et al., 2006a). In VSM, TRPC6 expression plays a key role in

enhanced VSM reactivity associated with mineralocorticoid-induced hypertension in rats (Bae et al., 2007; Abramowitz and Birnbaumer, 2009). TRPC6 heterodimerises with other TRPCs. In cardiac hypertrophy, TRPC6 and TRPC3 regulates the activation of the calcium-calcineurin-nuclear factor of activated T cells (NFAT) signaling pathway (Wilkins and Molkentin, 2004). TRPC6 also enhances the transcription factor NFAT activity in rat cardiomyocytes and has two functional NFAT binding sites. TRPC4 and TRPC6 play a significant role in regulating endothelial cell permeability (Abramowitz and Birnbaumer, 2009). TRPC6 and TRPC5 activation cascade is important for endothelial cell migration (Chaudhuri et al., 2008). Mutations in TRPC6 results in development of familial focal segmental glomerulosclerosis, which leads to kidney failure (Winn et al., 2005). Hence TRPC6 maintains the normal protein filtration function of the kidney. TRPC1 and TRPC6 expression are upregulated by hypoxia, hence involved in hypoxia-induced hypertension in pulmonary artery smooth muscle (Wang et al., 2006).

2.5.7. TRPC7

Little is known about TRPC7's role in regulating the function of various tissues. TRPC7 is expressed in the nervous system (dorsal root ganglion cells), keratinocytes, uterine myometrium and in leukemia cells (Abramowitz and Birnbaumer, 2009). During pregnancy, in the uterus (Babich et al., 2004) and during development in dorsal root ganglion cells the TRPC7 expression changes (Elg et al., 2007). TRPC7 in rodents via a calcineurin dependent pathway mediates the angiotensin II-induced myocardial apoptosis (Satoh et al., 2007). PGE2-induced apoptosis in K562 human leukaemia cells is regulated by TRPC7 (Föller et al., 2006).

2.6. TRPC channels and lipid signaling.

TRPC channels are sensitive to lipids, including diacylglycerols, phosphatidylinositol bisphosphate, arachidonic acid and its metabolites, oxidized phospholipids, lysophospholipids, cholesterol, sphingosine-1-phosphate and some steroidal derivatives (Beech, 2012). Hence, by lipid sensing, TRPC channels are enabled to integrate with other signaling systems.

TRPC6 and TRPC1 are found to bind with Phosphatidylinositol 4, 5-bisphosphate (PIP_2) or Phosphatidylinositol 3,4, 5-trisphosphate (PIP_3) with high affinity (Tseng et al., 2004; Kwon et al., 2007). PIP_2 inhibits TRPC4α but not the

TRPC4β channels (Otsuguro et al., 2008), but PIP_2 inhibits TRPC5 (Trebak et al., 2009). Hence there are contradictory and complex effects of PIP2 on TRPC channels

DAG activates the TRPC2/3/6/7 subgroups of TRPCs (Hofmann et al., 1999; Lucas et al., 2003). The N-terminal section of TRPC3/6/7 has a DAG-sensing domain (van Rossum et al., 2005). The exogenous DAG concentration required to stimulate the channels is high (Hofmann et al., 1999). TRPC4 and TRPC5 are not activated by DAG (Beech, 2012). TRPC1 is not directly activated by DAG (Hofmann et al., 1999). Therefore, DAG acutely and directly stimulates TRPCs and DAG also activates or inhibits TRPCs by triggering protein kinase C-dependent phosphorylation. Lysophospholipids relatively directly stimulate TRPC channels and the effects depends on the endogenous concentration of lysophospholipids (Beech, 2012).

TRPC5 is stimulated by sphingosine-1-phospphate (S1P) and hence considered to be an intracellular target for S1P (Xu et al., 2006). TRPC3 translocation depends on its interaction with PLCγ through an intermolecular pleckstrin homology (PH) domain. S1P bounds to a putative TRPC3-PLCγ1 intermolecular domain but the functional relevance of this binding is not known (van Rossum et al., 2005).

Cells loaded with cholesterol showed a positive effect on TRPC3 activation (Graziani et al., 2006). TRPC1 has been associated with cholesterol-containing caveolae and other lipid rafts (Lockwich et al., 2000). Podocin-dependent activation of TRPC6 channels requires cholesterol (Huber et al., 2006). TRPC2 in rodents was activated by sulphated steroids from urine (Nodari et al., 2008). TRPC channels are capable of sensing various lipids and are involved in lipid signaling (Beech, 2012).

2.7. Calcium binding proteins

Calcium binding proteins are proteins that participate in calcium signaling pathways by binding to calcium. Since calcium is an important second messenger in all type of cells, these calcium binding proteins are present in every cell and play an important role in regulating their function. Calcium binding proteins are diverse but they are generally classified into three subclasses, EF-hand protein, Annexins and C2-domain proteins.

2.7.1 EF-hand proteins

EF-hand proteins are proteins involved in regulating $[Ca^{2+}]_i$ and have a common helix-loop-helix structure motif in their calcium binding sites (Kretsinger and Nockolds, 1973; Nelson and Chazin, 1998). These proteins have high selectivity for calcium. They play an important role in calcium uptake, transport and buffering. EF-hand

proteins are classified into calcium signal sensors and modulators (Nelson et al., 2001). EF-hand calcium binding proteins regulate high voltage-activated voltage gated calcium channels (Dick et al., 2008). The best characterized EF-hand protein is calmodulin (CaM). CaM contains four functional EF-hand motifs (Klevit et al., 1984). Calmodulin is inactive when not bounded to calcium and changes confirmation when it binds to calcium. Another important EF-hand calcium binding protein is calpain, which is a non-lysosomal cysteine protease and is important for cell mobility and cell cycle progression (Murachi, 1989; Croall and DeMartino, 1991; Blanchard et al., 1997; Sorimachi and Ono, 2012).

2.7.2. C2-domain proteins (Protein kinase C)

Protein kinase C (PKC) was the one of the first kinases to be discovered (Takai et al., 1977) and is a family of serine/threonine kinases. PKCs are proteins having a C2 domain which binds to calcium. PKCs after binding to calcium translocate to the plasma membrane and bind phosphatidylserine. C2-domain proteins where first studied in conventional PKCs. PKCs are activated in specific intracellular compartments and depending upon the various lipid metabolites and calcium. PKCs play a distinct role in the control of major cellular functions. The PKC family consists of thirteen isozymes (Rosse et al., 2010). They are classified into three subfamilies, based on their second messenger requirements. These are the conventional (require calcium, DAG and phospholipids as activator), novel (requires DAG, but do not require calcium) and atypical (do not require either calcium or DAG) isoforms (Nishizuka, 1995). All PKCs consist of a regulatory domain and a serine/threonine kinase catalytic domain. The C1 domain of all isoforms of PKCs have a binding site for DAG and phorbol esters (Freeley et al., 2011). PKCs have multiple functions and are achieved by PKC mediated phosphorylation of various other cellular proteins. PKC's are involved in regulating cell growth, mediating immune responses, regulating transcription and various other cellular functions such as contraction, secretion and aggregation (Mellor and Parker, 1998).

2.7.3. Annexins

Annexins comprise of a multigene family of calcium-regulated membrane binding proteins and have a conserved calcium binding unit (Gerke and Moss, 2002). Annexins are involved in changing cell shape and membrane curvature by modulating actin

rearrangement. Annexins are also involved in membrane trafficking and the organization of compartment membranes (Gerke and Moss, 2002).

2.8. Calcium pumps

High levels of $[Ca^{2+}]_i$ are toxic to cells. The major role of calcium pumps and exchangers is to lower the $[Ca^{2+}]_i$. The plasma membrane calcium -ATPase (PMCA) and the sodium calcium exchanger (NCX) are the main regulators of $[Ca^{2+}]_i$. The sacro/endoplasmic reticulum calcium-ATPase (SERCA) tries to replenish the intracellular stores by transferring Ca^{2+} from the cytosol into the lumen of endoplasmic reticulum (Arruda et al., 2007; Carafoli, 2011). The PMCA and NCX moves calcium depending upon the electrochemical gradient (Strehler and Zacharias, 2001). Mammalian cells express 4 different PMCAs, out of which two are housekeeping isoforms (Carafoli, 2011). The PMCA is located mainly in PM, SERCA in the ER and secretory pathway calcium-ATPase (SPCA) in the golgi apparatus (Grover and Khan, 1992). Mammalian cells express three SERCA genes (Larsen et al., 2001). SERCA1 and SERCA2 are stimulated by phospholamban and SERCA3 may be regulated by PKA (Grover and Khan, 1992). Many cancers are associated with a remodeling of calcium pump expression (Curry et al., 2011). Deafness and balance disorder was observed in the PMCA2 knockout mice and the males were infertile in PMCA4 knockout mice (Prasad et al., 2004).

2.9. Calcium and TRPCs as regulators of proliferation and migration.

Calcium plays a fundamental role in the regulation of a large number of cellular processes such as proliferation, migration, invasion, adhesion, motility, gene transcription and apoptosis (Borle, 1981; Berridge, 1997; Berridge et al., 2003). To make such a broad spectrum of different actions possible, the cells have evolved multiple mechanism regulating cellular calcium levels, mainly by regulating the function of calcium channels, pumps and exchangers. The TRPC family have been related to several calcium-dependent physiological processes in different cell types, ranging from proliferation to contractility, under both physiological and pathological conditions (Abramowitz and Birnbaumer, 2009; Shapovalov et al., 2011).

In pulmonary artery smooth muscle cells TRPC1 expression enhances proliferation (Zhang et al., 2007). In vascular injury, TRPC1 is involved in atherosclerosis and neointimal formation and TRPC1 is up-regulated in neointima (Kumar et al., 2006). TRPC1 is also important in neural stem cell proliferation

(Abramowitz and Birnbaumer, 2009). TRPC1 and TRPC3 levels are increased when H19-7 hippocampal neuronal cells are grown in differentiating conditions (Wu et al., 2004). TRPC1 upregulation is a general feature of the smooth muscle cells in occlusive vascular disease (Kumar et al., 2006). TRPC1 also plays an important role in keratinocyte proliferation and differentiation (Cai et al., 2006).

TRPC6 also regulates the proliferation of the pulmonary artery smooth muscle (PASMCs) cells of patients with idiopathic pulmonary arterial hypertension (Yu et al., 2004). Hypoxic condition in human pulmonary artery endothelial cells increased the expression of TRPC4. Enhanced TRPC1 expression leads to the production of growth factors and increase proliferation in smooth muscle cells (Fantozzi et al., 2003; Stenmark et al., 2006). TRPC4 is also involved in EGF-induced proliferation of corneal epithelial cells (Yang et al., 2005). Proliferation of the human hepatoma cell line Huh-7 is controlled by TRPC6 (El Boustany et al., 2008). Phenylephrine treatment of prostate cancer epithelial cells augmented TRPC6 expression (Abramowitz and Birnbaumer, 2009). These findings suggest that TRPC channels play a central role in regulating proliferation in many normal and malignant cells.

Increased expression of TRPC1 increased the SOCE and enhanced cell migration in intestinal epithelial cells. (Rao et al., 2006). In renal epithelial cells, TRPC1 knockdown made the cells to moderately lose their polarity and also the ability to steadily migrate in a given direction (Fabian et al., 2008). Human (Ng 108-15) vascular smooth muscle cells express both TRPC1 and TRPC5 and treatment with antibodies against TRPC5 inhibited S1P-induced migration (Wu et al., 2007), suggesting that TRPC5 mediates the S1P-induced smooth muscle migration (Xu et al., 2006).

2.10. Role of calpain and matrix metalloproteinase-2 (MMP2) activity in migration and invasion

Calcium-dependent proteins, such as calpain also have been shown to regulate cellular migration and invasion (Wells et al., 2005). Calpains, are family of thiol proteases that are widely expressed (Perrin and Huttenlocher, 2002). In general calpains have two putative mechanism of action during migration which includes its role as signaling intermediate and regulation on focal adhesion structure and disassembly (Perrin and Huttenlocher, 2002). Calpain activity is essential in skin wound healing and contributes to scar formation (Nassar et al., 2012). Calpain cleaves and activates the TRPC5 channel to participate in semaphorin 3A-induced neuronal growth cone collapse (Kaczmarek et al., 2012).

Matrix metalloproteinase-2 (MMP-2), a zymogen requiring proteolytic activation for catalytic activity, has been implicated broadly in the invasion of various cells (Thompson et al., 1994). Enhanced translocation of TRPV2 to the plasma membrane results in increase migration in PC-3 cells by induction of key invasion markers like matrix metalloproteinase 2 and 9 (MMP2, MMP9) and cathepsin B (Monet et al., 2010). Calpain is required in cellular invasion as a regulator of MMP2 function (Jang et al., 2010). Expression of dominat negative TRPC6 in human microvasular endotheial cells changes the VEGF –mediated migration and sprouting (Hamdollah Zadeh et al., 2008). Recent studies by Tian et al., (2010) showed that TRPC5 and TRPC6 are critical regulators of cell migration through differential coupling to Rac and Rho A, respectively. The TRPC6-TRPC5 activation cascade is a critical element in the inhibition of endothelial cell migration evoked by lysophosphatidylcholine (Chaudhuri et al., 2008). The constitutive function of native TRPC3 channels modulates vascular cell adhesion molecule-1 expression in coronary endothelial cells (Smedlund et al., 2010).

Calpain has been shown to be a main molecular control point in adhesion of cells to the matrix (Wells et al., 2005). Calpain is also involved in the proteolytic cleavage of the adhesion protein E-cadherin in prostate and mammary epithelial cells (Rios-Doria et al., 2003).

2.11. Thyroid gland, structure and function

The thyroid gland, one of the largest endocrine organs in humans, regulates systemic metabolism (Stathatos, 2012). The primary function of the thyroid gland is to produce hormones T3 (Triiodothyronine) and T4 (Thyroxine), thereby regulating the rate of metabolism. The hormones regulate growth and development of many other organs. The thyroid gland is a butterfly-shaped organ and composed of two lobes. There are three types of cells found in the thyroid; follicular, endothelial and parafollicular cells (Miller, 2003).

2.11.1 Thyroid Stimulating Hormone (TSH) and Thyroid hormone production.

The thyroid stimulating hormone (TSH) is an anterior pituitary hormone which primarily controls the growth and function of thyroid follicular cells and it is regulated by the thyrotropin-releasing hormone (TRH) from the hypothalamus (reviewed by Economidou et al., 2011). By binding to a G-protein coupled receptor, TSH activates

the G_s alpha subunit which leads to intracellular cyclic adenosine monophosphate (cAMP) production (Köhrle, 1990). cAMP mediates the effect of TSH on the function of thyroid follicular cells such as proliferation, differentiation, iodine uptake, synthesis and secretion of hormones. Forskolin, which increases the cAMP production, can mimic the effects of TSH in thyroid follicular cells (Brandi et al., 1984). The role of cAMP and cAMP signaling is explained further.

Thyroid hormone production and release is a complex process as reviewed by Mansourian, (2011). The thyroid hormone biosynthesis starts with the iodine metabolism which occurs in 3 sequential steps (Mansourian, 2011). Iodination of tyrosyl residues of thyroglobulin (Tg) and enzymatic cleavage of Tg are important steps in thyroid hormone biosynthesis. Tg synthesis is regulated by thyroid-specific transcription factors. The ability of thyrocyctes to concentrate iodine is the rate-limiting step in thyroid hormone biosynthesis (Mansourian, 2011). The iodine oxidation of Tg is mediated by the thyrodperoxidase enzyme (TPO) which itself is activated by TSH. T4 and T3 are subsequently synthesized after the iodination. That leads to the enzymatic cleavage of Tg into idotyrosines monoidotyrosine (MIT) and diiodothyrosine (DTT). Thyroid hormones thus produced are released into the circulation through Tg pinocytosis. Deregulation of thyroid hormone production leads to hypothyroidism and hyperthyroidism (Di Jeso et al., 1998). A negative feedback loop monitors the thyroid function, in which the elevated levels of T4 suppresses TSH receptors (Chiamolera and Wondisford, 2009; Mansourian, 2011).

2.12. cAMP –signaling

The adenylyl cyclase pathway or cyclic adenosine monophosphate pathway is activated by G protein coupled receptors and the G_s alpha subunit. Adenylyl cyclase catalyzes the conversion of ATP into cyclic adenosine monophosphate (cAMP). Studies show the presence of nine adenylyl cyclase isoforms in mammals (Cooper, 2003). Among the nine of adenylyl cyclases AC5 and AC6 are inhibited by calcium and AC1 and AC8 are stimulated by calcium (Halls and Cooper, 2011). cAMP acts as an intracellular second messenger. Many of the effects mediated by the thyroid stimulating hormones can be mimicked by the activation of adenylyl cyclase enzymes (Medina and Santisteban, 2000). The increase in cAMP activates protein kinase A (PKA) which in turn modulates various diverse proteins such as transcription factor CREB (cAMP response element-binding protein), p70 ribosomal S6 kinase, P13K and triggers calcium entry (Medina and Santisteban, 2000; Mayr and Montminy, 2001; Gratschev et al., 2004). In thyroid follicular cells, cAMP and growth factor have signaling cross-talk and have positive regulation in the proliferation of cells (Medina

and Santisteban, 2000). cAMP also has signaling cross-talk between MAP kinase signaling and regulates cellular proliferation either by positive or negative regulation of ERK cascades (Stork and Schmitt, 2002). Ras superfamily guanine nucleotide binding protein, Rap1A, directly gets activated by cAMP but in a PKA-independent manner (Kawasaki et al., 1998).

2.13. Fisher rat thyroid low serum- 5% (FRTL-5) cells

The most commonly used rat thyroid cell line is the Fisher rat (FRTL-5) cells which was acquired from a primary culture of rats of the NIH Fisher 344 inbred strain (Ambesi-Impiombato et al., 1980). The primary thyroid cell culture was completely dependent on insulin and TSH for survival and growth (Medina and Santisteban, 2000). The FRTL-5 cells grow in monolayer. The FRTL-5 cells maintain highly differentiated features, such as thyroglobulin (Tg) and TPO synthesis, iodine uptake and hormone dependence, typical of the thyroid phenotype (Medina and Santisteban, 2000). TSH, insulin and IGF-1 are the main regulators of FRTL-5 cell growth and function (Dumont et al., 1992). The simplicity of the FRTL-5 cell system allows stable transfections and genetic experiments, which make them the preferred model in most of the in vitro studies of thyroid biology (Medina and Santisteban, 2000).

3. AIMS

The main aim of this study was to elucidate the importance and role of TRPC2 in FRTL-5 cell function.

The specific aims were:

1. To study the importance of the TRPC2 channel as a regulator of calcium homeostasis.
2. To discover what function TRPC2 has in thyroid cell signaling and is there communication between the calcium and cAMP-mediated signaling pathways.
3. To study the importance of the TRPC2 channel in cellular processes such as proliferation, migration, invasion and adhesion.

4. MATERIALS AND METHODS

The materials and methods used for the study are briefly described. Additional information can be found in the original publications and manuscripts, which are referred to by their Roman numerals.

4.1. Cell culture

The Fischer rat thyroid cell line FRTL-5, originally from the Interthyr Foundation (Bethesda,MD) were grown in Coon's modified Ham's F12 medium supplemented with 5% newborn calf serum, transferrin (5 µg/ml), hydrocortisone (1nM), TSH (0.3 mU/ml), insulin (10µg/ml), somatostatin (10ng/ml), gly-L-his-L-lys (10ng/ml) and 50U/ml penicillin and 50µg/ml streptomycin. The cells were kept in a water-saturated atmosphere of 5% CO2 and 95% air at 37°C.

4.2. Generation of stable TRPC2 knockdown cell lines

FRTL-5 cells were transfected using Fugene HD (Roche, Basel, Switzerland) and shRNA plasmids (SABiosciences, Frederick, MD, USA). The shRNA plasmids were designed to specifically knock down the expression of individual genes by RNA interference and create a stable puromycin resistant knockdown cell line. The transfection was done according to the manufacturers' instructions. 48 h post-transfection untransfected cells were removed with 1 µg/ml puromycin. Hence, the medium for the TRPC2 knockdown cells and their controls contained puromycin (1µg/ml) from here on. Out of the four shRNA plasmids provided by the manufacture the best knockdown construct (measured using qPCR) was used for further studies. The representative sequence used to design the shRNA is GGAATCTCATTCGATGCATAC for Negative control (shC cells) and CAGCAATGCGCGCGCCGARARGACC for the TRPC2 knockdown (shTRPC2 cells) (**I, II, III**).

4.3. Measurement of intracellular calcium

4.3.1. Measurement of $[Ca^{2+}]_i$: cell suspension experiments

Cells were detached using EGTA and trypsin and harvested with HBSS buffer. The cells were incubated with 1 µM Fura 2-AM for 30 min at 37°C in HBSS, and the

fluorescence was measured with a Hitachi F2000 fluorometer (excitation at 340 and 380 nm and emission at 510 nm). The signal was calibrated by the addition of 1 mM $CaCl_2$ and Triton X-100 to obtain maximum fluorescence and 5 mM EGTA and Tris base to obtain minimum fluorescence by chelating the extracellular calcium. $[Ca^{2+}]_i$ was calculated using a computer program designed for the fluorometer with a Kd value of 224 nm. All the experiments were repeated 4-6 times and at least 3 different batches of cells were used.

4.3.2. Measurement of $[Ca^{2+}]_i$: Single cell-imaging experiments

Cells were grown for 48 hrs in 35-mm cell culture dishes on polylysine-coated coverslips. The cells were then washed in HBSS and incubated with Fura 2-AM (final concentration 2 μM) for 30 min at 37°C. After washing the cells, the coverslip was mounted on an Axiovert 35- inverted microscope. The excitation filters were set at 340 nm and 380 nm. Excitation light was obtained using an XBO 75W/2 xenon lamp. Emission was measured at 510 nm. Fluorescence images were collected with a SensiCam 12BIT charge-coupled-device (CCD) camera. Images were taken every 3 s to avoid bleaching and the images were processed using Axon Imaging Workbench (6) software. The results are given as the 340 nm/380 nm ratio. All the data shown are calculated from the changes in $[Ca^{2+}]_i$ from all cells measured, i.e. both responding and non-responding cells (**I**, **II**, **III**).

4.5. Confocal microscopy and image analysis

The cells were transfected and cultured for 48 hrs. After incubating with 0.1mM EGTA for 10 min, the coverslips were washed three times with PBS, fixed using 4% PFA and mounted on a slide with Mowiol. Fifteen confocal optical sections with optimized image acquisition settings were acquired of each sample. All images were acquired with exactly the same settings and in one continuous session. All images were taken of the cell membrane near the glass surface. Experiments were performed with AxioObserver Z1 (63 × /1.4 oil immersion objective) equipped with LSM510 (Carl Zeiss Inc., Jena, Germany). Image analysis was carried out with the BioImageXD software. The final results were statistically analyzed with a two-tailed heteroscedastic t-test (**II**).

4.6. Cell transfection using electroporation

FRTL-5 cells (4,000,000) were transfected with 5 µM siRNA using electroporation with an exponential protocol of 300 voltages and a capacitance of 975 µF using a Bio-

Rad Gene Pulser Xcell (Bio-Rad Laboratories). The transfected cells were plated on 35-mm dishes and were used for calcium measurement and other experiments after 48 hrs (**I, II and III**).

4.7. Cell Proliferation Assay

4.7.1. [^{3}H]-thymidine incorporation assay

FRTL-5 cells (100,000) were plated on 35-mm plates and grown for the times indicated, with the presence of 0.4 mCi/ml ^{3}H-thymidine during the last 4 hrs. The assay was terminated by washing the cells three times with ice-cold PBS. DNA was precipitated with ice-cold 5% trichloroacetic acid (TCA). The precipitate was dissolved in 0.1 M NaOH, and mixed with Optiphase Hisafe 3 scintillation liquid. Radioactivity was measured using a Wallac 1410 liquid scintillation counter (**III**).

4.7.2. Cell counting assay

Cells from different time points were stained with trypan blue and counted using haemocytometer chamber. The chamber was viewed with 40X magnification under a light microscope (**III**).

4.8. Fluorescence-activated cell sorting (FACS) analysis

Cells were detached using EDTA-trypsin solution and washed with PBS. The cells were incubated for 15 minutes with propidium iodide (0.05 mg/ml propidium iodide, 3.8 mM sodium citrate, 0.1 % triton x-100 in PBS) at room temperature. The samples were then analyzed by flow cytometry using FACSCalibur and CellQuest Pro software (BD Biosciences, San Jose, CA, USA) (**III**).

4.9. Invasion Assay

For invasion experiments the membranes were coated with 5 µg/cm^2 collagen IV, and reconstituted with serum-free medium for 1 h at 37°C prior to the experiment. A 200 µl portion of cell suspension was added to the upper well and 800 µl of serum-containing

media was added into the lower well. The cells were allowed to migrate for 16 hrs. The assay was terminated and unmigrated cells were wiped off with a cotton swab. The migrated cells were fixed in paraformaldehyde, stained with crystal violet and counted using light microscope (**III**).

4.10. Wound healing assay.

Cells were grown on a 12-well plate until 95% confluency and 0.5 µg/ml of mitomycin C was added to inhibit further proliferation of the cells. The cell monolayer was scratched using a 200 µl pipette tip and images were taken using a Leica microsystems camera and frame work software (Leica Microsystem Wetzlar, Germany) immediately after the scratch, marked as 0 hr and after 24 and 48 hrs (**III**).

4.11. Cell adhesion assay

To perform cell adhesion experiments 200,000 cells were plated on collagen IV (5µM/cm^2) coated 96-well plates for 24 hrs and incubated with serum at 37°C. The cells were allowed to attach for 3 hrs. The cells were fixed with 70% (v/v) ethanol for 20 min and stained with 1mg/ml Crystal Violet in 20% (v/v) methanol for 10 min. After washing with water the wells were allowed to dry and the Crystal Violet was dissolved in 10% (v/v) acetic acid and the absorbance was measured at 570 nm (**III**).

4.12. H_2O_2 measurement

For H_2O_2 production measurement, the homovanillic acid fluorescence assay was used (Guilbualt et al., 1967). Cells were grown on 60-mm plates in 6H until confluent, and then with 5H medium without TSH (5H) for 5 days. On the day of the experiment the cells were washed with HBSS, and stimulated with 100 µM ATP in HBSS containing 0.44 mM homovanillic acid, 0.5 units/ml horseradish peroxidase and 0.01 % NaN_3 and incubated for 2 hrs at 37°C. The experiment was terminated by placing the plates on ice. Fluorescence was measured with a Hitachi 2000 fluorimeter (excitation at 315 nm and emission at 425 nm) (**unpublished data**).

4.13. ^{125}I uptake

The assay was made according to the protocol given by Weiss et al., (1984). 80% confluent cells were used for ^{125}I uptake assay. The cells were washed three times with HBSS, they were then incubated for 1h with HBSS containing 0.1 µCi ^{125}I and 10 µM

NaI at 37°C to allow iodine uptake. Washing three times with ice-cold HBSS terminated the experiment. To distinguish between active uptake and background, a potent NIS inhibitor (NaClO4, 10 µM) was included as control (Radović et al., 2005). The cells were solubilized with 0.1 M NaOH and radioactivity was measured. The radioactivity measured was normalized to protein content.

4.14. Western blotting

Cells were lysed in 200 µl of lysis buffer and the lysates were incubated on ice for 15 minutes and centrifuged at 10,000 x g for 15 minutes. Proteins were separated by 6-15 % SDS-PAGE, depending on the size of the protein of interest, and then transferred onto a nitrocellulose membrane. Membranes were incubated with primary antibody of interest and HRP-linked secondary antibody. Protein bands were detected using enhanced chemiluminescence (**I, II, III**).

4.15. Qualitative RT-PCR

RNA was isolated using the Aurum Total RNA Mini Kit and the RNA quality and integrity was tested by absorbance spectrometry and by agarose gel electrophoresis. Reverse transcriptase reactions were performed on 0.25 mg RNA using SuperScript III Reverse Transcriptase and Oligo(dT)$_{15}$ primers following the manufacturer's instructions. The PCR was performed in 50 µl reactions (5 µl cDNA, 1 mM primers, 200 nM each of dATP, dCTP, dGTP and dCTP, 0.5 U DynaZyme EXT DNA polymerase) on a Mastercycler gradient thermal cycler with activation step at 94°C for 5 min followed by 30 cycles with strand separation at 94°C for 30 s and annealing for 60s. The PCR products was separated by gel electrophoresis and visualized with ethidium bromide under UV-light. (**I, II, III**).

4.16. Primary thyroid cell isolation

Rat thyroid cells were isolated according to Eggo et al., (1996). Rat thyroid tissue was digested with 0.2% collagenase, and follicles were plated on poly-L-lysine coated culture dishes in Coon's modified Ham's F12 medium supplemented with 1% serum, TSH and insulin to allow the cells to attach. After 3 days, the medium was changed to serum-free Coon's modified Ham's F12 medium with 0.3 mU/ml TSH and 1µg/ml insulin to promote thyroid cell survival (**I**).

4.17. Quantitative real-time RT-PCR

qPCR assays were designed using the Universal ProbeLibrary Assay Design Center. The probes for TRPC2 and Tg were obtained Universal ProbeLibrary. ACTB and GAPDH were used as reference genes. The ACTB and GAPDH probes contained in addition to the fluorescent FAM dye also a TAMRA quencher. qPCR rections of 10 µl were used, containing 100 nM UPL probe (for TRPC2 or Tg) or 200 nM normal length probe (for ACTB or GAPDH), 300 nM forward and reverse primers, 1x ABsolute QPCR Rox Mix (Abgene) and cDNA. The Absolute QPCR Rox Mix contains Thermo-start DNA polymerase, proprietary reaction buffer; dNTP's and ROX reference dye. The cDNA was diluted 1/10 with MQ and 1 µl of the dilution was used per reaction. The qPCR was performed with the Applied Biosystems 7900HT Fast Sequence Detection System with a 15 min activation step at 95°C and 40 cycles with a strand separation step in 95°C for 15 s and an annealing and elongation step at 60°C for 1 min. The amplification results were analyzed using SDS and RQ Manager Programs (**I, II, III**).

4.18. SERCA activity assay

For measuring SERCA activity the cells were grown on 35-mm plates, harvested, lysed using lysis buffer and centrifuged. The precipitate was sonicated in HBSS buffer. SERCA activity was measured according to the manufacturer’s instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing City, P.R China). The colorimetric reading at 590 nm was normalized with the respective total protein concentrations (**I**).

4.19. Calpain activity assay

2,000,000 cells were grown on 35-mm plates and the cells were treated with 10 µM ATP for 30 minutes. The cells were lysed and the supernatant was used for protein concentration measurement using enhanced chemiluminescence. The cells were lysed and were diluted using extraction buffer and the activation was measured according to the manufacturer’s instructions (Abcam, Boston, MA). The colorimetric reading at 590nm was normalized with the respective total protein concentrations

4.20. Electrophysiology

FRTL-5 cells were plated at low density on to 13-mm coverslips kept in 24-well plates and grown in Coon´s 6H medium as above for 2-6 days before the experiments.

Recordings were done at room temperature of 23-25°C in a recording chamber (volume 200 µl) and continuously perfused (flow rate 700 µl/min).

Whole-cell membrane currents (Hamill et al., 1981) were recorded with an EPC–9 amplifier and Pulse software (HEKA Elektronik, Lambrecht, Germany). Currents were allowed to stabilize for at least 5 minutes before the experiments were started. The voltage-clamp protocol used had three segments (step-ramp-step): a 50-ms step to a holding voltage (V_h) of –90 mV was followed by an ascending ramp of 200 ms in duration (+0.5 mV ms^{-1}), after which the end voltage of 10 mV was maintained for 50 ms. The protocol was applied at 2-s intervals, the cells were held at −10 mV between the stimulation. Whole-cell capacitance and series resistance were at least 80% compensated for, membrane currents were sampled at 2 kHz and low pass filtered off-line at 1 kHz (**I, II, III**).

4.21. Statistics

All the experiments were repeated at least three times. A two-tailed *t*-test was used for comparison of a single experimental group against the control. For experiments where three or more groups were compared, one-way ANOVA was done. A P-value of less than 0.05 is considered significant (**I, II, III**).

5. RESULTS AND DISCUSSION

5.1. Expression of TRPC2 in FRTL-5 cells and primary rat thyroid cells (I and II)

In the present studies, we show that, of the TRPC family of cation channels, only TRPC2 was expressed in rat thyroid FRTL-5 cells. In rodents, TRPC2 was thought to be exclusively expressed in the vomeronasal organ (Liman et al., 1999; Birnbaumer, 2009). TRPC2 was found in both primary rat thyroid cells (isolated according to Eggo et al., 1996) and in freshly isolated rat thyroids (I, Figure 1A and II, Figure 1A). We also studied the splice variants of TRPC2 expressed in FRTL-5 cells. Based on the PCR products, FRTL-5 cells express only the F1MAE4 and Novel splice variants (II, Figure 1B and 1C). When we compared different rat TRPC2 variant sequences available, we observed that the F1MAE4 sequence of the Ensembl database is nearly identical to the rat TRP2 sequence cloned from the rat vomeronasal organ (Liman et al., 1999; GenBank accession number AF136401). The splice variant F1MAE4 may work similarly to the mouse TRPC2 variant smTRPC2, (Chu et al., 2005). Novel represents a short N-terminal sequence of the predicted full length TRPC2 and due to minimal trans-membrane structure is perhaps unlikely to form a functional channel (II, Supplementary Figure 1A).

5.2. Knockdown of TRPC2 in FRTL-5 cells

To investigate the physiological importance of the channel, we developed stable TRPC2 knockdown cells using short hairpin RNA (shTRPC2). On the mRNA level, the knockdown was 90% as measured with quantitative real-time PCR (I, Figure 1B) and the knockdown was 66% on the protein level (II, Figure 1D). In order to study the specificity of the antibody used, we transfected TRPC2 to HEK-293 cells. HEK cells have no endogenous TRPC2 expression, but after transfections, a band of a correct size was detected (II, Figure 1E).

Neither sodium/iodide symporter (NIS) nor TPO expression was affected by TRPC2 knockdown. The expression of thyroid-specific transcription factor TTF-1 and paired box gene 8 (PAX8) were also not altered (I, Supplementary Figure 2). Moreover the total Tg expression in control (shC) shTRPC2 cells were equal (I, Figure 6A). These results indicates that knockdown of TRPC2 did not dedifferentiate the FRTL-5 cells.

5.3. TRPC2 mediates calyculin A-evoked calcium entry (unpublished data)

Previous studies in our lab detected a novel calcium entry mechanism in FRTL-5 cells that was dependent on a phosphatase and protein kinase A (Gratschev et al., 2004, 2009). This pathway is unmasked by the phosphatase inhibitor calyculin A (caly A). This calcium influx was mediated by a DAG-activated channel (Gratschev et al., 2009). The caly A-evoked calcium entry was studied in the shTRPC2 cells. The caly A-evoked calcium entry was attenuated in the shTRPC2 cells when compared to shC cells (Figure. 2A and 2B), indicating that TRPC2 mediates the caly A-evoked calcium entry.

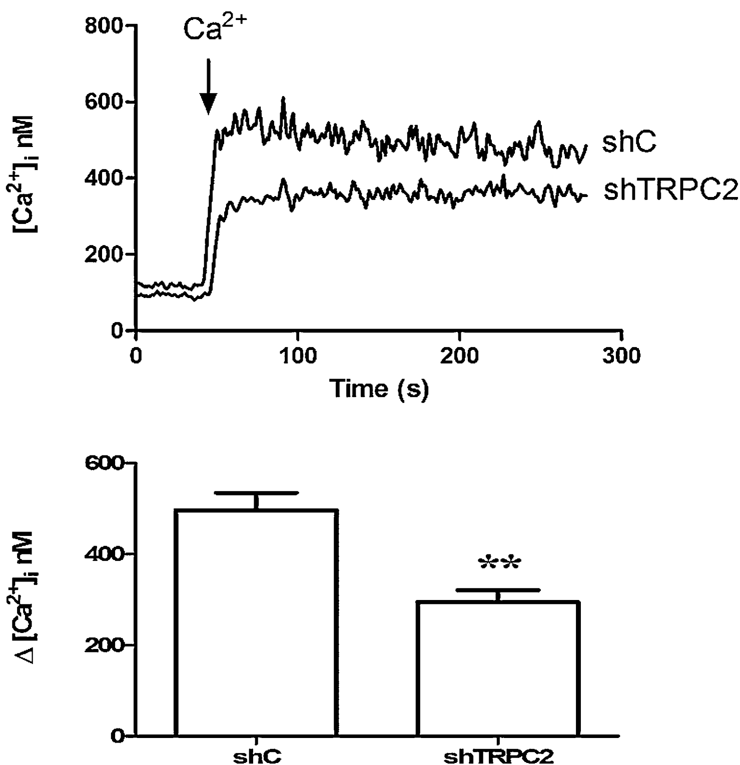

Figure 3. TRPC2 knockdown attenuates caly A-evoked increase in $[Ca^{2+}]_i$. A. Representative traces of control (shC) cells and TRPC2 knockdown (shTRPC2) cells pretreated with 100 nM caly A for 15 min, and resuspended in calcium-free buffer. The arrow indicates where calcium (final concentration 1 mM) was added. B. Bar diagram showing a summary of 6 experiments as described in (A). Each bar gives the mean ± SEM. **, $P < 0.01$.

5.4. TRPC2 knockdown attenuated the ATP-evoked calcium peak amplitude and ATP-evoked inward current in FRTL-5 cells (I and II)

ATP is a well-known agonist that evokes calcium transients in FRTL-5 cells (Okajima et al., 1987; Törnquist, 1992). However, the channels responsible for ATP-evoked calcium entry in FRTL-5 cells are unclear. TRPC2 have been shown to be activated downstream of purinergic receptor activation (Jungnickel et al., 2001). As FRTL-5 cells express several purinergic receptors (Ekokoski et al., 2001), we used ATP as an agonist to determine if TRPC2 is functional in FRTL-5 cells. When stimulating shC and shTRPC2 cells with ATP in a calcium-containing buffer there was a marked reduction in the ATP-evoked calcium peak amplitude in shTRPC2 cells (I, Figure 1C, and II, Figure 2A and Figure 2B). There are reports showing that reduction of the expression of TRPC3 affects mobilization of calcium from intracellular stores (Bandyopadhyay et al. 2008). However, we saw no differences in the ATP- evoked calcium peak amplitude in shC and shTRPC2 cells in a calcium-free buffer (II, Figure 3A and Figure 3B). Thus, a decreased expression of TRPC2 in FRTL-5 cells does not affect the mobilization of intracellular calcium from the ER. In sperm, which endogenously express TRPC2, an antibody against TRPC2 decreased both thapsigargin- and zona pellucida 3-induced calcium entry (Jungnickel et al., 2001). Inward currents were increased in HEK-293 cells, transiently overexpressing TRPC2 after stimulation of purinergic receptors with ATP (Jungnickel et al., 2001). Patch-clamp experiments revealed a pronounced reduction in the ATP-evoked inward current in shTRPC2 cells, compared with shC cells (II, Figure 2D).

5.5. TRPC2 as a receptor-operated channel in FRTL-5 cells (II)

In thyroid cells, including rat thyroid FRTL-5 cells, several investigations have shown that changes in $[Ca^{2+}]_i$ regulates a multitude of central processes. This includes the regulation of iodide efflux (Corda et al., 1985; Berman et al., 1987; Freichel et al., 2001), and the regulation of both proliferation and synthesis of DNA (Törnquist et al., 1996; Ekokoski et al., 2001).

TRPC2 can mediate both ROCE and SOCE, (Villereal, 2006; Yildirim and Birnbaumer, 2007). ROCE pathways are present in the FRTL-5 cells, they express several P2X ionotropic receptors (Ekokoski *et al.*, 2001). Many agonists, e.g. ATP, ADP, UTP, sphingosine 1-phosphate (S1P) and also TSH, evoke potent changes in intracellular free calcium through activation of G-protein coupled receptors in FRTL-5

cells (Raspé et al., 1991; Sho et al., 1991; Törnquist, 1992; Okajima et al., 1997; Törnquist et al., 1997; Grasberger et al., 2007). ATP-induced calcium entry and OAG (DAG analogue) -induced calcium entry was hampered in shTRPC2 cells (II, Figure 2A and Figure 5B). These results suggest that TRPC2 can act as a receptor-operated calcium channel in FRTL-5 cells. Studies with the SERCA inhibitor, thapsigargin-revealed that there is no difference in SOCE between shC and shTRPC2 cell (II, Figure 3E). Thus, in our cells the activation of TRPC2 seems to be exclusively receptor-mediated.

5.6. Importance of TRPC2 in regulating the basal calcium influx and ER calcium content in FRTL-5 cells (II).

TRPC2 can bind to the IP_3 receptor, either directly (Lucas et al., 2003; Yildirim et al., 2003; Yuan et al., 2003; Tong et al., 2004) or through Homer1 (Stiber et al., 2008; Mast et al., 2010). In our cells, TRPC2 is apparently not involved in the receptor-evoked release of calcium from the ER, as we did not observe any differences in the ATP- evoked calcium peak amplitude in shC and shTRPC2 cells when stimulated with ATP in a calcium-free buffer (II, Figure 3A). However, a careful analysis showed that in calcium-free buffer, the area under the curve of both the ATP-evoked and Thapsigargin-evoked calcium release was slightly, but significantly, smaller in shTRPC2 cells, compared with shC cells (II, Figure 3A and Figure 3E). Readdition of calcium to cells in a calcium-free buffer resulted in an enhanced calcium influx in shTRPC2 cells, compared with shC cells (II, Figure 4A). This suggests that TRPC2 may participate in regulating ER calcium content.

Recent investigations have suggested that the ER calcium sensor STIM2 is the master regulator of basal calcium entry, whereas STIM1 is of importance in SOCE (Huang et al., 2006; Brandman et al., 2007). We expressed STIM1-cherry and STIM2-YFP in our cells to determine if there is a difference in translocation of these proteins to the plasma membrane. The confocal images obtained were analyzed with BioImageXD software (Kankaanpää et al., 2012). In these experiments, no effect was seen on the translocation of STIM1-cherry but we observed a significant increase in STIM2-YFP puncta in shTRPC2 cells (II Figure 7A and Figure 7B). Increased STIM2 puncta in shTRPC2 cells suggests that ER calcium stores may be affected.

5.7. PKC regulates the basal calcium influx in FRTL-5 cells (II)

PKC is a regulator of calcium entry in several cell types (Mellor and Parker, 1998). Recent studies show that PKCβI phosphorylates Orai1, which attenuate SOCE (Kawasaki et al., 2010). PKC plays an important role in the translocation of STIM1 (Kawasaki et al., 2010). Previous studies (Park et al., 2002; Ramström et al., 2004) have revealed the expressions of PKC isoforms α, βI, βII, γ, η, ε, ζ and δ in FRTL-5 cells. The expressions of these isoforms were studied in shC and shTRPC2 cells (II, Figure 6A). In shTRPC2 cells, the expression of PKCβI and PKCδ were decreased (II, Figure 6A). By reducing the expression of PKCβ1 and PKCδ in native FRTL-5 cells, basal calcium entry was enhanced (II, Figure 8B).

SERCA function may be regulated by PKC (Colyer, 1998). In our cells, both the SERCA activity and PKCβ1 and PKCδ expression is clearly decreased in shTRPC2 cells, compared to the shC cells (II, Figure 8E). The decreased SERCA activity probably explains the smaller ER calcium content in shTRPC2 cells. This, in turn may also induce STIM2 puncta formation, and an increased basal calcium entry in shTRPC2 cells.

5.8. TRPC2 regulates cAMP production (I).

Stimulating the thyroid stimulating hormone receptor (TSHR) will activate $G\alpha_s$, which results in increased cAMP production by activating the adenylyl cyclases (Raspé et al., 1991; Kimura et al., 2001; Rivas and Santisteban, 2003). By knocking down the expression of TRPC2 with shRNA, we saw a marked increase in agonist-induced production of cAMP (I, Figure 2A). The TSHR was upregulated both on mRNA and protein level in shTRPC2 (I, Figure 2C and Figure 2D). Adenylyl cyclases AC5 and AC6, which are inhibited by calcium, are present in FRTL-5 cells (Tsuzaki et al., 1991; Bell et al., 2002) (I, Figure 2F). When the intracellular calcium was chelated using BAPTA, there was no difference in cAMP production in shc and shTRPC2 cells. This could imply that calcium transients mediated by TRPC2 attenuates the cAMP production by inhibiting adenylyl cylase 5 and 6. Increased TSHR could partly also explain the increase in cAMP production in shTRPC2 cells.

We investigated the phosphorylation of ERK1/2 in shTRPC2 and shC cells. The basal phosphorylation of ERK1/2 was increased in shTRPC2 cells (I, Figure 3A). Chelating the extracellular calcium by EGTA also increased the ERK1/2 phosphorylation and TSHR expression in wild type FRTL-5 cells (I, Figure 4A and 4B). TSH can increase the phosphorylation of ERK1/2 through cAMP/Rap1 (Iacovelli et al., 2001). Expression of a constitutively active mutant of MEK1 in wild type FRTL-

5 cells increased the TSHR expression (I, Figure 4F). Taken together, we propose a model where communication between the calcium and cAMP pathways regulate the expression of TSHR and TRPC2 plays a key role in regulating this communication.

5.9. Regulation of glycosylation and secretion of thyroglobulin by TRPC2 (I).

Calcium is necessary for the maturation and dimerization of thyroglobulin (Tg) (Di Jeso et al., 1997). Di Jeso et al., (1998) showed that, interfering with calcium signaling in FRTL-5 cells interferes with the glycosylation, dimerization and secretion of Tg. We investigated the expression of Tg in shC and shTRPC2 cells. A marked decrease in thyroglobulin (Tg) secretion was shown in shTRPC2 cells (I. Figure 6A). The total Tg expression on protein and mRNA level in shC and shTRPC2 cells were equal (I, Supplementary Figure 2H). In shTRPC2, we detected an additional lower molecular weight band on the western blot, which we hypothesized to be an immature form of Tg (I, Figure 6A). Treatment with 1µM Tunicamycin, (inhibitor of GlcNAc phosphotransferase, which catalyses the first step in N-Linked glycoprotein synthesis) resulted in mobility shift of all Tg bands on western blots. This indicates that the additional lower molecular weight band in shTRPC2 was glycosylated (I, Figure 6C). The additional lower molecular weight band in shTRPC2 cells was sensitive to EndoH treatment, suggesting that the immature form of Tg is retained in the ER in shTRPC2 cells (I, Figure 6D). Attenuated Tg secretion was observed in shTRPC2 cells when the secreted Tg from the shC and shTRPC2 cells were immunoprecipitated with an anti-Tg antibody and (I, Figure 6E). Hence, our results show that decreasing the expression of TRPC2 had major effects Tg maturation and secretion.

5.10. Knockdown of TRPC2 reduces cellular proliferation (III)

Calcium regulates various cellular processes including proliferation (Croall and DeMartino, 1991). Knockdown of the TRPC2 channel in rat thyroid FRTL-5 cells affected cellular proliferation (III, Figure 1A). Several other members of the TRPC family, mainly TRPC1 (Li et al., 2012) and TRPC3 (Woo et al., 2010) are important for regulating proliferation (Abramowitz and Birnbaumer, 2009). We observed an accumulation of shTRPC2 cells in the G1/S phase of the cell cycle when analyzed using FACS (III, Figure 1B). The G1 phase progression is actually inhibited if cellular cAMP is maintained at a high level (Villone et al., 1997). It is thus feasible that the

accumulation of shTRPC2 cells in the G1 phase is the result of both an increased production of cAMP and decreased calcium entry. In shTRPC2 cells the tumor suppressor p53, cyclin-dependent kinase inhibitors p27 and p21 were upregulated, which may also have resulted in decreased proliferation in these cells (III, Figure 1E-H). The phosphorylation of cdk6, cyclin D1, cyclin D2 and Erk 1/2 (I, Figure 4A and III, Supplementary Figure 1A) were upregulated in shTRPC2 cells. This may likely be a rescue effect to keep the cells proliferating, as (Cheng et al., 1998) reported that increased levels of cyclin D1-cdk complexes sequester p27, relieving the inhibitory effect of p27 and thus allowing a progression of the G1 phase. In conclusion, TRPC2 is important for the proliferation of FRTL-5 cells.

5.11. TRPC2 knockdown affects cellular migration and invasion (III)

Calcium-dependent proteins, such as calpain have also been shown to regulate cellular migration and invasion (Wells et al., 2005). Calpain activity is essential in skin wound healing and contributes to scar formation (Nassar et al., 2012). Calpain is also involved in the proteolytic cleavage of the adhesion protein E-cadherin in prostate and mammary epithelial cells (Rios-Doria et al., 2003). Calpain cleaves and activates the TRPC5 channel to participate in semaphorin 3A-induced neuronal growth cone collapse (Kaczmarek et al., 2012). Calpain also regulates the MMP2 activity (Jang et al., 2010). In shTRPC2 the decreased secretion and activity of MMP2 may be due to less activity of calpain (III, Figure 4G and 4 B-E). Reduced calpain activity in shTRPC2 cells may have resulted in reduced invasion, migration and adhesion in shTRPC2 cells (III, Figure 3A-E).

5.12. Knockdown of TRPC2 increases iodine uptake

In FRTL-5 cells, the iodine uptake is rapidly enhanced in response to TSH and purinergic stimulation, which all increase the intracellular calcium concentration $[Ca^{2+}]_i$ indicating a strong interaction between $[Ca^{2+}]_i$ and iodine release (Weiss et al., 1984; Corda et al., 1985; Köhrle, 1990). The iodine uptake studies were done using radioactive I^{125} as described by Weiss et al., (1984). The results showed an increased uptake of I^{125} in shTRPC2 cells than in shC cells. To distinguish between active uptake and background, a potent NIS inhibitor (NaClO4, 10 μM) was included as control (Radović et al., 2005). These results were correlated with a lower calcium activated

chloride current (CaCC) in shTRPC2 cells which effected the iodide homeostasis (Viitanen et al., 2012).

5.13. H_2O_2 production is not affected by TRPC2 knockdown (unpublished data).

Calcium is an important factor in regulating hydrogen peroxide production (Björkman and Ekholm, 1984) and H_2O_2 is needed for the iodination of Tg (Chiamolera and Wondisford, 2009). We did not observe any differences in basal production of H_2O_2, nor in response to 10 µM ATP or 5 µM forskolin, in shTRPC2 cells compared to shC cells (Figure 3). H_2O_2 is required for thyroid peroxidase (TPO) which catalyses the thyroid hormone formation (Ohye and Sugawara, 2010). We could not detect a difference in TPO expression between shC and shTRPC2 cells. Taken together, the knockdown of TRPC2 did not affect H_20_2 production.

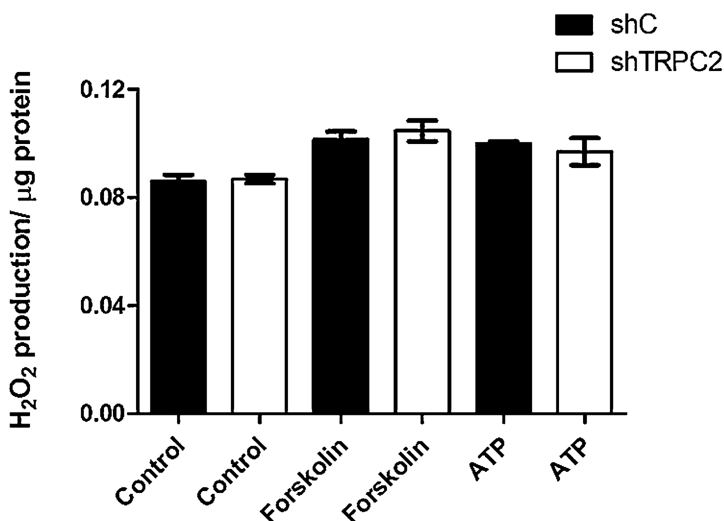

Figure 4: H_2O_2 production measurement - H_2O_2 production was measured in shC and shTRPC2 cells, with and without stimulation with 10 µM ATP or 5 µM forskolin. Bar diagram gives the mean ± SEM of three separate experiments.

5.14. The effect of TRPC2 knockdown was reversible (I, II and III).

To conclude that the effects seen by knocking down the TRPC2 channels in FRTL-5 cells were specific, we conducted a series of rescue experiment where exogenous TRPC2-GFP were added back to the shTRPC2 (shTRPC2-TRPC2) cells. Studies were also done with expression of a dominant negative form of TRPC2 in wild type FRTL-5 cells (TRPC2-DN). Differences in ATP-evoked calcium peak amplitude in shC and shTRPC2 cells were rescued in shTRPC2-TRPC2 cells (II, Figure 2A I, Figure 7A). ATP-evoked calcium peak amplitude and current were attenuated in TRPC2-DN cells (III, Figure 6E and 6F). The increased phosphorylation of ERK1/2 and increased TSHR expression was successfully reversed by exogenous expression of TRPC2 and RTP1 in shTRPC2 cells (I, Figure 7A-H). Decreased invasion and proliferation was observed in TRPC2-DN cells when compared to the control (III, Figure 2B and 6E). The attenuated effect on proliferation and invasion was rescued in shTRPC2-TRPC2 cells (III, Figure 2A and Figure 2F). Taken together, all the effects observed by knocking down the TRPC2 channel in FRTL-5 cells could be reversed by TRPC2-GFP expression in shTRPC2 cells and could be mimicked by TRPC2-DN expression in wild type FRTL-5 cells.

6. CONCLUSIONS

Based on the results obtained in three original publications, the following conclusions may be drawn:

TRPC2 channels in FRTL-5 cells function as receptor-operated channels and knockdown of TRPC2 channels attenuated the ATP-evoked calcium peak amplitude in FRTL-5 cells. TRPC2 is important in regulating the cytosolic basal calcium levels and ER calcium content in FRTL-5 cells. Thereby, TRPC2 is an important regulator of calcium homeostasis in FRTL-5 cells.

TRPC2 regulates cAMP production by inhibiting AC activity. The increased cAMP in shTRPC2 cells results in activation of Rap1 and the MEK/ERK1/2 pathway. The expression of the TSHR is increased due to activation of the ERK1/2 pathway. TRPC2 also regulates the glycosylation and secretion of thyroglobulin in FRTL-5 cells.

Knockdown of TRPC2 decreased calpain and MMP2 activity, which decreased the migration and invasion of shTRPC2 cells. Furthermore, the proliferation in shTRPC2 cells was attenuated due to a prolonged G1/S cell cycle phase.

The effects of TRPC2 knockdown were reversible by expressing TRPC2-GFP in shTRPC2 cells and the effects seen in shTRPC2 cells could be mimicked by TRPC2-DN expression in wild type FRTL-5 cells.

Collectively, we show for the first time the presence of TRPC2 as the only TRPC channel present in rat thyroid FRTL-5 cells and the results give novel insight into how TRPC2 channels function in rat thyroid FRTL-5 cells. Figure 5 represent the schematic presentation of the collective results shown in the Thesis.

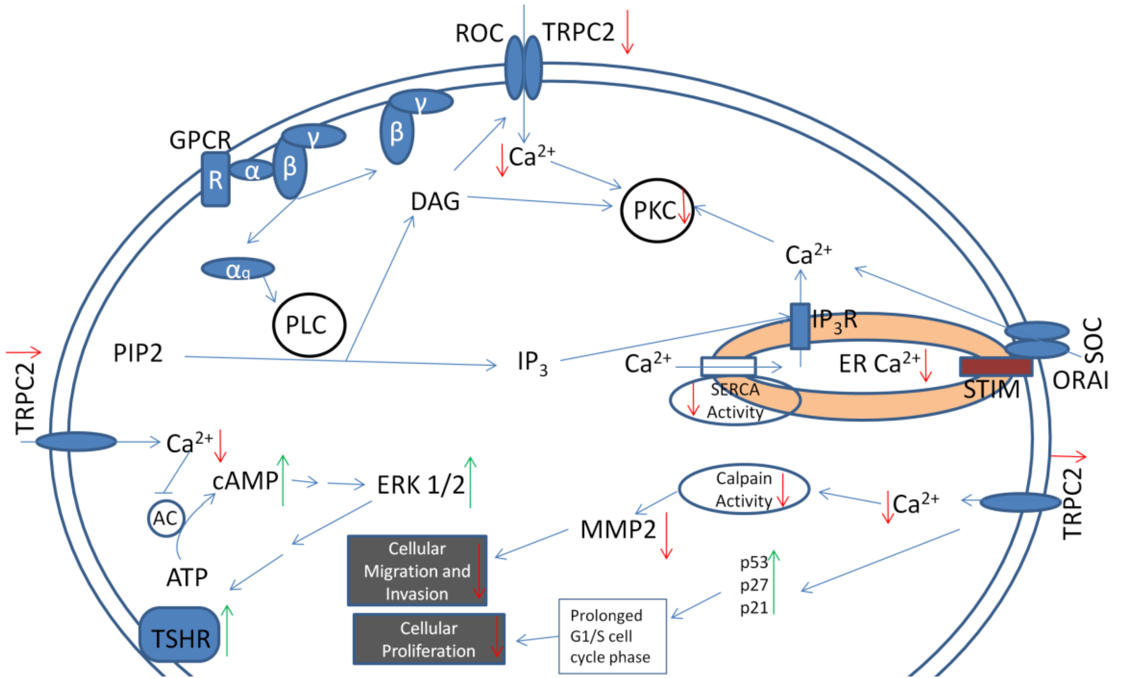

Figure 5: Schematic representation of the results presented in the Thesis. Red arrows indicate a downregulation of expression or reduction in the activity and green arrows indicate an upregulation of expression or increased activity.

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Åbo

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