

TR(i)P, TR(i)P, HOORAY: NEW FUNCTIONS FOR TRPC2 AND TRPC3

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To my family

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ABSTRACT

Calcium signaling affects almost every aspect of cell biology, from fertilization of the egg cell to growth and differentiation of all cell types. The transient receptor potential canonical (TRPC) channels are calcium permeable cation channels that are activated downstream of G-protein coupled receptors through various mechanisms. Effects for TRPCs in regulation of proliferation and cell movement have been reported. Based on this, the TRPCs might have potential roles in diseases.

The results presented here show novel functions for TRPC2 and TRPC3. Overexpression of TRPC3 revealed a possible role for this channel as an intracellular calcium release channel in the endoplasmic reticulum. In contrast to some reports, TRPC3 was not involved in store-operated calcium entry. However, TRPC3 was activated directly by a diacylglycerol-analog, which implies that the channel is putatively involved in receptor-operated calcium entry.

TRPC2 has important functions in regulating pheromone-sensing and social behavior in mice. Here we show that TRPC2 is involved in autocrine sphingosine-1-phosphate (S1P) signaling. S1P produced in the cells by sphingosine kinase 1 was transported to the extracellular space where it activated S1P₂-receptors and calcium entry through a calcium channel. Results indicate that TRPC2 most likely was responsible for this calcium entry. Moreover, TRPC2 was found to have important downstream effects in a thyroid cell model. TRPC2 negatively regulates cAMP production in the cells, which suppress the extracellular signal-regulated kinase (ERK) pathway. When TRPC2 expression is reduced the activity of the cAMP/ERK pathway is enhanced, which increased the expression of thyroid-stimulating hormone receptor (TSHR). We speculate that the cAMP pathway might regulate TRPC2 by activation of protein kinase A. Taken together, TRPC2 and the cAMP pathway seem to cross-communicate to keep the expression of the TSHR at an appropriate level. Lastly, we provide evidence for the importance of TRPC2 in regulating glycosylation and secretion of the precursor to thyroid hormones.

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals. In addition, unpublished results are also included in the thesis.

- I. **Löf, C., Blom, T. and Törnquist, K.** (2008) Overexpression of TRPC3 reduces the content of intracellular calcium stores in HEK-293 cells. *J Cell Physiol* **216**, 245-252.
- II. **Gratschev, D., Löf, C., Heikkilä, J., Björkbom, A., Sukumaran, P., Hinkkanen, A., Slotte, J. P. and Törnquist, K.** (2009) Sphingosine kinase as a regulator of calcium entry through autocrine sphingosine 1-phosphate signaling in thyroid FRTL-5 cells. *Endocrinology* **150**, 5125-5134.
- III. **Löf, C., Sukumaran, P., Viitanen, T., Vainio, M., Kemppainen, K., Pulli, I., Näsman, J., Kukkonen, J. and Törnquist, K.** (2012) Communication between the cAMP and calcium pathways regulates the expression of the thyroid-stimulating hormone receptor: TRPC2 in the center of action (In revision).

ABBREVIATIONS

AC	adenylyl cyclase
AKAP	A-kinase anchoring protein
Caly A	calyculin A
CaM	calmodulin
CaMKII	calmodulin kinase II
cAMP	cyclic adenosine monophosphate
CRE	cAMP response element
CREB	cAMP response element binding protein
DAG	diacylglycerol
DOG	1,2-dioctanoyl-sn-glycerol
EPAC	exchange protein activated by cAMP
ER	endoplasmic reticulum
ERK1/2	extracellular signal-regulated kinase 1/2
GAP	GTPase activating protein
GEF	guanine-nucleotide exchange factor
GPCR	G-protein coupled receptor
IP ₃	inositol 1,4,5-trisphosphate
IP ₃ R	IP ₃ -receptor
MAPK	mitogen-activated protein kinase
MPK	MAPK phosphatase
NCX	sodium calcium exchanger
NFAT	nuclear factor of activated T-cells
NIS	sodium iodide symporter
OAG	1-oleoyl-2-acetyl-sn-glycerol
Pax8	paired box 8
PDE	phosphodiesterase
PH	pleckstrin homology
PI3K	phosphoinositide 3-kinase
PIP ₂	phosphatidylinositol 4,5-bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PMCA	plasma membrane calcium ATPase
PP2A	protein phosphatase 2A
RA	Ras association
RAF	rapidly accelerated fibrosarcoma
ROC	receptor-operated channel
RTK	receptor tyrosine kinase
RTP	receptor transporting protein
S1P	sphingosine-1-phosphate
SERCA	sarco-endoplasmic reticulum calcium ATPase
SH	Src homology
SK	sphingosine kinase
SOC	store-operated channel

SOCE	store-operated calcium entry
SPCA	secretory-pathway calcium ATPase
STIM1	stromal interaction molecule 1
T3	3,5,3'-triiodothyronine
T4	3,5,3',5'-tetraiodothyronine
Tg	thyroglobulin
TPO	thyroid peroxidase
TRH	thyrotropin-releasing hormone
TRP	transient receptor potential
TRPC	transient receptor potential canonical
TSH	thyroid-stimulating hormone
TSHR	thyroid-stimulating hormone receptor
TTF	thyroid transcription factor
VOC	voltage-operated channel

1 INTRODUCTION

Calcium signaling is involved in almost every major decision that cells make. Calcium ions use a wide array of different effectors to control everything from the fertilization of an egg cell to the growth and differentiation of all types of cells. With the fate of cells tightly intertwined with the regulation of calcium fluxes, it is not surprising that deregulated calcium signaling is involved in many diseases.

There are two major pools of calcium ions in multicellular organisms, a major extracellular and a minor intracellular pool. Calcium channels are gateways for extracellular calcium ions to enter cells where they bind to calcium-binding proteins that translate increased calcium levels to cellular effects. One class of channels that mediate calcium entry is transient receptor potential canonical (TRPC) channels. They are activated by a multitude of different stimuli, however, a unifying factor is that they are activated downstream of G-protein coupled receptors (GPCRs). The importance of the localization of these channels has been emphasized in many studies and misdirected proteins might explain differences reported.

Several sphingolipids have important cell signaling properties, while others have structural roles. Sphingosine-1-phosphate (S1P) functions as a mitogen and activation of its plasma membrane receptors have important effects on cell signaling, including calcium signaling.

The research presented herein, attempts to elucidate new functions and regulation mechanisms for TRPC2 and TRPC3. The importance of TRPC3 has previously been investigated in many different systems. Conflicting evidence regarding activation mechanisms for TRPC3 have been reported. By studying how TRPC3 functions in an overexpression system, I describe why peculiarly the intracellular calcium stores are negatively affected. TRPC2 has been studied extensively in mouse models, where the channel is important for pheromone recognition and social behavior. Furthermore, TRPC2, like TRPC3, seems to be activated downstream of GPCR-activation. In this thesis, the mechanism by how the bioactive lipid sphingosine-1-phosphate (S1P) augments calcium entry by an autocrine mechanism through a S1P-receptor is described. In addition, initial evidence for TRPC2 mediating the calcium entry regulated by S1P is presented. Lastly, I show evidence for the importance of TRPC2 in thyroid cells. The results show that TRPC2 regulates the cAMP pathway and subsequently the extracellular signal-regulated kinase 1/2 (ERK1/2), which controls the expression of the thyroid-stimulating hormone receptor. Moreover, TRPC2 might have a role in regulating thyroid hormone production and secretion.

Taken together, this thesis presents novel insights into how TRPC2 and TRPC3 channels regulate and influence calcium homeostasis in eukaryotic cells. For TRPC2, the influence of calcium signaling on downstream effects in a thyroid cell model was investigated.

2 REVIEW OF THE LITERATURE

2.1 Calcium signaling

The field of calcium signaling has made tremendous strides since Sidney Ringer's pioneering work on the importance of calcium for the contraction of frog hearts in the 1880s (Ringer, 1883). It took decades until the findings of Ringer would be understood on a molecular level (Katz, 2001). Nonetheless, it is remarkable how an experiment mistakenly made with contaminated tap water, instead of distilled water, would start a research field that we today call calcium signaling.

Another great advance in calcium signaling was made when Michael Berridge, working with fluid secretion from insect salivary glands, showed that inositol phosphates were involved in releasing calcium ions from intracellular calcium stores (Berridge, 2005). In a now classic *Nature* paper from 1983, Berridge and coworkers eventually showed that it was inositol 1,4,5-trisphosphate (IP₃) that releases calcium from intracellular stores (Streb et al., 1983).

The intracellular calcium stores are limited in comparison to the almost unlimited pool of extracellular calcium ions. Extracellular calcium can enter cells through three classes of calcium channels, i.e. voltage-operated channels (VOCs), receptor-operated channels (ROCs), and store-operated channels (SOCs) (Berridge, 1997).

How does the simple calcium ion assert its effects on cellular functions? To answer this question, one has to look at how proteins are regulated. The functions of proteins are regulated by their shape and charge. When calcium binds to specific sites on calcium-binding proteins it changes both their charge and shape, and thus regulates their function (Clapham, 2007). There are three classes of proteins that bind calcium ions and thus work as calcium effector proteins; they are proteins that contain an EF-hand domain (Nakayama and Kretsinger, 1994) or a C2 domain (Lemmon, 2008), and a third class of proteins called annexins (Gerke et al., 2005).

Calcium ions are mobilized from both intra- and extracellular stores, after which they regulate downstream effector proteins. Thereafter, the calcium signal must be terminated and intracellular calcium concentrations are lowered (Figure 1). Typical resting calcium ion concentrations in eukaryotic cells are ~100 nM, but they may transiently increase up to 20,000-fold, into the mM range. A calcium ion concentration this high is highly toxic for cells and must be lowered quickly. As calcium ions cannot be broken down, they must be compartmentalized and buffered or extruded from the cells (Clapham, 2007). Calcium is removed from the cytoplasm by various pumps and exchangers. The sarco-endoplasmic reticulum ATPase (SERCA) pumps calcium into the endoplasmic reticulum (ER), where it is sequestered by calcium binding proteins and kept in storage awaiting the next stimulus. Calcium is also transported out of the cell by plasma membrane calcium ATPase (PMCA) and the sodium/calcium exchanger (NCX) (Berridge et al., 2000; Berridge et al., 2003). Also mitochondria have important functions in modulating calcium signals. The mitochondrial calcium uniporter serves to take up calcium into mitochondria and thus lowers cytosolic calcium levels (Walsh et al., 2009). Also Golgi organelles can apparently sequester calcium by uptake mediated by the secretory-pathway calcium ATPases (SPCAs) (Berridge et al., 2003).

Calcium signaling is crucial for many key events in the life cycle of eukaryotic cells. They include fertilization of the egg cell (Machaca, 2011), gene transcription (Alonso and Garcia-Sancho, 2011), secretion of proteins (Hay, 2007), movement (Prevarskaya et al., 2011), proliferation (Roderick and Cook, 2008), and finally the death of the cell (Decuyper et al., 2011). With a signaling repertoire such as this, it comes as no surprise that deregulated calcium signaling could be involved in the development and progression of diseases. In the chapters to come, relevant literature for understanding general calcium signaling is reviewed. In addition, the cAMP and ERK1/2 pathways will be discussed.

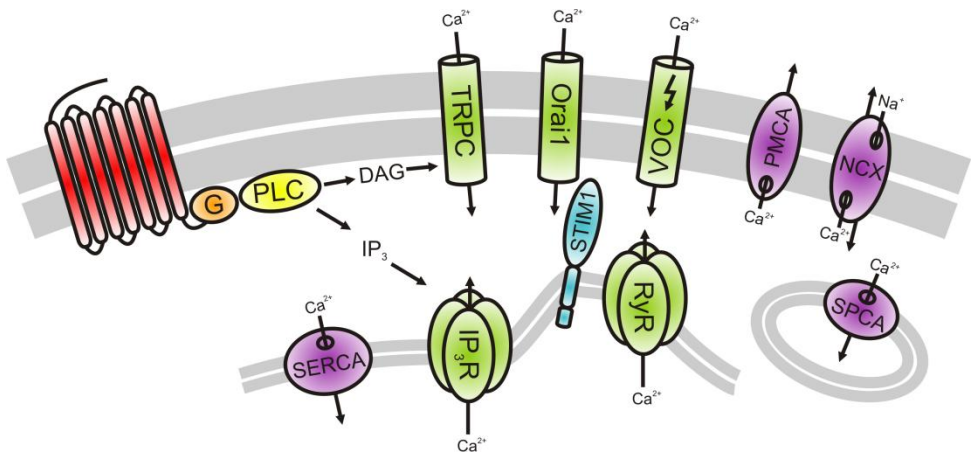


Figure 1. The general effectors that shape the calcium signal in cells. A G-protein coupled receptor is depicted; however, this schematic is the same for any receptor that couples to phospholipase C (PLC). Following activation of PLC, diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP₃) are produced. IP₃ activates its receptor (IP₃R) on the ER, which results in calcium release from the ER into the cytosol. Furthermore, DAG is able to activate transient receptor potential canonical (TRPC) channels in the plasma membrane, which allow calcium ions to enter the cell. Activation of IP₃R will lower the concentration of calcium ions in the ER; this is sensed by stromal interaction molecule 1 (STIM1). STIM1 oligomerizes and activates store-operated calcium entry through Orai1 channels in the plasma membrane. Excitable cells have voltage-operated calcium channels (VOCs); these channels are activated by depolarization of the plasma membrane. Calcium entry through VOCs is amplified by calcium release from the ER through ryanodine receptors (RyRs), which are activated by calcium. When the calcium concentration is at peak level, calcium pumps and exchangers start to lower the intracellular free calcium concentration. The SERCA uses energy stored in ATP to pump calcium into the ER, whereas the plasma membrane calcium ATPase (PMCA) pumps calcium out of the cell. Moreover, secretory pathway calcium ATPase (SPCA) transports calcium ions into the Golgi apparatus. The sodium calcium exchanger (NCX) removes one calcium ion for every three sodium ions that flows down the electrochemical gradient.

2.1.1 Calcium release

There are a few known second messengers that have well documented effects on calcium mobilization from intracellular stores. However, since cyclic adenosine diphosphate ribose- and nicotinic acid dinucleotide phosphate-mediated release of calcium are not relevant for the understanding of the findings presented here, they will not be discussed further. There are also some reports of intracellular calcium release mediated by sphingosine-1-phosphate (S1P). At present, there is no known intracellular receptor for S1P that would mediate calcium release. For this reason, the focus is on explaining calcium release mediated by IP₃.

2.1.1.1 Activation of phospholipase C and the production of IP₃

Activation of phospholipase C (PLC) generates the main intracellular calcium releasing agent, IP₃. PLC produces two important secondary messengers by hydrolyzing phosphatidylinositol 4,5-bisphosphate (PIP₂) to IP₃ and diacylglycerol (DAG). IP₃ releases calcium from intracellular stores by activating IP₃-receptors (IP₃R) on the ER membrane, while DAG activates protein kinase C (PKC) and also activates some TRPC channels (Berridge et al., 2003).

There are six classes of PLC isoforms that are grouped together based on sequence similarities. All classes contain several members and even more variants can be formed through alternative splicing. Almost all PLC isoforms have four conserved domains; the catalytic domain, a pleckstrin homology (PH) domain for PIP₂ binding, and both EF-hands and a C2 domain for calcium binding (Katan, 2005). Emphasis will be put on explaining how the GPCR signaling pathway controls IP₃-production, as it is more relevant for this study.

PLC beta (β) is activated by many G-protein coupled receptors (GPCRs). When the receptor is activated, it catalyzes the exchange of GDP for GTP on the α-subunit of the G-protein heterotrimer. Once in a GTP-bound state, the α-subunit dissociates from the βγ-subunits and both the α- and the βγ-subunits are free to activate PLCβ (Rebecchi and Pentylala, 2000). PLCβ is mainly activated by α_{q/11}-family G-protein subunits (Smrcka et al., 1991), with different members of the Gα_{q/11}-family activating different PLCβ-isoforms to a various degrees (Lee et al., 1992). In addition to this pertussis toxin-insensitive pathway, there is also a pertussis toxin-sensitive pathway to activate PLCβ. α_{i/o}-type G-protein subunits, which are inhibited by pertussis toxin, activate PLCβ (Moriarty et al., 1990). In addition, βγ-subunits of Gα_{i/o}-type G-proteins can also activate PLCβ by binding to a different site than the Gα-subunits (Smrcka and Sternweis, 1993). There are many levels of cross-communication between the cAMP and calcium pathways, also on the level of IP₃-production. Evidence for protein kinase A (PKA) - mediated inhibition of PLCβ2 (Liu and Simon, 1996) and PLCβ3 (Ali et al., 1997) has been reported. Phosphorylation of PLCβ2 was found to inhibit Gβγ-mediated activation, while phosphorylation of PLCβ3 inhibited its activation by a G_{i/o}-coupled receptor.

PLC gamma (γ) is activated by receptor tyrosine kinases (RTKs). In order to interact with RTKs, PLCγ has an additional PH domain, two Src homology (SH) 2 domains and a SH3 domain (Katan, 2005). PLC delta (δ) is activated by the atypical G-protein, G_h. PLCδ is also activated by calcium concentrations in the μM range, which indicates that it could function as an amplifier of the calcium signal initiated by other PLCs (Rebecchi and Pentylala, 2000). PLC zeta (ζ) is a sperm-specific PLC-isoform that is important for fertilization and induces calcium oscillations in the egg (Saunders et al., 2002). PLC epsilon (ε) has a unique mode of regulation, as it is regulated by small GTPases of the Ras superfamily. In addition to the conserved domains, PLCε contains a Cdc25 domain that has guanyl-nucleotide exchange factor activity and two Ras

association (RA) domains (Bunney and Katan, 2006). PLC η (η) is the newest class, which has two members. At least PLC η 2 is, like PLC β , activated by $G_{\beta\gamma}$ -subunits downstream of GPCR activation (Zhou et al., 2005).

After IP_3 has been formed by the action of PLCs, IP_3 is free to activate its receptor, the IP_3R , on the ER membrane. Mammals, and all other vertebrates, have three types of IP_3R s that are ubiquitously expressed and are primarily located on the ER membrane (Foskett et al., 2007). The IP_3R s form a massive homo- or heterotetramer complexes, which are mainly regulated by calcium. Gating of the channel is activated at low and inhibited at high calcium concentrations. IP_3 -binding to the IP_3R removes the inhibitory action of calcium and enables calcium to bind to activating sites on the receptor (Mak et al., 1998). Activation of one IP_3R and the release of calcium can in turn activate another IP_3R ; this feed-forward mechanism is called calcium-induced calcium release (Berridge, 1997). Regulation of the IP_3R is complex and there are multiple interacting proteins and kinases affecting its activity (Foskett et al., 2007). One rather well defined regulator of the IP_3R 1 is PKA. There are two phosphorylation sites for PKA on IP_3R 1 at serine 1589 and serine 1755 (Ferris et al., 1991). When these residues are phosphorylated, calcium release by IP_3R 1 is potentiated (Wagner et al., 2004). Second messenger molecules are usually rapidly broken down in cells. The enzymes responsible for IP_3 breakdown or modulation are inositol polyphosphate 5-phosphatases and IP_3 3-kinase, respectively. The former will produce an inactive inositol phosphate that ultimately is stripped down to free inositol. IP_3 3-kinase phosphorylates IP_3 to form an inositol phosphate with other potential signaling properties (Pattni and Banting, 2004).

2.1.2 Calcium entry

There are three groups of plasma membrane calcium channels. They are grouped according to their activation mechanism into VOCs, SOCs and ROCs (Berridge, 1997). Emphasis is here put on explaining calcium entry mediated by ROCs, as TRPC channels are important conveyors of receptor-operated calcium entry.

2.1.2.1 Voltage-operated calcium entry

Voltage-operated or voltage-gated calcium channels are, as the name implies, activated by a change in membrane potential. They are rapidly activated upon membrane depolarization and inactivated upon repolarization. There are five types of VOCs that belong either to low voltage activated or high voltage activated channels (Tsien and Tsien, 1990). T-type channels are low voltage activated and L-, N-, P/Q-, and R-type are high voltage activated. VOCs consist of α_1 -, β - and $\alpha_2\delta$ -subunits (Catterall, 2000). Calmodulin (CaM) is also considered an essential component of VOCs, since CaM also binds VOCs independently of calcium (Halling et al., 2006). The α_1 -subunit forms the voltage-sensing pore and consists of four homologous repeats. Each homologous repeat has six transmembrane segments, the first four segments convey voltage-sensing, while the two last form the calcium-selective pore (Van Petegem and Minor, 2006). VOCs are inactivated by voltage-dependent inactivation and calcium-dependent inactivation. However, VOCs can also be primed for activation by calcium-dependent facilitation (Catterall, 2000). The β -subunit binds to the first two segments of the α_1 -subunit and affects gating and

trafficking of the channel. The α_2 -subunit is linked to the transmembrane δ -subunit through disulfide bonds and it enhances channel function (Tsien and Tsien, 1990; Catterall, 2000).

2.1.2.2 Store-operated calcium entry mediated by STIM1 and Orai1

The molecular components of store-operated calcium entry (SOCE) were unknown until 2005. Stromal interaction molecule 1 (STIM1) was first identified as an essential component of SOCE (Roos et al., 2005). Later in the same year, STIM1 and STIM2 were found to be calcium sensors in the ER (Liou et al., 2005). Following calcium depletion, STIM1 translocates and forms puncta close to the plasma membrane (Liou et al., 2005; Zhang et al., 2005). A mutant form of Orai1 was discovered as the cause of hereditary severe combined immune deficiency, where the patients' immune cells lack SOCE (Feske et al., 2006). Simultaneously, another group found Orai1 to be essential for SOCE in an RNA interference screen (Vig et al., 2006b). SOCE was largely unaffected by overexpression of either STIM1 or Orai1. However, SOCE was massively increased when STIM1 and Orai1 were overexpressed together (Peinelt et al., 2006; Soboloff et al., 2006). It was concluded that STIM1 functions as the calcium sensor in the ER and Orai1 tetramers function as the pore-forming subunit of SOCs (Prakriya et al., 2006; Vig et al., 2006a; Mignen et al., 2008). The N-terminus of STIM1 contains a calcium-binding EF hand and a sterile alpha motif, which are two domains necessary for STIM1 to function as ER luminal calcium sensor (Stathopoulos et al., 2008). STIM1 oligomerizes in response to a drop in ER calcium concentration and this is the first step in activating SOCE (Luik et al., 2008). After activation, STIM1 tetramers translocate to ER sites close to the plasma membrane and activate SOCE through tetramerization of Orai1 (Penna et al., 2008). Gating of Orai1 is regulated by a ~100 amino acid fragment in STIM1 which was described simultaneously by two groups as STIM1 Orai activating region (SOAR; amino acids 344-442) (Yuan et al., 2009) or CRAC activation domain (CAD; amino acids 342-448) (Park et al., 2009). SOCE is inactivated by calcium-dependent inactivation, which requires both a short domain C-terminal to the CAD domain in STIM1 and CaM binding to Orai1 (Lee et al., 2009; Mullins et al., 2009). In a recent report, STIM1 was reported to also regulate the ion selectivity of Orai1 (McNally et al., 2012).

2.1.2.3 Receptor-operated calcium entry mediated by TRPC channels

The transient receptor potential cation channels are named after the *Drosophila* mutant, whose eyes had a transient response to light (Cosens and Manning, 1969). It took 20 years until *Drosophila* TRP was cloned and its tentative identification as a channel (Montell and Rubin, 1989). The TRP superfamily of cation channels consist of seven subfamilies, six of which are present in mammals (Montell, 2005). The classical or canonical TRPs, the TRPCs, are the closest relatives to the *Drosophila* TRP channel. Activation of the GPCR-G_q-PLC β cascade results in a transient increase in intracellular calcium. This transient increase is followed by a sustained calcium increase which is dependent on extracellular calcium. TRPC channels were discovered when the identity of channels responsible for sustained calcium increases were investigated (Abramowitz and Birnbaumer, 2009; Birnbaumer, 2009).

TRPC channels have six transmembrane segments with the pore being located between the last two (Figure 2) (Vannier et al., 1998). The N-terminus contains several ankyrin motifs that are important for protein-protein interactions. The N-terminus and the ankyrin repeats are involved in TRPC subunit tetramerization and are thus critical for channel function (Lepage et

al., 2006; Lepage et al., 2009). The C-terminus contains one or more CaM-binding sites, one or more IP₃R binding sites, and a PDZ domain. The IP₃R- and CaM-binding sites are important for activation and inhibition of the channel, respectively (Birnbaumer, 2009). The PDZ domain is, like the ankyrin motifs, important for protein-protein interactions (Harteneck, 2003). TRPCs also have a highly conserved TRP domain after the channel forming transmembrane domain (Venkatachalam and Montell, 2007; Birnbaumer, 2009).

With a wealth of knowledge on what TRPC channels are important for, it is rather surprising that the gating mechanisms are not better characterized (Birnbaumer, 2009). Direct activation of TRPCs by DAG is the most studied. TRPC3 and 6, but not TRPC1, 4 and 5, were first shown to be activated by DAG-analogs (Hofmann et al., 1999a). The cloning and characterization of TRPC7 revealed another DAG-activated TRPC (Okada et al., 1999). TRPC2 is also activated by DAG-analogs (Lucas et al., 2003). TRPCs are phosphorylated and inactivated by PKC. As DAG also is a potent activator of conventional and novel PKC isoforms, this revealed a classical negative-feedback regulatory mechanism (Venkatachalam et al., 2003). Moreover, PKC-inhibition prior to stimulation with a DAG-analog revealed that also TRPC4 and 5 are activated by DAG (Venkatachalam et al., 2003). In contrast, there are no reports for DAG-mediated activation of TRPC1 (Birnbaumer, 2009; Albert, 2011).

PIP₂ is not only the precursor to IP₃ and DAG, it also possesses signaling properties (Suh and Hille, 2005). More often PIP₂ has an activating effect on channel function, although, some TRP channel members are inhibited by PIP₂ (Suh and Hille, 2005; Kukkonen, 2011). As often is the case in the TRPC field, conflicting reports regarding the regulatory function of PIP₂ on channel function have been published. At least in portal vein vascular smooth muscle cells, PIP₂ has an inhibitory effect on DAG-activated TRPCs (Albert, 2011). Furthermore, TRPC4 was shown to be inhibited by PIP₂. Interestingly, only the full-length protein was inhibited, and not TRPC4 β , which lacks 84 amino acids in the C terminus (Otsuguro et al., 2008). In contrast, TRPC6 is activated by phosphoinositides, most potently by PIP₃ and PIP₂ (Kwon et al., 2007). It was reported that phosphoinositides compete for the CaM-binding site and thus remove channel inhibition (Kwon et al., 2007). This would group at least TRPC6 with other TRP channels such as TRPMs, which are activated by PIP₂ (Nilius et al., 2006). This is still a matter of debate, however; a stimulatory action by DAG and inhibitory action by PIP₂ would fit nicely with the GPCR-G_q-PLC β pathway: activation of PLC would remove inhibition and produce the agonist for activating the channel.

Another possible way of activating TRPCs is through conformational coupling with ER calcium release channels, mainly IP₃Rs (Venkatachalam and Montell, 2007; Birnbaumer, 2009). In this model, activation of IP₃Rs would subsequently activate TRPCs in the PM. Such interactions have been shown for heterologously expressed IP₃R and TRPC3 and 6 (Kiselyov et al., 1998; Boulay et al., 1999). There is evidence for the involvement of the Homer scaffolding proteins in the conformational coupling model (Yuan et al., 2012). Homer1 binds to the N-terminus of the IP₃R and to the C-terminus of TRPCs. It seems that Homer1 keeps TRPCs inactive until IP₃Rs are activated, where after the IP₃R-Homer1-TRPC complex dissociates and TRPCs are activated (Yuan et al., 2003). Furthermore, Homer1 has been shown to aid the translocation of TRPC3 to the PM (Kim et al., 2006). TRPCs, like many other calcium channels, are negatively regulated by CaM. Activation of TRPCs through displacement of CaM by IP₃R has been reported (Zhang et al., 2001).

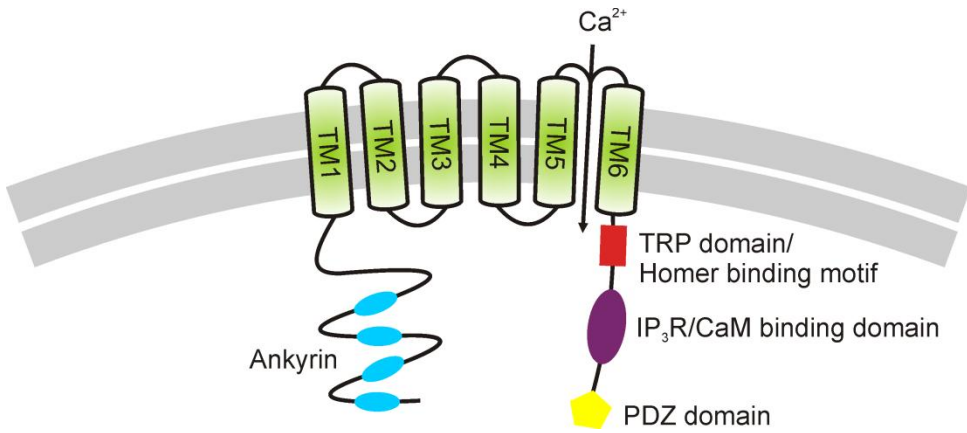


Figure 2. Schematic picture of TRPC structure. TRPC channels have six transmembrane (TM) domains. The pore is located between TM5 and TM6. TRPC channels contain several domains that are important for protein interactions and channel regulation, among them the Homer binding motif in the TRP domain and the IP₃R/CaM binding domain [modified from (Vazquez et al., 2004)].

2.1.3 Calcium binding proteins

The calcium ion needs a sensor that conveys changes in intracellular calcium concentrations into cellular responses. There are three main groups of proteins that bind calcium and confer calcium-mediated responses; EF-hand proteins, annexins, and C2-domain proteins.

2.1.3.1 EF-hand proteins

EF-hand proteins are named after the calcium-binding domain formed by helices E and F of parvalbumin (Kretsinger and Nockolds, 1973; Gifford et al., 2007). Several well-known calcium-binding proteins are EF-hand proteins, e.g. CaM, troponin C, and aequorin. The EF-hand consists of an α -helix, a loop around the calcium ion, which is followed by a second α -helix (Nakayama and Kretsinger, 1994). The calcium binding domain is rich in negatively charged amino acids such as aspartic acid and glutamic acid. In most cases it is aspartic acid side chains that provide the oxygen ligands to which the calcium ion bind (Gifford et al., 2007). EF-hand proteins usually consist of two calcium binding domains, however, some contain three or more. The best known EF-hand protein is CaM. CaM has two calcium binding EF-hand domains connected with a flexible linker (Bhattacharya et al., 2004). CaM is inactive when not bound to calcium and changes conformation when the calcium concentration increases. The conformation change enables CaM to interact with downstream effectors (Nakayama and Kretsinger, 1994). The conformational change reveals hydrophobic side chains that form the

protein interaction domain (Zhang et al., 1995a; Gifford et al., 2007). However, not all EF-hand proteins are important for signaling. For example, parvalbumin chelates calcium and thus functions as a calcium buffer and prevents the cytotoxic effects of high calcium concentrations (Gifford et al., 2007).

2.1.3.2 Annexins

Annexins are another class of calcium-binding proteins. The annexins are curved disc-shaped proteins, which in a calcium-dependent manner are able to interact with cellular membranes with their convex surface (Huber et al., 1990; Gerke et al., 2005). The annexin core is comprised of ~310 amino acids and contains four annexin repeats. One annexin repeat is made of five α -helices with calcium-binding loops separating them (Lemmon, 2008). Calcium binding to calcium binding sites on this outer convex surface gives rise to a conformational change in the annexins. This conformational change reveals carbonyl and carboxyl groups that are able to interact with phosphoryl moieties of the phospholipids in the cell membrane (Gerke et al., 2005). The concave surface faces the cytoplasm and functions as a docking site for other proteins. Calcium-bound annexin A5 has been shown to interact with membranes through insertion of a tryptophan residue into the membrane (Concha et al., 1993). The N-terminus on the concave surface houses domains to which several EF-hand proteins bind. The N-terminus is also subject to post-translational modifications. Phosphorylation of tyrosine residues modulates the calcium and membrane binding of annexins A1 and A2. EGF receptor kinase and Src kinase phosphorylate tyrosine residue 20 in annexin A1 and tyrosine residue 23 in annexin A2, respectively (Gerke and Moss, 2002). Furthermore, phosphorylation of annexins by serine/threonine kinases has been reported. For example, phosphorylation of annexin A2 by PKC is essential for calcium-regulated exocytosis (Sarafian et al., 1991; Gerke and Moss, 2002). Annexins are also involved in changing membrane curvature and cell shape by modulating actin rearrangement (Gerke et al., 2005). Furthermore, evidence for annexins regulating calcium fluxes has also been shown (Gerke and Moss, 2002).

2.1.3.3 C2-domain proteins

The third class of calcium-binding proteins, C2-domain proteins, share functional characteristics with annexins but are structurally different (Lemmon, 2008). The C2-domain was first studied in conventional PKCs and is therefore named after the second homology region in PKC (Cho and Stahelin, 2006; Lemmon, 2008). The ~130 amino acid C2-domain contains eight antiparallel β -strands with three loops that connect the β -strands. The inter-strand loops are responsible for binding calcium ions and membrane lipids (Cho and Stahelin, 2006; Lemmon, 2008). The general consensus is that, upon calcium binding to the C2 domain, the protein translocates to cellular membranes and binds phosphatidylserine. However, many C2-domains bind calcium with low affinity and some C2-domain proteins function independently of calcium (Cho and Stahelin, 2006). Studies have shown that calcium changes the electrostatic potential of the C2-domain and thus favors binding to negatively charged membranes (Shao et al., 1997; Lemmon, 2008). Furthermore, calcium ions seem to function as bridges between the C2-domain and the anionic phospholipids (Ubach et al., 1998; Lemmon, 2008). There is also evidence that calcium-mediated conformational changes could trigger C2-domain interactions with the plasma membrane (Sutton et al., 1995).

Lipid selectivity varies between different C2-domains. The C2-domains in conventional PKCs bind phosphatidylserine with high affinity. However, other C2-domains bind all negatively charged phospholipids, while some prefer zwitterionic phospholipids (Cho and Stahelin, 2006; Lemmon, 2008).

2.1.4 Decreasing intracellular calcium

The primary task of calcium pumps (ATPases) and exchangers is to lower intracellular calcium to avoid the toxic effects of high calcium concentrations. However, the pumps and exchangers also affect the amplitude and duration of the calcium signal. After the rapid increase of intracellular calcium that follows upon e.g. activation of a GPCR, SERCA tries to replenish the intracellular stores in the ER and PMCA and NCX function to lower intracellular calcium levels by extruding calcium.

2.1.4.1 P-type calcium ATPases

Three classes of calcium pumps (P-type calcium ATPases) are expressed in membranes of mammalian cells. The SERCA is located mainly in the ER, the SPCA in the Golgi, and PMCA in the plasma membrane. As the name foretells, P-type calcium ATPases hydrolyze ATP to pump calcium ions against their electrochemical gradient. P-type ATPases exist transiently as a phosphorylated intermediate, after which they are named (Pedersen, 2007; Brini and Carafoli, 2009). The P-type ATPase superfamily has eight subfamilies and hundreds of members (Brini and Carafoli, 2009). The P-type ATPases have high affinity for calcium on the cytoplasmic side. When calcium binds, a series of conformational changes take place, which favors phosphorylation of a catalytic residue. The phosphorylated form has lower affinity for calcium and calcium is released in the ER or outside of the cell. Thereafter, the phosphate group is hydrolyzed and the high affinity form is regenerated (Brini and Carafoli, 2009). SERCA has two calcium-binding sites in the transmembrane helices, while PMCA and SPCA only have one. Both PMCA and SPCA lack an essential acidic residue in transmembrane domain 5, which explains why these two only transport one calcium ion for every ATP hydrolyzed (Clarke et al., 1989; Clarke et al., 1990; Brini and Carafoli, 2009).

Mammals have three SERCA genes. Their transcripts undergo tissue-specific alternative splicing which increases the number of isoforms. SERCA1a and 1b are expressed in fast-twitch skeletal muscle cells. SERCA2a is expressed in the heart and in slow-twitch muscle cells. SERCA2b is considered a housekeeping isoform and is ubiquitously expressed. The SERCA3 isoforms are not that well known, however, they seem specialized for relaxation of vascular and tracheal smooth muscle cells (Brini and Carafoli, 2009). The activity of SERCA1a, SERCA2a, and SERCA2b are regulated by phospholamban (James et al., 1989; Fujii et al., 1990). The inhibitory action of phospholamban is reversed if phospholamban is phosphorylated by PKA (Tada et al., 1979; Toyofuku et al., 1993) or CaM kinase II (CaMKII) (Mattiuzzi et al., 1998; Kuschel et al., 1999; Brini and Carafoli, 2009). In a recent study, sumoylation was found to stabilize and increase the activity of SERCA2a (Kho et al., 2011).

The SPCA is the newest member of the calcium ATPases. Two human SPCA isoforms have been described (Hu et al., 2000; Xiang et al., 2005; Brini and Carafoli, 2009): SPCA1 is ubiquitously expressed while SPCA2 expression is more restricted (Brini and Carafoli, 2009).

Interestingly, the SPCAs are also efficient transporters of manganese (Van Baelen et al., 2001). There are several enzymes in the Golgi lumen that require manganese as a co-factor, which could explain why SPCAs have evolved this function (Brini and Carafoli, 2009).

There are four isoforms of PMCA and numerous splice variants. PMCA1 and 4 are ubiquitously expressed. PMCA2 and PMCA3 are mainly expressed in the nervous system and muscle cells (Strehler and Zacharias, 2001). PMCA has high calcium affinity but low transport capacity, and are thus minor regulators of intracellular calcium concentrations (Brini and Carafoli, 2009). The PMCA is inhibited in the absence of CaM, when the C-terminal tail binds two intracellular loops and inhibits activity. When the calcium concentration rises, calcium-CaM binds a domain in the C-terminal. This induces a conformational change that frees the catalytic domain (Strehler et al., 2007). PMCA is phosphorylated in the C-terminal by PKA (Neyses et al., 1985) or PKC (Wang et al., 1991). This has been reported to facilitate PMCA activity (Strehler et al., 2007). The 14-3-3 ϵ protein, on the other hand, has an inhibitory effect on pump activity when it interacts with the N-terminal of PMCA4 (Rimessi et al., 2005).

2.1.4.2 Sodium/calcium exchanger

When it comes to removing calcium from the cytosol, the low-affinity high capacity NCX complements the high-affinity low capacity PMCA very well (Clapham, 2007). The NCX can move calcium into or out of cells depending on the electrochemical driving force (Blaustein and Lederer, 1999). In the forward mode of action, one calcium ion is extruded for every three sodium ions that flow along the electrochemical gradient (Hilgemann et al., 1991). Three isoforms of NCX have been cloned, but additional isoforms are made through alternative splicing (Guerini et al., 2005). The NCX1 isoform is ubiquitously expressed while NCX2 and NCX3 are mostly expressed in excitable cells (Blaustein and Lederer, 1999). The NCXs are activated by high intracellular calcium, with a central cytosolic loop being critical for calcium-mediated activation (Levitsky et al., 1994; Matsuoka et al., 1995). A high intracellular sodium concentration, on the other hand, has been reported to inhibit the NCX (Hilgemann et al., 1992). Although there are reports of phosphorylation-mediated regulation of NCX activity, it is still a matter of debate (Lytton, 2007).

2.1.5 Calcium signaling and cancer

With the aforementioned review on how intracellular calcium concentrations are regulated, the end of the chapter will be dedicated to discussing potential implications of rogue calcium signaling in cancer.

Calcium is needed at many stages of the cell cycle and for the movement of cells; deregulated calcium signaling is therefore of interest in cancer (Cook and Lockyer, 2006; Parkash and Asotra, 2010; Prevarskaya et al., 2011). Intracellular calcium concentrations are tightly regulated. Calcium is kept low during the early G1-phase and S-phase, but it rises at the G1/S transition and in the G2-phase (Pande et al., 1996). In addition, calcium is needed for phosphorylation of the master regulator of the cell cycle, the retinoblastoma protein (Takuwa et al., 1993). Calcium-CaM has many important effects on cell proliferation. CaMK regulates cyclin D1 expression, which is important for cyclin-dependent kinase (CDK) 4 and CDK2. Moreover, Calcium-CaM regulates calcineurin which dephosphorylates and thus activates nuclear factor of activated T-cells (NFAT) (Parkash and Asotra, 2010). Calcium influx through

the SOC channel Orai1 is important for activation of NFAT (Feske et al., 2006). Which transcription factors are activated is largely dependent on the amplitude and duration of the calcium signal. In B lymphocytes, NFAT is activated by a low amplitude sustained calcium signal, while nuclear factor κ B and c-Jun N-terminal kinase require a large transient increase in calcium to be activated (Dolmetsch et al., 1997). The TRP channel TRPV6 has been implicated in prostate cancer. TRPV6 is overexpressed in prostate cancer and is connected with increased NFAT activation and cell survival (Lehen'kyi et al., 2007). Moreover, TRPV6 expression correlates with the aggressivity of the cancer, thus making it an interesting target (Fixemer et al., 2003). Alterations in calcium pumps have also been investigated in relation to cancer susceptibility. Mutations in SERCA2 have been reported to associate with colon and lung cancer (Korosec et al., 2006). Downregulation of SERCA2b and resistance to apoptosis was seen in neuroendocrine-differentiated prostate cancer cells (Vanoverberghe et al., 2004). Reduced calcium uptake to the ER will affect calcium transients negatively; however, it may confer resistance to cell death. Reduced IP₃-mediated release of calcium from the ER protects cells from apoptosis (Szado et al., 2008). Also reports exist for the involvement of PMCA in cancer. The expression of PMCA was increased in gastric and colon cancer (Ribiczey et al., 2007). Moreover, a mutation in PMCA3 has been found in pancreatic cancer cells (Jones et al., 2008; Brini and Carafoli, 2009).

The migration of cells requires calcium and the involvement of calcium channels in metastatic behavior have gathered interest (Prevarskaya et al., 2011). When cells move, the intracellular calcium concentration is lowest at the leading edge of the cell and highest at the rear of the cell (Brundage et al., 1991; Hahn et al., 1992). This polarization of calcium is thought to facilitate rear-end retraction (Prevarskaya et al., 2011). The internalization and recycling of integrins at the rear end of a cell is needed for the cell to move forward. This process is regulated by the calcium-CaM-activated protein phosphatase calcineurin (Lawson and Maxfield, 1995). Focal adhesion kinase is an important regulator of cell migration, which calcium can activate through CaMKII (Fan et al., 2005). STIM1 and Orai1 are important for focal adhesion turnover and thus migration of breast cancer cells in vitro. Furthermore, RNA interference of either STIM1 or Orai1 inhibited metastasis in a mouse model (Yang et al., 2009). Members of the TRP channels are frequently reported as crucial for cell migration (Lee et al., 2011; Prevarskaya et al., 2011). Interestingly, TRPC5 and 6 have been reported to activate and inhibit cell migration, respectively. TRPC5 activated the small GTPase Rac1 and TRPC6 the small GTPase RhoA, which had diametrically different effects on cell migration (Tian et al., 2010).

2.2 GPCR signaling

GPCRs are a large group of plasma membrane receptors with over 800 members. Many key physiological functions are controlled by GPCRs; among them are neurotransmission, hormone release from endocrine glands, and cardiac contraction. There are three families of GPCRs, which are grouped together based on sequence similarities. The sequences vary between families to a great extent, but they all share the seven transmembrane domain structure. GPCRs convey information to intracellular effectors through G-proteins. The G-proteins are heterotrimeric and are comprised of α - and $\beta\gamma$ -subunits. The α -subunit binds GTP and GDP and has intrinsic GTPase activity. However, a GTPase activating protein (GAP), regulator of G-

protein signaling, accelerates the hydrolysis of GTP. The β and γ subunits form a tightly bound complex. There are at least 16 α , five β , and 12 γ subunits. There are four subfamilies of α -subunits: α_s , $\alpha_{i/o}$, α_q , and $\alpha_{12/13}$. G-proteins are named on the basis of their α -subunit. For example, a heterotrimeric G-protein containing α_s is a G_s -protein. To simplify greatly, G_s -proteins activate adenylyl cyclases (ACs), $G_{i/o}$ -proteins couple to inhibition of ACs, G_q -proteins couple to activation of PLC β , and $G_{11/12}$ -proteins activate guanine-nucleotide exchange factor (GEF) for Rho. When a ligand is bound to the GPCR, it induces a conformational change that enables the receptor to interact with its cognate G-protein and promotes GDP dissociation and GTP binding. The GTP-bound α -subunit dissociates from the $\beta\gamma$ -subunit and activates downstream effectors. In addition, there is also evidence that the $\beta\gamma$ -subunit activates effectors (Neves et al., 2002; Pierce et al., 2002).

2.2.1 Sphingolipid signaling

S1P, sphingosine, and ceramide are three sphingolipids with well-documented effects on cell signaling. Ceramide is a central molecule in sphingolipid biosynthesis and sphingosine is a direct breakdown product of ceramide; its formation is catalyzed by ceramidase (Figure 3). The phosphorylation of sphingosine and hence the production of S1P is catalyzed by sphingosine kinase (SK). All steps are reversible and S1P can be dephosphorylated by S1P phosphatase, and ceramide can be re-synthesized from sphingosine by the action of ceramide synthase. However, S1P lyase irreversibly degrades S1P to hexadecenal and phosphoethanolamine (Pitson, 2011). Ceramide synthesis increases in cells in response to many cytotoxic treatments and is thus linked to apoptosis. Similarly, sphingosine is a PKC inhibitor and inhibits cell growth and induces apoptosis. In contrast, S1P inhibits apoptosis and promotes cell survival. It has been proposed that the balance between these three lipids determines the fate of the cell (Maceyka et al., 2002).

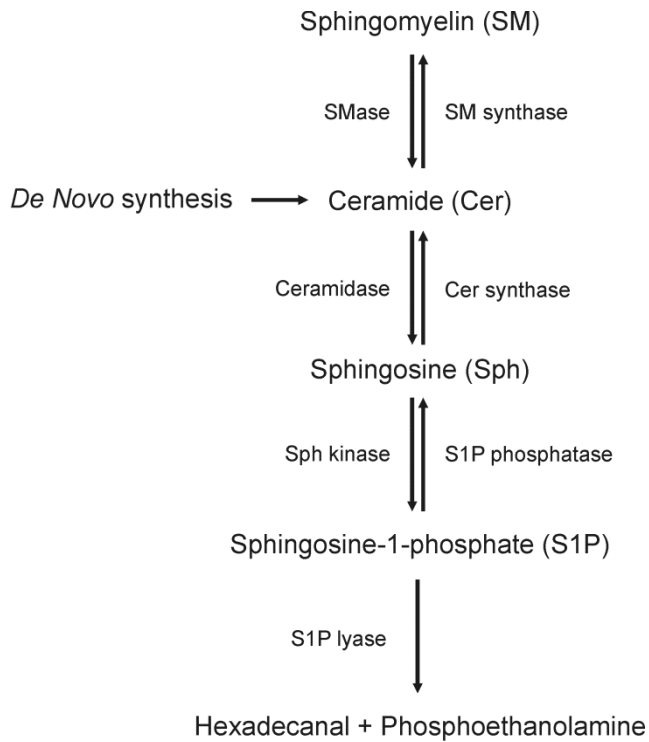


Figure 3. Sphingolipid metabolism. Ceramide is a central molecule in sphingolipid metabolism. Ceramide is made by breakdown of sphingomyelin or by *de novo* synthesis. Sphingosine is a direct breakdown product of ceramide. Sphingosine is phosphorylated by SK to S1P. S1P is irreversible broken down by S1P lyase, all other steps in sphingolipid biosynthesis are reversible.

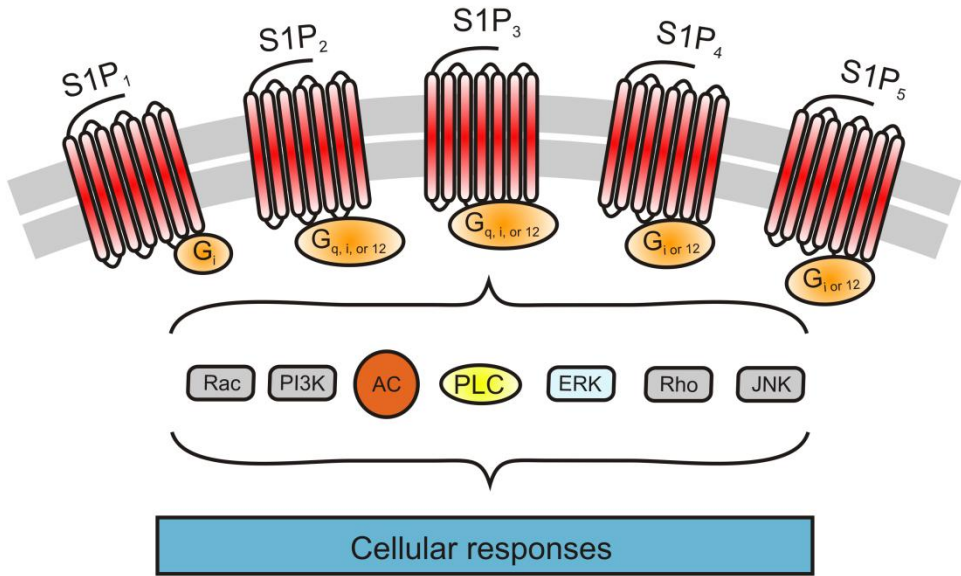
2.2.1.1 *Sphingosine-1-phosphate receptors*

There are five GPCRs, termed S1P₁₋₅, that specifically bind S1P. Depending on the receptor isoforms expressed, S1P can regulate a wide array of physiologically important processes. In addition, the G-proteins add additional complexity, since several S1P-receptors couple to more than one family of G-proteins (Figure 4A). However, S1P₁ couples exclusively to G_i-proteins. S1P₂ and S1P₃ are more promiscuous and couple to G_i, G_q, and G_{12/13}. S1P₄ is able to couple to both G_i and G_{12/13} (Spiegel and Milstien, 2003; Sanchez and Hla, 2004). The least studied S1P-receptor, S1P₅, has also been shown to couple to G_{i/0} and G₁₂ (Malek et al., 2001). Downstream effectors activated by S1P₁ include: ERK, phosphoinositide 3-kinase (PI3K) and PLC. Furthermore, S1P₁-activation results in inhibition of cAMP accumulation. ERK and PLC are also activated by S1P₂ and S1P₃. Moreover, Rho is regulated by these receptors (Sanchez and Hla, 2004; Pyne and Pyne, 2010). The downstream effectors of S1P₄ and S1P₅ are less well known, but there is evidence for S1P₄ activating Cdc42 and S1P₅ activating c-Jun N-terminal kinase (Malek et al., 2001; Kohno et al., 2003).

2.2.1.2 *Autocrine/paracrine signaling*

An interesting model of action for S1P is that, S1P produced inside the cell is exported and activates S1P-receptors on the same or neighboring cell in an autocrine or paracrine fashion, respectively (Figure 4B) (Spiegel and Milstien, 2003; Rosen and Goetzl, 2005; Pyne and Pyne, 2010). Johnson and colleagues were the first to show that SK1 was translocated to the plasma membrane via a PKC-dependent mechanism and that the S1P produced was secreted (Johnson et al., 2002b). The involvement of ATP binding cassette (ABC) transporters has been implicated in the export of S1P in mast cells. Downregulation of ABCC1 in mast cells reduced S1P secretion and migration (Mitra et al., 2006). Another ABC transporter, namely ABCA1, transports S1P in astrocytes (Sato et al., 2007). This inside-out signaling for S1P is important also in cancer cells. In addition to ABCC1, ABCG2 was shown as critical for the release of S1P from breast cancer cells in response to estradiol (Takabe et al., 2010). Interestingly, it was reported that SPNS2 is important for embryogenesis in zebrafish. A mutation in this gene caused the development of two hearts, which is the same phenotype as seen in S1P₂-mutants. The authors linked SPNS2 to the secretion of S1P and the migration of myocardial precursor cells (Kawahara et al., 2009). Moreover, SPNS2 is involved in transport of several S1P-analogs in human cells, among them the S1P-receptor agonist FTY720 (Hisano et al., 2011). In contrast, there were no changes in plasma S1P concentrations between wild-type and ABCC1 or ABCA1 knockout mice (Lee et al., 2007). However, it is not known whether ABC transporters have overlapping functions or whether some compensatory mechanism was involved (Pyne and Pyne, 2010).

A



B

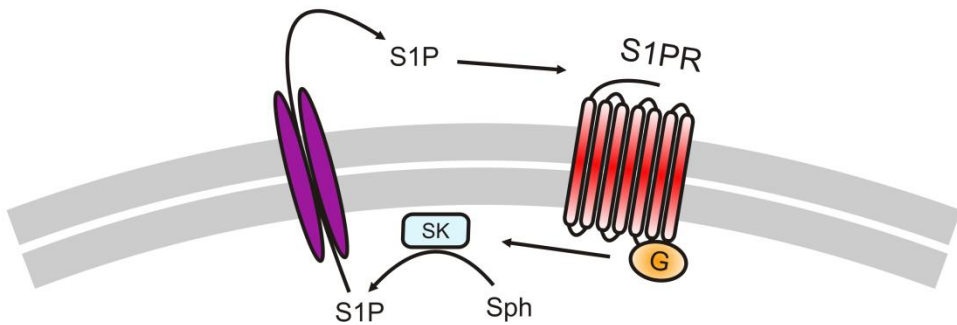


Figure 4. S1P signaling mediated by S1P-receptors. (A) There are five GPCRs that are specifically activated by S1P. S1P₁ only couples to G_i-proteins, whereas the other receptors couple to two or three classes of G-proteins. A sample of downstream effectors that are activated or inhibited by S1P-receptors are depicted. These pathways give rise to a multitude of different cellular responses [modified from (Spiegel and Milstien, 2003)]. (B) S1P produced inside the cell can be transported out where it activates S1P-receptors through autocrine signaling.

2.2.2 Thyroid-stimulating hormone receptor signaling

Thyroid-stimulating hormone (TSH) is a ~30-kDa glycoprotein secreted by the anterior pituitary in response to thyrotropin-releasing hormone (TRH) from the hypothalamus. Like all cysteine-knot growth factors, TSH is comprised of a common α -subunit and a hormone-specific β -subunit. TSH is a glycosylated protein with human β -subunits having one and human α -subunits having two carbohydrate chains linked to asparagine residues, respectively. In addition to giving species-specificity, the carbohydrate chains seem to improve stability and receptor binding (Szkudlinski et al., 2002). TSH controls thyroid hormone production and thyroid growth through its G-protein coupled receptor, the TSH receptor (TSHR). Like all GPCRs, the TSHR has a C-terminal tail domain, an N-terminal extracellular domain, and seven transmembrane domains connected by intra- and extracellular loops (Kleinau and Krause, 2009). The large N-terminal extracellular domain is sufficient for high-affinity hormone binding (Da Costa and Johnstone, 1998). Unlike many closely related GPCRs, the TSHR has a high basal activity, which leads to cAMP production in the absence of ligand (Cetani et al., 1996). Furthermore, deletions or degradation of parts of the extracellular domain result in receptor activation (Zhang et al., 1995b; Van Sande et al., 1996). Point mutations of the TSHR have been linked to increased receptor activity and the incidence of thyroid adenomas (Parma et al., 1993). This is in line with reports showing links between activating mutations in G_{α_s} and thyroid adenomas (Lyons et al., 1990). Many thyroid diseases are caused by defective or over-active TSHR signaling. Hyperthyroidism can result from activating mutations in the TSHR or from thyroid-stimulating antibodies acting on the TSHR as an agonist, which is the case in the autoimmune disease Graves' disease (Paschke et al., 1996). If the TSHR loses its ability to respond to TSH, the outcome is hypothyroidism. This is the case in a mouse model where a point mutation results in loss of function in the TSHR (Stein et al., 1994). The TSHR is subject to post-translational modifications, with documented functional effects for glycosylation of asparagine residues 77 and 113. Glycosylation of these residues was important for expression of functional TSHR at the plasma membrane (Russo et al., 1991). Studies with TSHR-knockout mice concluded that the TSHR is important for growth and development and critical for thyroid hormone production. Unless supplemented with thyroid hormone the mice died within one week of weaning. However, the TSHR is not needed during embryogenesis or for thyroid development (Marians et al., 2002; Postiglione et al., 2002).

Most of the effects mediated by the TSHR are transduced by G_s -proteins (Rivas and Santisteban, 2003). However, evidence for the TSHR coupling to $G_{i/o}$, G_q , and $G_{12/13}$ has been reported (Laugwitz et al., 1996). However, a relatively high concentration of TSH is required to stimulate G_q -proteins (Rivas and Santisteban, 2003).

2.3 cAMP signaling

That cAMP is an intracellular secondary messenger was first discovered in the 1950's. Research in the field of cAMP has resulted in many groundbreaking discoveries (Beavo and Brunton, 2002). Many of the effects mediated by the TSHR result from activated G_s -proteins and AC enzymes, which produce cAMP (Medina and Santisteban, 2000). Peculiarly, cAMP acts as a mitogen or anti-mitogen depending on the cell system used. However, thyroid cell proliferation is positively regulated by cAMP (Medina and Santisteban, 2000; Rivas and Santisteban, 2003).

2.3.1 cAMP production by adenylyl cyclases

The enzymes catalyzing the production of cAMP from ATP are ACs. To date nine ACs have been cloned (Sunahara et al., 1996; Cooper, 2003; Willoughby and Cooper, 2007). In addition, one soluble AC has been described, and seems to be expressed specifically in the testis (Buck et al., 1999). The membrane-bound ACs have tissue-specific distribution patterns. However, all nine isoforms are expressed in the brain (Hanoune and Defer, 2001). All membrane-bound isoforms have two hydrophobic domains, each consisting of six transmembrane domains. Two cytoplasmic domains dimerize and form the catalytic site (Cooper, 2003; Willoughby and Cooper, 2007). All isoforms are stimulated by $G_s\alpha$ -activation, while all ACs are inhibited by $G_i\alpha$ except ACs 2, 4 and 7 (Sunahara et al., 1996). There are also reports of ACs being regulated by $G\beta\gamma$ -subunits. AC1 and 8 are inhibited, while AC3, 5, 6, and 9 are insensitive to regulation by $G\beta\gamma$ (Willoughby and Cooper, 2007).

The diterpene forskolin, which is extracted from the plant *Coleus Forskohlii*, is a potent activator of all ACs except AC9 (Seamon et al., 1981; Hanoune and Defer, 2001). Forskolin binds to the catalytic cleft and promotes assembly of the two cytoplasmic domains, which activates the enzyme (Tesmer et al., 1997; Zhang et al., 1997).

Calcium has well-documented regulatory effects on AC activity (Sunahara et al., 1996; Hanoune and Defer, 2001; Cooper, 2003; Willoughby and Cooper, 2007). AC1 and 8 are stimulated by calcium by a CaM-dependent mechanism. Mutation of selected residues in the CaM-binding domain of AC1 inhibited calcium-stimulated activity (Wu et al., 1993). AC8 has two CaM-binding domains, one in the N-terminus and one in the C-terminus. The N-terminal domain had little effect on AC8 function when mutated. However, mutation of the C-terminal domain decreased calcium-mediated AC8 activity. Interestingly, removal of the domain resulted in a hyperactive enzyme. This implies that the C-terminal CaM-domain in AC8 is involved in autoinhibition of the enzyme (Gu and Cooper, 1999). Later, it was shown that the N-terminal CaM-binding domain is important in recruiting CaM. Furthermore, AC8 binds partially calcium-bound CaM, but only fully calcium-bound CaM stimulates the activity of AC8 (Simpson et al., 2006). In contrast to AC1 and 8, AC5 and 6 activities are inhibited by calcium. Interestingly, the mechanism of calcium-mediated inhibition is still largely unresolved and is not CaM-dependent (Willoughby and Cooper, 2007). One hypothesis is that calcium ions compete with magnesium ions in the catalytic site, the latter being critical for the catalytic activity (Guillou et al., 1999).

Phosphorylation-mediated regulation has been shown for some ACs. The activity of AC5 and AC6 is reduced by PKA-mediated phosphorylation (Premont et al., 1992; Iwami et al., 1995; Chen et al., 1997). The PKA phosphorylation site in AC 6, which lies within the catalytic domain at serine 674, is responsible for inhibition of activity (Chen et al., 1997). There is also evidence for PKC-mediated activation or inhibition of several ACs. However, the significance of PKC-mediated phosphorylation of ACs in intact cells remains inconclusive (Willoughby and Cooper, 2007). AC3 is the only isoform that has been implicated in direct phosphorylation-mediated inhibition by CaMKII (Willoughby and Cooper, 2007). Wei and colleagues showed that serine 1076 in AC3 was a direct phosphorylation site for CaMKII (Wei et al., 1996).

2.3.2 cAMP breakdown by phosphodiesterases

Phosphodiesterase (PDE) -mediated breakdown of cAMP is the main pathway for reducing cellular cAMP concentrations. The cyclic nucleotidase superfamily consists of 11 families of PDEs. Dual-specificity PDEs hydrolyze the cyclic phosphate bond in both cAMP and cGMP and produce 5'-AMP and 5'-GMP, respectively. PDE1, 2, 3, 10, and 11 belong to this group of PDEs. However, PDE4, 7, and 8 are cAMP-specific PDEs, while PDE5, 6, and 9 are cGMP-specific PDEs. PDE1-4 are expressed in many tissues, while other cAMP-hydrolyzing PDEs are not widely expressed (Francis et al., 2011). The cAMP-specific PDEs will be discussed in more detail below.

Four genes generate the PDE4 family and they are termed PDE4A-PDE4D. More than 20 members have been described in the PDE4 family and they are produced from these four genes by alternative start sites and alternative splicing (Bender and Beavo, 2006). The ability of PDE4 isoforms to affect cellular signaling is largely dependent on the localization and phosphorylation-regulated activity of the enzyme (Houslay and Adams, 2003; Bender and Beavo, 2006). In addition, dimerization is important for the regulation and autoinhibition of PDE4 (Francis et al., 2011). The localizations of several PDE4 isoforms have been reported to be regulated by different scaffold proteins. PDE4D5 localization is regulated by RACK1 (Yarwood et al., 1999). PDE4D3 is localized to signaling complexes orchestrated by A-kinase anchoring protein (AKAP) (Dodge et al., 2001). Furthermore, β -arrestins have been shown to recruit PDE4 isoforms to β_2 -adrenoceptors. This negative feedback counteracts the cAMP formed downstream of the activated β_2 -adrenoceptor (Perry et al., 2002). Regulatory domains and phosphorylation sites are located in the N-terminus. PDE4A has a domain in the N-terminus that allows the enzyme to bind the membrane lipid phosphatidic acid in a calcium-dependent manner (Baillie et al., 2002). The activity of PDEs was increased in response to TSH and forskolin in FRTL-5 cells. Moreover, it was concluded that the increase in PDE activity was a result of PKA-mediated phosphorylation at serine 54 of PDE4D3 (Sette et al., 1994; Sette and Conti, 1996). In contrast, an inhibitory effect on PDE4D3 has been reported by ERK2-mediated phosphorylation of serine 579 (Hoffmann et al., 1999). PDE4D3 has docking sites for ERK2, which are crucial for ERK2 binding and phosphorylation of PDE4D3 (MacKenzie et al., 2000). Interestingly, sumoylation of PDE4 was shown to augment activation by PKA and to repress inhibition by ERK (Li et al., 2010).

The PDE7 family has two genes, called PDE7A and PDE7B. Not much is known about the regulation and importance of this family of PDEs. It has been hypothesized that PDE7 is important for maintaining basal cAMP levels (Bender and Beavo, 2006). PDE7 seems to be of importance in immune cells (Smith et al., 2003). Furthermore, inhibition of the PKA pathway via PDE7-activation is important for T-cell activation (Li et al., 1999). AKAPs have been shown to bind and regulate the translocation of several PDEs (Bender and Beavo, 2006). The AKAP myeloid translocation gene translocates PDE7 to the Golgi (Asirvatham et al., 2004). A potential role for PDE7B in cancer was shown when increased expression of PDE7B was measured in chronic lymphocytic leukemia cells. The authors reported that the increased PDE7B expression hampered PKA-dependent apoptosis in these cells (Zhang et al., 2008).

Like the PDE7 family, the PDE8 family contains two genes termed PDE8A and PDE8B (Bender and Beavo, 2006). PDE8 has the highest affinity for cAMP of all PDEs (Gamanuma et al., 2003). PDE8 has two regulatory domains (PAS and REC), however, no clear function have been described for either of them (Bender and Beavo, 2006). One report shows that PDE8A is important for regulating excitation-contraction coupling in the heart (Patrucco et al., 2010).

Patrucco and colleagues showed that myocytes from PDE8A knockout mice had lesser calcium currents coming through voltage-gated channels than wild-type myocytes. Moreover, the importance of PDE8A has been studied in the testosterone-producing Leydig cells of the testis. The authors reported that the sensitivity of steroidogenesis for luteinizing hormone was increased four-fold in PDE8A knockout mice (Vasta et al., 2006).

2.3.3 Protein kinase A

PKA is a direct downstream effector of cAMP and one of the most studied serine/threonine protein kinases (Pearce et al., 2010). In the inactive state PKA has four subunits, two catalytic subunits and two regulatory subunits. When ACs are activated and cAMP is produced, cAMP binds to the regulatory subunits and the catalytic subunits are free to phosphorylate substrates (Chin et al., 2002; Taylor et al., 2005; Taylor et al., 2008; Pearce et al., 2010).

There are several isoforms of cAMP-binding regulatory subunits, termed RI α , RI β , RII α , and RII β . However, they all have a similar structure with the same domains but are not functionally redundant. In the N-terminus they have a D/D domain that orchestrates dimerization and binding to AKAPs. Following the D/D domain is an inhibitor sequence and two cAMP-binding domains. The inhibitor sequence functions as pseudosubstrate for the catalytic site in the catalytic subunit, thus suppressing activity (Taylor et al., 2005; Taylor et al., 2008).

There are three isoforms of the catalytic subunits called C α , C β , and C γ . The major isoforms are the C α and C β -isoforms (Taylor et al., 2005; Taylor et al., 2008). In addition to cAMP binding to the regulatory subunits, threonine 197 of the activating loop in the catalytic subunit must be phosphorylated by phosphoinositide-dependent protein kinase 1 (PDK1) (Cheng et al., 1998). PKA does not have intrinsic domains that control their localization, rather PKA requires the scaffolding protein AKAP to localize to specific sites. AKAP controls the localization of PKA by binding to the regulatory subunits. There are over 50 different AKAPs that localize PKA to the plasma membrane, mitochondria, or the cytoskeleton. Furthermore, AKAPs bring together PKA with other signaling molecules such as receptors, kinases, and channels (Wong and Scott, 2004).

The best characterized PKA substrate is the transcription factor cAMP response element (CRE) binding protein (CREB), which binds to CRE sequences in gene promoters and subsequently regulates transcription. The first step in CREB activation is the transport of PKA into the nucleus. This is mediated by A-kinase interacting protein 1 (AKIP1) (Sastri et al., 2005). CREB is activated upon phosphorylation of serine 133 by PKA. Phosphorylation of CREB augments its association with the co-activators CREB-binding protein (CBP) and p300. CBP and p300 catalyzes histone acetylation in the promoter, thus aiding transcription by recruiting DNA polymerase II complexes. CREB is regulated by co-activators called cAMP-regulated transcriptional co-activators (CRTCs). In the inactive state, CRTCs are bound to 14-3-3 proteins in a phosphorylation-dependent manner. When cAMP and calcium levels rise, calcineurin dephosphorylates the CRTCs, which triggers nuclear translocation of the CRTCs and subsequent activation of CREB. In addition, the elevated cAMP inhibits salt-inducible kinases that phosphorylate and thus inhibit CRTCs. This model where the calcium and cAMP pathways cooperate may explain results where CREB-phosphorylation on serine 133 has no effect on gene expression (Altarejos and Montminy, 2011). In thyroid cells, many but not all effects leading to proliferation by activation of the TSHR/cAMP/PKA-pathway are mediated by CREB (Rivas and Santisteban, 2003).

2.3.4 Exchange protein activated by cAMP

All effects mediated by cAMP are not a result of activated PKA. The exchange protein activated by cAMP (EPAC) is a GEF for the small GTPase Rap1 (de Rooij et al., 1998). There are two isoforms of EPAC; EPAC1 is ubiquitously expressed while EPAC2 is mostly expressed in the brain. Both EPACs have a N-terminal regulatory domain and a C-terminal catalytic domain. The regulatory domain directly interacts with and inhibits the catalytic domain when cAMP is not present. Upon cAMP-binding to the regulatory domain, the conformation changes and inhibition is removed (Borland et al., 2009; Breckler et al., 2011).

EPAC/Rap1 controls integrin-mediated adhesion to fibronectin in response to stimulation of G_sα-coupled receptors (Rangarajan et al., 2003). Moreover, EPAC/Rap1 increases adhesion to other cells through vascular endothelial cadherin (Fukuhara et al., 2005). One possible mechanism for increased adhesion is that EPAC/Rap1 promotes activation of Rac1-specific GEFs (Birukova et al., 2007). Moreover, EPAC/Rap1 mediates the anti-inflammatory effects of cAMP in vascular endothelial cells (Sands et al., 2006).

Another downstream effector of EPAC/Rap is PLCε. EPAC1 induces calcium mobilization from intracellular stores by activating Rap2b, which subsequently activates PLCε (Schmidt et al., 2001). However, results showing PLCε-activation by Rap1 have also been reported (Song et al., 2002; Baljinnyam et al., 2010). The importance of EPAC-activated PLCε has been shown in several model systems. For example, PLCε was involved in regulating both the migration of melanoma cells (Baljinnyam et al., 2010) and the acrosome reaction of human sperm cells (Branham et al., 2009).

Like for many other signaling molecules, not only the activity but also the localization of EPAC is important for the function (Breckler et al., 2011). The muscle-specific AKAP, mAKAP has been implicated in coordinating cAMP signaling by providing a platform for PKA, PDE4D3, and EPAC1 (Dodge-Kafka et al., 2005). Furthermore, AKAP9 was shown to be important for EPAC1-mediated integrin adhesion (Sehrawat et al., 2011). The translocation of EPAC1 to the plasma membrane requires the activation of ezrin-radixin-moesin proteins, which function as scaffold proteins that directly bind to EPAC1 upon receptor activation. In addition, the translocation of EPAC1 to the plasma membrane was crucial for EPAC1-mediated cell adhesion (Ponsioen et al., 2009; Gloerich et al., 2010).

2.4 ERK1/2 signaling

Cells respond to a multitude of different stimuli by activating the mitogen-activated protein kinase (MAPK) pathways (Meloche and Pouyssegur, 2007; Raman et al., 2007). There are three major pathways in the MAPK family, the ERK1/2, the p38 kinase, and the c-jun N-terminal kinase pathway. The regulation and significance of the ERK1/2 pathway will be discussed here. However, a comprehensive overview of the ERK1/2 pathway is beyond the scope of this thesis, only the general regulation mechanisms are discussed herein. The ERK1/2 pathway is activated by a three-module phosphorylation cascade starting with RAF, then MAP/ERK kinase (MEK) and ending with the phosphorylation of ERK1/2. Many of the signaling pathways leading to activation of ERK1/2 go through the small GTPases of the Ras family and G-proteins (Figure 5).

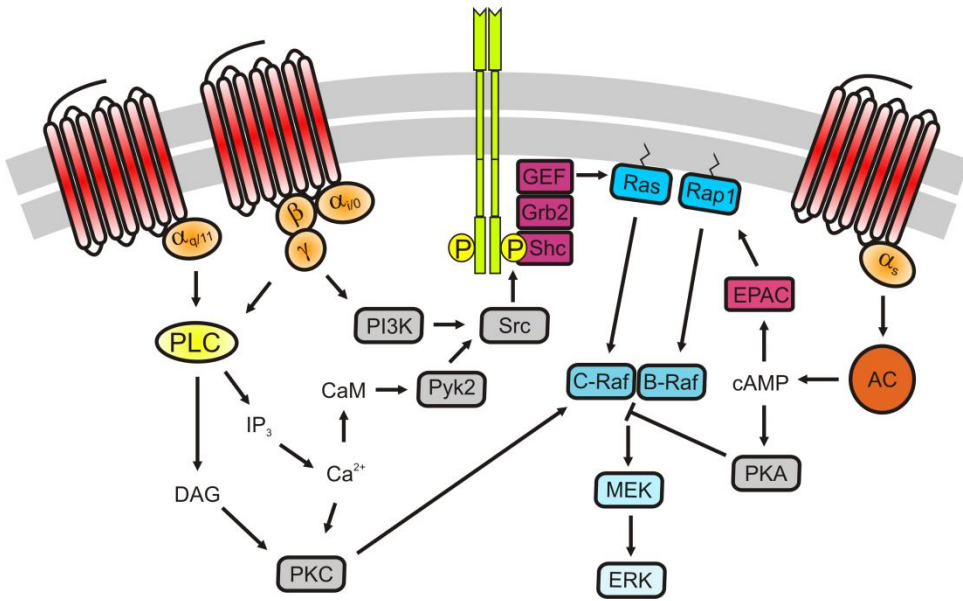


Figure 5. ERK signaling regulated by GPCRs and RTKs. Activation of PLC increases the availability of calcium, which activates Src through CaM and Pyk2. Src phosphorylates tyrosine residues on RTKs, thereby activating them. Activation of RTKs generates phosphorylated tyrosine residues, which are binding sites for Shc. Shc binds Grb2 and RasGEFs that activate the ERK pathway through Ras. $\beta\gamma$ -subunits of G_i -proteins are able to activate Src through PI3K. Activation of PLC also generates DAG that activates PKC. PKC circumvents Ras by directly activating C-Raf. Activation of ACs generates cAMP, which activates ERK through EPAC and Rap1 [modified from (May and Hill, 2008)].

2.4.1 Ras

Rap GTPases function as cellular switches, activated when bound to GTP and inactive when bound to GDP. The activation and inactivation of Ras is controlled by GEFs and GAPs, respectively. RasGEFs stimulate the exchange of GDP for GTP and RasGAPs stimulate the GTPase activity of Ras, thus inactivating it (Karnoub and Weinberg, 2008; Vigil et al., 2010).

A series of post-translational modifications is crucial for the membrane association of Ras and thus its activation (Karnoub and Weinberg, 2008). In a series of steps, farnesylation takes place on a C-terminal CAAX-motif. First, cysteine 186 is farnesylated (Hancock et al., 1989), then the remaining AAX amino acids are removed, and finally the now C-terminal cysteine

undergoes methylation (Gutierrez et al., 1989). Moreover, Ras has a palmitoylation domain next to the CAAX-motif. Together, these two post-translational modifications support the translocation of Ras to the plasma membrane (Hancock et al., 1990). In the early 1990's, several groups reported simultaneously how Ras is coupled to receptor tyrosine kinases and activated. The scaffold protein, growth factor-bound protein 2 (GRB2) binds to phosphorylated tyrosine residues on the receptor with its SH2-domain. Furthermore, receptor-bound GRB2 functions as scaffold and binds RasGEFs with a SH3-domain (Egan et al., 1993; Gale et al., 1993; Li et al., 1993; Rozakis-Adcock et al., 1993).

Calcium is able to directly activate a class of RasGEFs called RasGRF1 and RasGRF2, which stimulate the activity of Ras. There are also calcium/DAG regulated GEFs, called CalDAG-GEFs, which have a C1 domain that binds DAG and a calcium-binding EF-hand. However, only CalDAF-GEF2 activates Ras. Moreover, calcium regulates the shutdown of Ras signaling by activating two RasGAPs, p120 RasGAP and calcium-promoted Ras inactivator (Cullen and Lockyer, 2002).

2.4.2 Rap1

The Rap1 GTPase is another member of the Ras family that activates the ERK1/2 pathway through B-RAF (Vossler et al., 1997). Like Ras, Rap1 switches between an active GTP-bound state and an inactive GDP-bound state, which is regulated by GEFs and GAPs (Hattori and Minato, 2003; Gloerich and Bos, 2011).

Rap1 has the same C-terminal CAAX domain as Ras (Kawata et al., 1988), but the domain seems to be geranylgeranylated instead of farnesylated like Ras (Kawata et al., 1990). It has been shown that geranylgeranylation induces membrane association in the same manner as farnesylation. However, the membrane binding affinity is stronger when modified by the 20-carbon geranylgeranyl group, than the 15-carbon farnesyl group (Hancock et al., 1991). The importance of geranylgeranylation and translocation of Rap1 to membranes has been shown (Sato et al., 2001). Moreover, Rap1 is translocated to the plasma membrane from endosomes in response to mitogens (Bivona et al., 2004).

Interestingly, in addition to the already discussed EPACs, two isoforms of CalDAG-GEFs activate Rap1. There are reports showing DAG-mediated translocation of CalDAG-GEF3 and the activity of CalDAG-GEF1 is modulated by calcium (Guo et al., 2001; Hattori and Minato, 2003; Gloerich and Bos, 2011). However, the regulation of CalDAG-GEFs by calcium and DAG is still controversial. CalDAG-GEF1 is not directly regulated by DAG, and calcium has had only minor modulatory effects on CalDAG-GEFs in some studies (Gloerich and Bos, 2011).

2.4.3 The RAF protein kinase family

Directly downstream of Ras and Rap1 is the serine/threonine kinase rapidly accelerated fibrosarcoma (RAF). There are three isoforms of RAF called A-RAF, B-RAF, and C-RAF (also known as RAF-1). Only the regulation of B-RAF and C-RAF will be discussed here. RAF kinases have three conserved regions, which contain the Ras-binding domain, a regulatory serine/threonine rich domain that binds the 14-3-3 protein, and the protein kinase domain, respectively. In addition to the inhibitory binding site for the 14-3-3 protein in the conserved region 2, there is a stimulatory site for the 14-3-3 protein after the kinase domain in the C-terminus (Wellbrock et al., 2004; Roskoski, 2010).

In the inactive state, the 14-3-3 protein is bound to two phosphorylated serine residues of RAF and is located in the cytosol. The binding of a GTP-bound Ras to C-RAF and B-RAF recruits them to the plasma membrane. However, it is not enough to fully activate these kinases. The RAF proteins undergo conformational changes and require phosphorylation and dephosphorylation to acquire full activity (Wellbrock et al., 2004; Roskoski, 2010). C-RAF has several serine residues that are phosphorylated by PKA, namely serines 43, 233, and 259. Phosphorylation of these sites inhibits C-RAF activity. The serine/threonine kinase AKT has also been shown to phosphorylate serine 259. There are several phosphorylation sites that augment C-RAF activity. Phosphorylation of serine 621 is important for 14-3-3 binding to the C-terminus, which stabilize the active conformation. Phosphorylation of serine 338 and tyrosine 341 are crucial for the activity of C-RAF. Src kinase is the kinase responsible for the phosphorylation of tyrosine 341. Furthermore, the phosphorylation of threonine 491 and serine 494 in the kinase domain is needed for activation (Chong et al., 2001). Like for serine 338, the kinase responsible for threonine 491 and serine 494 phosphorylation is still unclear. However, one explanation is that they are autophosphorylated (Wellbrock et al., 2004). Serine 471 in the kinase domain of C-RAF is critical for kinase activity and interaction with MEK (Zhu et al., 2005). Mutation of the corresponding B-RAF site, serine 578, resulted in an inactive kinase. The authors speculate that this phosphorylation site is mimicked by the B-RAF V600E mutation, which is often seen in cancers (Zhu et al., 2005). Serine 728 in B-RAF, which is equivalent to serine 621 in C-RAF, is believed to have the same function in B-RAF, i.e. 14-3-3-binding to the C-terminus (Wellbrock et al., 2004). Phosphorylation of threonine 598 and serine 601 in the activation segment is essential for B-RAF function (Zhang and Guan, 2000). In contrast to C-RAF, serine 445 in B-RAF, which is equivalent to serine 338 in C-RAF, is constitutively phosphorylated in B-RAF. As a result, B-RAF has high basal activity (Mason et al., 1999). Membrane recruitment is the crucial activation step for B-RAF. It is thought that the high basal activity and the need for only a single point mutation is the reason why B-RAF mutations often are reported in cancer (Wellbrock et al., 2004).

RAF dimerization is important for kinase activity. Both homo- and heterodimers are formed. Serine 621 in C-RAF is important for dimerization and C-RAF/B-RAF heterodimers are more active than either homodimer or monomers. In addition, threonine 753 in B-RAF is phosphorylated by ERK, which has been shown to decrease dimerization (Rushworth et al., 2006).

Feedback inhibition from ERK is a common inhibitory signal for both C-RAF and B-RAF (Wellbrock et al., 2004; Roskoski, 2010). ERK phosphorylates C-RAF on five sites (Dougherty et al., 2005), while B-RAF has four inhibitory ERK-phosphorylation sites that inhibit Ras-binding and B-RAF heterodimerization with C-RAF (Ritt et al., 2010).

2.4.4 The MEK protein kinases

The only well-characterized and widely accepted substrates for RAF are MEK1/2. RAF activates MEK1 by phosphorylation of serine 218 and 222 within the activation segment (Zheng and Guan, 1994). The corresponding residues on MEK2 are serine 222 and 226. MEKs have a ~290 amino acid protein kinase domain and smaller N- and C-terminal segments of ~30 and ~70 residues, respectively. MEK1/2 are dual-specificity kinases as they phosphorylate both tyrosine and threonine residues. The N-terminus contains the ERK-binding domain, the nuclear export sequence, and an inhibitory segment (Roskoski, 2011). MEK1 is inhibited by phosphorylation

of four residues; serine 212 in the activation site (Gopalbhai et al., 2003), threonines 286 (Rossomando et al., 1994) and 292 (Eblen et al., 2004) in the the kinase domain, and threonine 386 in the C-terminus (Brunet et al., 1994). Phosphorylation of threonine residues 292 and 386 by ERK is important for negative feedback control (Brunet et al., 1994). The threonine 292 in MEK1 is essential for negative feedback control by ERK on MEK1/2 heterodimers, as MEK2 lacks an equivalent residue (Catalanotti et al., 2009).

2.4.5 ERK1/2

The last kinases in this tightly regulated pathway are the serine/threonine kinases ERK1/2. ERK1/2 are activated by MEK1/2-induced phosphorylation of threonine 202 and tyrosine 204 in ERK1 and threonine 185 and tyrosine 187 in ERK2, both of which are within respective kinase activation domain (Payne et al., 1991; Meloche and Pouyssegur, 2007). The phosphorylations are ordered, in the case of ERK2, phosphorylation of tyrosine 187 precedes phosphorylation of threonine 185 (Haystead et al., 1992). The nuclear export signal located in MEK functions as an anchor for ERK1/2 until it is activated. Upon phosphorylation, ERK1/2 is free to translocate to the nucleus (Adachi et al., 1999).

ERK1/2 phosphorylates serine and threonine residues residing in a Pro-Xaa-Ser/Thr-Pro motif. In addition, ERK1/2 substrates contain several different domains that augment ERK1/2 binding and thus phosphorylation. Examples of substrates that are phosphorylated by ERK1/2 are transcription factors such as Elk1 and c-Fos, protein kinases such as p90 ribosomal s6 kinase, and numerous other cytoplasmic targets (Pearson et al., 2001).

The MAPK phosphatases (MPKs) dephosphorylate ERK1/2 and thus inactivate them. MKPs are regulated by different mechanisms (Liu et al., 2007). MKP1 is induced upon activation of ERK1/2 by immediate early genes (Sun et al., 1993). Furthermore, the stability of MKP1 is increased upon phosphorylation by ERK1/2, thus facilitating dephosphorylation (Brondello et al., 1999). Moreover, MKP1 has been shown to translocate to the nucleus where it inhibits ERK1/2-induced phosphorylation of Elk1 (Wu et al., 2005). For MKP3, the binding to ERK1/2 is enough to increase phosphatase activity (Camps et al., 1998). The MKP, DUSP5, binds and anchors inactive ERK1/2 in the nucleus (Mandl et al., 2005). In contrast, MKP3 dephosphorylates ERK1/2 and anchors it to the cytoplasm (Karlsson et al., 2004).

2.4.6 Regulation of the ERK1/2 pathway by GPCRs

There are well-documented effects of GPCR-mediated activation of the ERK-pathway through various mechanisms (Goldsmith and Dhanasekaran, 2007; May and Hill, 2008). The G_sα-protein activates ERK1/2 by increasing cAMP which activates an EPAC/Rap1/B-Raf pathway (Vossler et al., 1997; Laroche-Joubert et al., 2002). Depending on cell type, another RapGEF than EPAC is responsible for G_sα-mediated activation of ERK1/2. In this pathway, PKA activates Src, which subsequently activates a RapGEF called C3G (Obara et al., 2004; Weissman et al., 2004; Wang et al., 2006). G_sα is further able to negatively regulate ERK1/2 by activating PKA that inhibits C-Raf by phosphorylating serine 259 (Dhillon et al., 2002).

The G_iα-subunit may inhibit or activate ERK1/2 in a cell type-dependent manner. If the cell type is inhibited by cAMP, G_i-proteins are able to reverse this by directly inhibiting ACs (Hanoune and Defer, 2001; Cooper, 2003). Furthermore, the G_iα-subunit binds and activates a Rap1GAP, thus inactivating Rap1 that antagonizes Ras-signaling (Cook et al., 1993; Okada et

al., 1998; Mochizuki et al., 1999). The $G_i\beta\gamma$ -subunit, on the other hand, is able to activate ERK1/2 via Src, which activates Ras by phosphorylating receptor tyrosine kinases. The phosphorylated tyrosine residue attracts the adaptor protein Shc, which subsequently binds to GRB2 and RasGEF. Two different pathways have been shown by which the $G_i\beta\gamma$ -subunit might activate Src. First, it has been shown that the $G_i\beta\gamma$ -subunit activates PI3K (Stephens et al., 1994; Lopez-Illasaca et al., 1997). Second, the $G_i\beta\gamma$ -subunit activates PLC, which subsequently activates calcium release and CaM-mediated activation of the protein tyrosine kinase Pyk2 (Dikic et al., 1996; Della Rocca et al., 1997).

The $G_{q/11}\alpha$ -subunit activates the ERK1/2 pathway using the same calcium-activated pathway as the $G_i\beta\gamma$ -subunit (Dikic et al., 1996; Della Rocca et al., 1997). Interestingly, the $G_q\alpha$ -subunit has been shown to activate ERK1/2 through a Rap1/B-Raf mediated pathway. Moreover, the authors showed that calcium activated Rap1 via CalDAG-GEF1 (Guo et al., 2001).

All G-proteins that activate PLC are able to positively regulate the ERK1/2 pathway through conventional and novel PKCs (Goldsmith and Dhanasekaran, 2007; May and Hill, 2008). PKC activates C-RAF directly without the involvement of Ras (Ueda et al., 1996). A possible mechanism could be that PKC phosphorylates and inhibits the C-Raf kinase inhibitory protein, which leads to activation of C-Raf (Corbit et al., 2003). Lastly, transactivation of receptor tyrosine kinases by GPCRs is also employed by cells, which is an additional pathway to ERK1/2 activation mediated by GPCRs (Wetzker and Bohmer, 2003).

2.5 The synthesis of thyroid hormones

The thyroid gland is a major endocrine organ, whose sole function is to produce the thyroid hormones 3,5,3',5'-tetraiodothyronine (T4) and 3,5,3'-triiodothyronine (T3) (Dunn and Dunn, 2001). Low circulating levels of thyroid hormones are sensed by the hypothalamus, which produces TRH. Subsequently, TRH stimulates the pituitary to produce TSH, which stimulates the thyroid to produce thyroid hormones (Chiamolera and Wondisford, 2009). The thyroid has two interconnected lobes, one on each side of the trachea. There are two hormone-producing cell types in the thyroid, the thyroid hormone-producing epithelial (also known as follicular) cells and calcitonin-producing parafollicular cells. Histologically, the thyroid consists of spherical structures called follicles surrounded by connective tissue and blood capillaries. The follicles are comprised of mostly epithelial cells that enclose the lumen of the follicle. The precursor of thyroid hormones, thyroglobulin (Tg), is stored in the lumen. The thyroid-specific transcription factors paired box (Pax)-8, thyroid transcription factor (TTF)-1, and TTF2 regulate the transcription of thyroid-specific genes beginning at embryonic day 8.5 in mice (Van Vliet, 2003).

Thyroid hormones are produced from the precursor Tg. After its synthesis, Tg forms stable dimers that are shuttled to the Golgi with the help of chaperones. In the Golgi, the glycosylated residues of Tg are processed where after it is transported in vesicles to the lumen of the follicle. Iodide is taken up into the epithelial cells at the basal side by the sodium iodide symporter (NIS). Iodide moves with the electrochemical gradient to the apical, lumen-facing, side of the epithelial cell. Iodination of Tg takes place at the apical membrane, where thyroid peroxidase (TPO) iodineates tyrosine residues on Tg and subsequently produces iodothyronines. Next, the newly iodinated Tg is taken up by micropinocytosis and goes

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through the endosomes and lysosomes where Tg is degraded and T4 and T3 are released from the now obsolete Tg scaffold. The newly formed thyroid hormones are then released into the bloodstream (Dunn and Dunn, 2001). The thyroid hormones elicit negative feedback on the hypothalamus and the pituitary. This results in lowered secretion of TRH and TSH, respectively (Chiamolera and Wondisford, 2009).

3 AIMS

The unifying factor for this study is calcium signaling, specifically conveyed by TRPC channels. The aims were to elucidate the functions of TRPC3 and TRPC2 channels and attempt to answer the following questions:

1. What roles have overexpressed TRPC3 in cells, apart from conveying entry of extracellular calcium?
2. Phosphatase inhibition revealed a calcium entry pathway in thyroid-derived FRTL-5 cells. Is this calcium entry regulated by autocrine S1P-signaling?
3. The aforementioned calcium entry in FRTL-5 cells was conducted by TRPC2. What function does TRPC2 have in thyroid cell signaling, and is there communication between the calcium- and cAMP-mediated signaling pathways?

4 EXPERIMENTAL PROCEDURES

4.1 Cell culture and cell lines (I-III)

Human embryonic kidney cells (HEK-293) were used for the study with overexpression of TRPC3 (I). HEK-293 cells were chosen as they are well suited for over-expression studies and show robust calcium signals in response to various agonists. MEM medium containing 10% fetal bovine serum, 2 mM L-glutamine, 50 U/ml penicillin and 50 µg/ml streptomycin was used for growing HEK-293 cells. When the cells reached 80-90% confluency, they were detached with PBS + 0.02% EDTA + 0.1% trypsin and reseeded at a ratio of 1:8.

Rat thyroid FRTL-5 cells were used for studying autocrine S1P signaling (II) and for elucidating the functions of TRPC2 in thyroid cells (III). FRTL-5 cells were grown in Coon's modified Ham's F12 medium supplemented with 5% calf serum, six hormones (insulin, 10 µg/ml; transferrin, 5 µg/ml; hydrocortisone, 10 nM; tripeptide gly-L-his-L-lys, 10 ng/ml; TSH, 0.3 mU/ml; somatostatin, 10 ng/ml), and 50 U/ml penicillin and 50 µg/ml streptomycin. FRTL-5 cells were detached with PBS + 0.02% EDTA + 0.1% trypsin and reseeded at a ratio of 1:4.

Primary rat thyroid cells were used for investigating what TRPC channels are expressed in vivo (III). Rat thyroid tissue was digested with 0.2% collagenase, and follicles were plated on poly-L-lysine coated plates in Coon's modified Ham's F12 medium supplemented with 1% FBS, 0.3 mU/ml TSH and 1 µg/ml insulin to allow cells to attach. After three days, the medium was changed to serum-free Coon's modified Ham's F12 medium supplemented with 0.3 mU/ml TSH and 1 µg/ml insulin to allow thyroid cell survival and prevent the survival of other cell types (Eggo et al., 1996). After two weeks in serum-free conditions, the cells were used for experiments.

All cells were grown in a water-saturated atmosphere of 5% CO₂ and 95% air at 37°C.

4.2 Plasmid constructs (I-III)

The YFP plasmid (pEYFP-N1) was from Clontech (Mountain View, CA, USA). The TRPC3 plasmids pCAGGSM2_GFP and pcDNA3h-TRP3topaz were kindly provided by Dr. Bernd Nilius (KU Leuven, Leuven, Belgium) and Dr. James Putney, Jr (NIH/NIEHS, Research Triangle Park, NC, USA), respectively. The expression vector pCMV6-XL4-SK2 was purchased from Origene (Rockville, MD, USA). SK2 was cut out with *Not*I and subcloned into a pcDNA3 vector. The SK2 insert was confirmed by sequencing. The truncated form of TRPC2 (TRPC2-DN) was a kind gift from Dr. Genevieve Bart (University of Eastern Finland, Kuopio, Finland). MEK1-S218E/S222D and MEK1-K97M was originally obtained from Dr. Natalie Ahn (University of Colorado, Boulder, CO, USA). The PDE4D3 construct was originally obtained from Dr. Marco Conti (University of California, San Francisco, CA, USA). N-EPAC was kindly provided by Dr. Daniel Altschuler (University of Pittsburgh, PA, USA). The Rap1A^{N17} construct was originally obtained from Dr. Jean de Gunzburg (Institut Curie, Paris, France). The RTP1 plasmid was kindly provided by Dr. Debra Fadool (Florida State University, Tallahassee, FL, USA). The rat GFP-TRPC2 construct was made as follows. The rat TRPC2 cDNA was a gift from Dr. Catherine Dulac

(Harvard University, Cambridge, MA, USA). The cDNA was cut out from the pBluescript cloning vector with BamHI and NcoI. After blunting the NcoI site, the fragment was subcloned into pEGFP-C3 (Clontech) cut with BglIII and SmaI. By using the BamHI site, the initiator methionine was deleted from the TRPC2 gene and the fusion generated the EGFP-TRPC2 construct.

4.3 Transfections (I-III)

4.3.1 Calcium phosphate precipitation (I)

HEK-293 cells are easily transfected cells and calcium phosphate precipitation is an affordable way of transfecting cells. Cells were seeded on poly-L-lysine coverslips in a 35-mm plate and allowed to attach over night. A DNA precipitate was made by mixing HEPES-buffered saline (280 mM NaCl, 10 mM KCl, 1.5 mM Na₂HPO₄, 50 mM HEPES, and 12 mM D-glucose, pH 7.05) with plasmid DNA dissolved in Tris-EDTA-CaCl₂ (1mM Tris base, 0.1 mM EDTA, and 0.26 M CaCl₂, pH 8.0). The resulting mixture was incubated for 20 min at room temperature to allow the precipitate to form. The precipitate was then resuspended and added to the cells. The medium was removed after 4 hours of incubation in the incubator. The cells were then treated with 15% glycerol in HEPES-buffered saline for 1 min to improve transfection efficacy. Normal growth medium was added after a wash with PBS, and the cells were thereafter returned to the incubator. Cells were used for experiments after 24h.

4.3.2 Fugene HD (II)

Fugene HD (Roche, Indianapolis, IN, USA) was used in some experiments to transfect FRTL-5 cells. Cells were grown to 70% confluency prior to starting the experiment. Plasmid DNA and transfection reagent was diluted in OptiMem (Invitrogen, Carlsbad, CA, USA). The amount of DNA and reagent was scaled to the size of the plate according to the manufacturer's instructions. Experiments were performed 24 h after transfection.

4.3.3 Electroporation (III)

Electroporation was the most reliable method to get a large amount of FRTL-5 cells transfected with various plasmid constructs. Cells from a 100-mm plate were detached and counted, 4×10^6 cells were centrifuged down and resuspended in 400 μ l OptiMEM (Invitrogen, Carlsbad, CA, USA) together with 20 μ g plasmid DNA. The cells were then electroporated in BTX cuvettes (975 μ F and 240 V) using a Bio-Rad Gene Pulser Xcell (Bio-Rad Laboratories, Hercules, CA, USA). The electroporated cells were used for experiments after 48-72 h.

4.3.4 Generation of a stable TRPC2 knockdown cell line (III)

FRTL-5 cells were plated on 12-well plates. The following day transfections were carried out with Fugene HD and shRNA plasmids (SABiosciences, Frederick, MD, USA) according to the manufacturers' instructions. 48 h post-transfection, puromycin (1 μ g/ml) was added to the growth medium to kill untransfected cells. Puromycin was included in the growth media from here on.

4.3.5 Transduction of FRTL-5 cells with SK1^{WT} and SK1^{G82D} (II)

Human SK1 cDNA was cloned and FLAGtagged at the 3' end according to Pitson et al. (Pitson et al., 2000a; Pitson et al., 2000b). The SK-FLAG fragment was PCR-amplified by using primers with 5' *MluI* and 3' *Sall* sites and cloned into the WPT-GFP lentiviral vector, which had been digested with *MluI* and *Sall* to remove the GFP gene. Lentiviral vectors expressing the SK-FLAG construct were produced by transient three-plasmid co-transfection into HEK 293T cells by using calcium phosphate precipitation. The three-plasmid mixture consisted of 14.5 µg WPT-SKFLAG, 8.3 µg pCMVΔR8.91, and 2.1 µg MD.G (all plasmids were a kind gift from Dr. D. Trono, Lausanne, Switzerland). The virus-containing medium was harvested 48 h later by filtering through a 0.45 µm filter and centrifugation at 16,000 × *g* for 2.5 h at 4°C. The resulting virus pellets were resuspended in 200 µl serum-free DMEM. For transduction, FRTL-5 cells were plated on six-well plates (1×10⁵ per well), and 24 h later, virus together with 8 µg/ml Polybrene was added at multiplicity of infection 10 and incubated for 6 h after which time the medium was replaced with fresh medium.

4.4 Calcium measurements (I-III)

4.4.1 Cell suspension (II)

The medium was aspirated, and the FRTL-5 cells were detached and washed three times in HBSS. After washing, the cells were incubated with 1 µM Fura-2 AM (Invitrogen, Carlsbad, CA, USA) (30 min, 37°C) in HEPES-buffered salt solution (HBSS) consisting of 118 mM NaCl, 4.6 mM KCl, 1 mM CaCl₂, 10 mM glucose and 20 mM HEPES (pH 7.4). The cells were then washed twice with HBSS buffer, incubated for 15 min at room temperature, and then washed once more. Fluorescence was measured with a Hitachi F2000 fluorometer using excitation wavelengths of 340 and 380 nm and detecting emission at 510 nm. The signal was calibrated by the addition of 1mM CaCl₂ and Triton X-100 to obtain maximum fluorescence. To obtain minimum fluorescence, Tris base was first added to elevate the pH above 8.3, then all calcium was chelated with 5mM EGTA. [Ca²⁺]_i was calculated as described (Grynkiewicz et al., 1985), using a K_d value of 224 nM for Fura-2.

4.4.2 Adherent cells (I, III)

Cells cultured on poly-L-lysine coated coverslips were washed twice with HBSS and incubated with 2 µM Fura-2 AM for 30 min at room temperature. The cells were then washed with HBSS. The coverslip was placed in a perfusion chamber that was mounted on a Zeiss Axiovert 35 microscope. The 340 nm and 380 nm excitation filters were used and emission was measured at 510 nm. Light was obtained from an XBO 75W/2 xenon lamp. The shutter was controlled by a Lambda 10-2 control device (Sutter Instruments, Novato, CA, USA), and images were collected with a SensiCam CCD camera (PCO/CD Imaging, Kelheim, Germany). The images were processed using Axon Imaging Workbench 5.1 software (Axon Instruments, Foster City, CA, USA). The experiments were performed at room temperature. For experiments in calcium-free HBSS, CaCl₂ was omitted and 150 µM EGTA added. The F₃₄₀/F₃₈₀ ratio was used as a measure of intracellular calcium concentrations.

Experimental Procedures

For experiments on permeabilized cells, cells were plated on poly-L-lysine coated coverslips. Cells were loaded with 3 μM mag-Fura-2 AM for 45 min at 37 °C and then washed with KCl rinse buffer (125 mM KCl, 25 mM NaCl, 10 mM HEPES and 0.2 mM MgCl_2 , pH 7.25). Cells were then permeabilized with intracellular buffer (KCl rinse buffer with 200 μM CaCl_2 and 500 μM EGTA, pH 7.25) containing 10 $\mu\text{g}/\text{ml}$ digitonin. The permeabilization process was monitored continuously. When approximately 80 % of the cells were permeabilized, excess digitonin was rinsed away by perfusion with intracellular buffer.

4.5 Qualitative and quantitative polymerase chain reaction (III)

Cells were grown on 60-mm plates. The plates were washed once with ice-cold PBS and RNA was isolated using the Aurum Total RNA Mini Kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. RNA quality and integrity was tested by absorbance spectrometry and by agarose gel electrophoresis, respectively. RNA concentrations were determined using the RiboGreen RNA Quantitation Reagent (Molecular Probes, Eugene, OR, USA). Reverse transcriptase reactions were performed on 0.25 μg RNA using SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) and Oligo(dT)₁₅ primers (Promega, Madison, WI, USA) following the manufacturers' instructions.

The PCR was performed in 50 μl reactions (5 μl cDNA, 1 mM primers, 200 nM each of dATP, dCTP, dGTP and dTTP, 0.5 U DynaZyme EXT DNA polymerase) on a Mastercycler gradient thermal cycler (Eppendorf, Hamburg, Germany) with an activation step at 94°C for 5 min followed by 30 cycles with strand separation at 94°C for 30 s and annealing for 60 s (temperatures listed in Supplementary Table 1) followed by elongation at 72°C for 60 s. Negative controls, where reverse transcriptase was omitted from the cDNA reaction, were performed to rule out that the RNA samples were contaminated with genomic DNA. The PCR products were separated by gel electrophoresis and visualized with ethidium bromide under UV-light.

Quantitative PCR assays were designed using the Universal Probelibrary Assay Design Center (Roche, Basel, Switzerland). qPCR reactions of 10 μl were used, containing 100 nM UPL probe or 200 nM normal length probe, 300 nM forward and reverse primers, 1x Absolute QPCR Rox Mix (Abgene, Rochester, NY, USA) and cDNA. The Absolute QPCR Rox Mix contains Thermo-start DNA polymerase, proprietary reaction buffer, dNTPs and ROX reference dye. 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 (Applied Biosystems, Foster City, CA, USA).

4.6 Immunocytochemistry (I)

For confocal microscopy, the cells were washed with cold PBS, and then fixed using 2% paraformaldehyde in PBS for 10 min. Next, the cells were washed three times with PBS and treated with 1% Triton-X in PBS for 3 min, followed by three washes with PBS. After incubation with blocking solution (PBS containing 1% serum and 0.2% Tween) for 20 min, the cells were incubated overnight at 4°C with primary antibodies in blocking solution. After three washes

with PBS, secondary antibodies were added and the coverslips were incubated in a humidified chamber for 1 h. The coverslips were washed three times with PBS, and were then mounted on a slide with Mowiol. For each staining there was a negative control that was not incubated with primary antibody. The images were acquired using a Leica TCS SP confocal microscope (Leica, Heidelberg, Germany) equipped with an Argon-Krypton laser (Omnichrome, Melles Griot, Carlsbad, CA, USA).

4.7 Sphingosine kinase activity assay (II)

The activity of SK1 and SK2 was assessed as described previously (Olivera et al., 2000). Cells were suspended in assay buffer containing 20 mM Tris-HCl (pH 7.4), 1 mM EDTA, 0.5 mM deoxy pyridoxine, 15 mM NaF, 1mM 2-mercaptoethanol, 1 mM sodium orthovanadate, 10µg/ml leupeptin, 10 µg/ml aprotinin, 8.64 mg/ml β-glycerophosphate, 4 mM phenylmethylsulfonyl fluoride, 20 % glycerol, and 20 µg/ml trypsin inhibitor. The cells were lysed by freeze-thawing followed by brief sonication. The lysate was transferred to centrifuge tubes and centrifuged (105,000×g at 4°C for 90 min). The supernatant was stored at -85°C. Total protein concentration was measured with the Bio-Rad protein assay. The activity of SK was measured with 200 µg total protein at 37°C for 30 min with [γ -³²P]ATP and either 50 µM D-erythro-sphingosine-BSA (1 mM D-erythro-sphingosine in 4 mg/ml BSA) complex or 50 µM D-erythro-sphingosine-Triton X-100 (1 mM D-erythro-sphingosine in 5% Triton X-100). The reaction was stopped by putting the tubes on ice and adding HCl followed by addition of chloroform/methanol/HCl, (100:200:1, vol/vol). The tubes were vortexed, and after 10 min, chloroform and KCl were added together with 10 nmol of D-erythro-sphingosine-1-phosphate (carrier) for phase separation. After 10 min, the tubes were centrifuged (600×g for 10 min), and an aliquot of the organic phase was applied to high performance thin-layer chromatography plate and eluted in a horizontal chamber with 1-butanol/H₂O /methanol/acetic acid, (80:20:20:10, vol/vol). The activity was assessed using autoradiography.

4.8 Sphingosine-1-phosphate production assay (II)

Prior to the experiment, cells were incubated overnight in medium with hormones and 0.2% fatty acid-free BSA. Cells were then stimulated with agonist or vehicle together with [³H]sphingosine (~200,000 cpm). Lipids were extracted by aspirating the culture medium and adding 500 µl ice-cold methanol to the cells, after which the cells were scraped from the Petri dishes and transferred to Eppendorf tubes. The tubes were sonicated for 5 min and then centrifuged (6000×g for 10 min) to remove cell debris. The supernatant was transferred to glass vials. S1P was added to each sample for identification, and the supernatant was evaporated. [³H]S1P was extracted from the medium as previously described (Johnson et al., 2002a). After re-dissolving in methanol, the samples were spotted onto high-performance thin-layer chromatography plates and separated with butan-1-ol/acetic acid/water (3:1:1, vol/vol). S1P was stained with ninhydrin, spots were scraped, and the formed [³H]S1P was counted using liquid scintillation.

4.9 Western blotting (III)

Cells were washed three times with ice-cold PBS, and scraped in lysis buffer (10 mM Tris/HCl (pH 7.7), 150 mM NaCl, 7 mM EDTA, 0.5 % NP-40, 0.2 mM PMSF, and 0.5 µg/ml leupeptin). The lysates were incubated on ice for 15 minutes and then centrifugated at 10,000 x g for 15 min. 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 the primary antibody of interest and an HRP-linked secondary antibody. Protein bands were detected using enhanced chemiluminescence. The Image J program (Abramoff et al., 2004) was used to quantify band intensities.

4.10 Immunoprecipitation of secreted thyroglobulin (III)

Cells were plated on 100-mm plates and grown in 6H medium. After 48 h, aliquotes of the medium were collected and incubated with goat anti-Tg antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The samples were incubated under rotation over-night at 4°C. The following day, protein A/G PLUS-agarose beads (Santa Cruz Biotechnology) were added and the samples where incubated for an additional 2 h. The beads where pelleted by centrifugation (1000 x g, 2 min) and washed three times with immunoprecipitation wash buffer (50 mM Tris pH 7.5, 250 mM NaCl, 0.1% NP-40, 0.05% sodium deoxycholate). After the last wash the beads where resuspended in Laemmli buffer and boiled for 5 min. The beads were pelleted by centrifugation and the supernatant was separated on a 6 % SDS-PAGE gel. Proteins were then transferred onto a nitrocellulose membrane and incubated with goat anti-Tg primary antibody and mouse anti-goat HRP-linked secondary antibody (Santa Cruz Biotechnology). The amount of secreted Tg was normalized to intracellular Tg from respective cell lysate.

4.11 cAMP assay (III)

Cells were plated on 24-well plates and grown in growth medium for 24 hours. The cells were stimulated for 30 min with agonists in a humidified 5 % CO₂ atmosphere at 37°C. A phosphodiesterase inhibitor (3-isobutyl-1-methylxanthine (IBMX), 100 µM) was included in the medium during the stimulations. The incubation was terminated by three washes with cold PBS. Cells were lysed with 300 µl/well 0.1 M HCl containing 0.1 % Triton X-100 for 10 min. Cyclic AMP concentrations in the samples were determined with Direct Cyclic AMP Enzyme Immunoassay Kit (AssayDesigns Inc., Ann Arbor, MI, USA) according to the manufacturer's instructions. Levels of cAMP in the samples were normalized to mg protein in the respective sample.

4.12 Electrophysiology (III)

Cells were plated at low density on 13-mm coverslips. Recordings were made at room temperature (23–25°C). Coverslips were placed in a chamber of 200 μl and continuously perfused at flow rate of 700 $\mu\text{l min}^{-1}$ with an extracellular solution comprised of 150 mM NaCl, 5.4 mM CsCl, 3.0 mM CaCl_2 , and 5 mM HEPES (pH 7.40 with NaOH). Patch pipettes (3–8 M Ω) were filled with a solution containing 135 mM Cs-Methanesulfonate, 10 mM CsCl, 4.25 mM Na_2ATP , 0.5 mM LiGTP, 125 μM EGTA, 10 mM HEPES (pH 7.20 with CsOH). The liquid junction potential of 15 mV was estimated with JPCalc software (Barry, 1994) and was subtracted off-line.

Whole-cell membrane currents (Hamill et al., 1981) were recorded with an EPC-9 amplifier and Pulse software (HEKA Elektronik, Lambrecht, Germany). Cells were stimulated with a series of rectangular voltage pulses (ranging from -135 mV to +105 mV, increment 10 mV and duration 100 ms) that was preceded and followed by 100 ms and 50 ms steps to -95 mV. The protocol was applied at 2 s intervals, during which cells were held at -15 mV. Whole-cell capacitance and series resistance were 80% compensated for and currents were sampled at 20 kHz and filtered at 10 kHz.

Analysis and graphics were done using PulseFit (HEKA Elektronik, Lambrecht, Germany) and Origin (OriginLab, Northampton, MA, USA) software. Mean current level in the end of voltage pulse series was measured (time window 10 ms).

4.13 Statistics (I-III)

Results are expressed as means \pm SEM from at least three independent experiments. Student's *t* test was used when two means were compared. One-way ANOVA and Bonferroni's *post hoc* test was used when three or more means were compared. Two-way ANOVA was used for comparing concentration-responses. A P-value less than 0.05 was considered statistically significant.

5 RESULTS AND DISCUSSION

5.1 Activation mechanisms of TRPC2 and TRPC3 (I, II, III, unpublished)

The activation mechanisms of TRPC2 and TRPC3 have been elucidated in many previous studies (Venkatachalam and Montell, 2007; Birnbaumer, 2009). Both channel types have basal activity that is increased upon stimulation of PLC. Overexpressed TRPC3 showed a high level of basal activity (I, Figure 1), while the basal activity of endogenous TRPC2 was measurable only in patch-clamp experiments (III, Figure 1). Basal activity of TRPC3 has been shown previously (Zitt et al., 1997; Albert et al., 2006; DeHaven et al., 2009), whereas the basal activity of TRPC2 has been reported to be smaller (Hofmann et al., 2000).

The involvement of TRPCs in SOCE is highly debated with numerous reports showing conflicting results (Abramowitz and Birnbaumer, 2009; Salido et al., 2009). The results presented here show no involvement of overexpressed TRPC3 in SOCE (I, Figure 3). Furthermore, SOCE was not affected in TRPC2-knockdown (shTRPC2) cells (Sukumaran et al. manuscript submitted). Taken together, neither TRPC2 nor TRPC3 seem to affect SOCE in a significant manner.

The most widely recognized TRPC activation mechanism is direct activation by DAG. Hofmann and colleagues were the first to show that TRPC3 is directly activated by the membrane-permeable DAG-analog 1-oleoyl-2-acetyl-sn-glycerol (OAG) (Hofmann et al., 1999b). In agreement with this, overexpressed TRPC3 was strongly activated by OAG (I, Figure 2). Like TRPC3, TRPC2 is activated by OAG (Lucas et al., 2003). In our hands, there were no TRPC2-mediated calcium transients in response to another DAG-analog, 1,2-dioctanoyl-sn-glycerol (DOG) (results not shown). However, we never tested the effects of a DAG-analog on membrane currents in FRTL-5 cells. DOG does not seem to activate TRPC3 as potently as OAG (Hofmann et al., 1999b), which could explain why there were no effects of DOG on FRTL-5 cells. In addition, overexpression of TRPC channels is necessary to acquire measurable effects of DAG-analogs (Hofmann et al., 1999b). In FRTL-5 cells there is a calcium influx unmasked by the serine/threonine phosphatase inhibitor calyculin A (caly A) (Gratshev et al., 2004). This calcium influx is mediated by a DAG-activated channel (II, Figure 4). The channel mediating this influx is TRPC2, as the caly A-mediated calcium influx was inhibited in shTRPC2 cells (Figure 6). Taken together, endogenously produced DAG might have a direct effect on TRPC2 under these special circumstances.

FRTL-5 cells express several types of purinergic receptors that potently stimulate calcium release when stimulated with ATP (Ekokoski et al., 2001). In shTRPC2 cells, the ATP-induced calcium influx was severely hampered (III, Figure 1). As purinergic receptors activate PLC, both IP₃ and DAG are produced. Although there is no direct evidence, it is feasible that DAG activates TRPC2 downstream of purinergic receptor activation, thus explaining why less calcium is entering the shTRPC2 cells when stimulated with ATP.

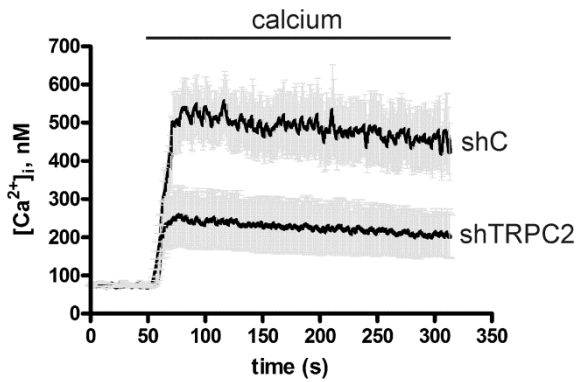


Figure 6. Caly A-evoked calcium entry is attenuated in shTRPC2 cells. Traces are the mean \pm SEM of 4 separate measurements, $P < 0.05$ for difference between cell populations.

5.2 Localization of TRPC2 and TRPC3

5.2.1 The effect of TRPC3 on intracellular calcium stores (I)

It was clearly visible that overexpression of TRPC3 affected release of sequestered calcium from intracellular calcium stores, both when stimulated with a GPCR-agonist (S1P) or the SERCA-inhibitor thapsigargin (I, Figure 3, 6). By permeabilizing the cells, the content of the intracellular stores was measured. The basal level of calcium was significantly higher in control cells, as compared to TRPC3-overexpressing cells (I, Figure 4, 6). Moreover, when stimulated with ionomycin or IP_3 , the sequestered calcium was released and the new basal levels were indistinguishable. This indicates that the ER calcium concentration is lower in TRPC3-overexpressing cells and that the result seen was not an effect of differences in the permeabilization process. Further, the localization of TRPC3 was investigated. TRPC3 was expressed in plasma membranes and in intracellular compartments, part of which localized to the ER, which was visualized with calnexin-staining (I, Figure 5). The plasma membrane localization of TRPC3 is not clearly visible in the images. In Figure 7, the localization of TRPC3 in both the plasma membrane and in an intracellular compartment is more readily apparent. An explanation for the reduced calcium content in TRPC3-overexpressing cells was not revealed. We speculated that TRPC3 might be functionally active in the intracellular compartment. In an attempt to test whether intracellular TRPC3 was functional, permeabilized cells were stimulated with OAG (results not shown). However, OAG elicited no response in permeabilized cells, which speaks against this hypothesis. The experiments on permeabilized cells are technically demanding and many intracellular proteins are presumably lost or damaged in the

permeabilization process. This could affect TRPC3 activation by OAG in the same manner as was seen with the lack of an effect of thapsigargin on permeabilized cells (1, Figure 4). Another plausible explanation is that OAG cannot reach the ER as it is trapped by the plasma membrane.

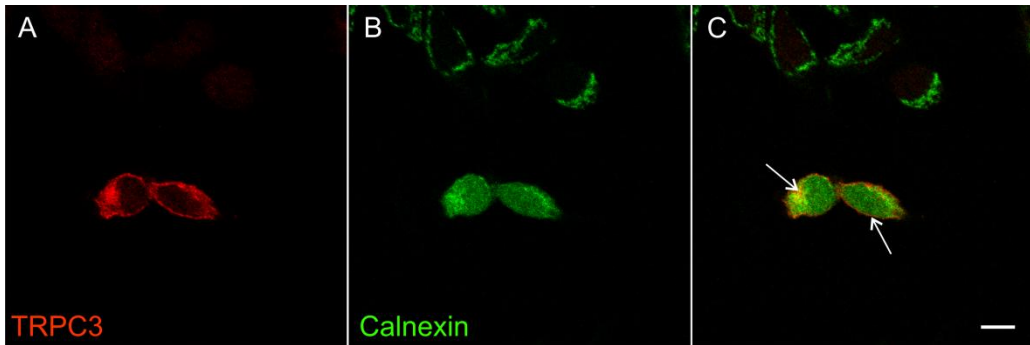


Figure 7. Localization of overexpressed TRPC3. (A) TRPC3 was transiently overexpressed in HEK-293 cells and visualized with anti-TRPC3 and Alexa Fluor 568-antibodies. (B) Calnexin was used as a marker for the ER. Endogenous calnexin was visualized with anti-calnexin and Alexa Fluor 488-antibodies. (C) Overlay image from both channels. Arrows show ER and plasma membrane localization of TRPC3. Scale bar represents 10 μ M.

Several studies showing TRP superfamily members located in intracellular compartments have been reported. TRPV1 have been shown to localize both to the ER and the plasma membrane (Castro et al., 2009). Interestingly, the activation of TRPV1 and subsequent release of ER calcium did not result in activation of SOCE or translocation of STIM1 to the plasma membrane (Turner et al., 2003; Wisnoskey et al., 2003; Castro et al., 2009). That TRPV1 was located in a subcompartment of the ER that does not affect SOCE was speculated as the reason for this. Moreover, the subcompartment important for SOCE was located closer to the plasma membrane (Castro et al., 2009). TRPC1 is located in intracellular membranes when transiently expressed in HEK-293 cells. However, TRPC1 was located in the plasma membrane when it was co-transfected with TRPC4 or TRPC5, which indicates that heteromultimerization is important for the plasma membrane localization of TRPCs (Hofmann et al., 2002; Salgado et al., 2008). When TRPC1 was expressed alone, it formed functional calcium release channels in the ER (Salgado et al., 2008). It seems that TRP channels are able to function as putative ER calcium release channels, which supports our finding with TRPC3. However as we have no direct evidence, our conclusion remains hypothetical. Another possible explanation for our finding is that overexpression of TRPC3 affects SERCA activity or expression.

An error in the experimental setup might explain the lack of activation downstream of GPCR-activation. In our study, TRPC3-overexpressing cells were compared to non-transfected cells on the same coverslip. The control cells were chosen based on visible fluorescence from green fluorescent protein. The control cells might overexpress TRPC3, albeit at low levels, which enhances calcium signals. This could explain why no differences were seen between control and TRPC3-overexpressing cells. Moreover, low levels of TRPC3-overexpression have been shown to be important for activation by the PLC pathway (Putney, 2004).

5.2.2 Requirement of RTP1 for the function of TRPC2 (III)

The localization of TRPC2 was not studied in FRTL-5 cells. However, the ATP-induced calcium influx was severely decreased in shTRPC2 cells (III, Figure 1), which implies that TRPC2 is located on the plasma membrane. A class of transmembrane proteins, called receptor transporting proteins (RTPs), that are expressed in olfactory neurons are important for plasma membrane insertion of odorant receptors (Saito et al., 2004). Recently, a report showed that RTP1 forms a complex with the scaffold protein Homer and TRPC2, and this increased plasma membrane localization of TRPC2 (Mast et al., 2010). This may explain a previous study that showed retention of TRPC2 in intracellular membranes when heterologously expressed in HEK-293 cells (Hofmann et al., 2000). When rescuing the effects of TRPC2 knockdown on calcium signaling, there was no requirement of additional proteins (III, Figure 7). In contrast, RTP1 was needed to reverse the effects seen on ERK1/2, TSHR, and Tg. Currently, I have no explanation for this. However, the calcium signal is an acute output, while protein phosphorylation and especially protein expression and post-translational modifications require correct signaling over a longer time-span. It is possible that RTP1 is important for sustained TRPC2 activity or transport back to the cell surface after internalization. At present there is no evidence for this, making this scenario highly hypothetical. In addition, the calcium signal in shTRPC2 cells expressing GFP-TRPC2 was only increased to the same level as in control cells. This indicates that additional proteins are probably needed to further enhance the calcium signal, e.g. the scaffold protein Homer.

5.3 Activation of calcium entry via autocrine S1P-signaling (II)

The ability of S1P to signal in an autocrine manner is believed to regulate such cellular processes as angiogenesis, survival, growth, and migration (Pyne and Pyne, 2010). The migration of cancer cells has been shown to be mediated by autocrine S1P signaling in several systems. In thyroid cancer cells, S1P is transported out in part by ABCC1 and activates migration through S1P-receptors (Bergelin et al., 2009).

5.3.1 Sphingosine inhibits caly A-evoked calcium entry (II)

The influence of autocrine S1P signaling on calcium signaling has not previously been reported. We showed that the sphingolipid rheostat and autocrine signaling of S1P affects calcium signaling in FRTL-5 cells (II). The serine/threonine phosphatase inhibitor caly A revealed a calcium entry pathway in FRTL-5 cells (Gratschev et al., 2004). Sphingosine has been shown to inhibit both VOCs (McDonough et al., 1994; Titievsky et al., 1998) and the I_{CRAC} current (Mathes

et al., 1998), which is the electrophysiological term for SOCE. More recently, sphingosine was shown to inhibit STIM1 and Orai1 interactions, thus hampering SOCE (Calloway et al., 2009). In our study, sphingosine inhibited the caly A-induced calcium influx in a concentration-dependent manner (II, Figure 1). Calcium entry was inhibited both with preincubation and with acute addition of sphingosine, the latter indicating that sphingosine has a direct effect on a channel. Other lipids had negligible effects on calcium entry, which shows that the effect is specific for sphingosine.

5.3.2 Dual effects of SK1 on caly A-evoked calcium entry (II)

Once the effects of sphingosine had been established, we investigated the possible involvement of SK. Overexpression of SK has previously been shown to remove inhibitory sphingosine from VOCs (Blom et al., 2006). In FRTL-5 cells, inhibition of SK had a similar effect on the calcium entry (II, Figure 2). Interestingly, addition of exogenous S1P rescued the calcium influx inhibited by an SK-inhibitor. This suggested that S1P is working through an autocrine mechanism. The effect of S1P on calcium signaling is not due to SOCE, as SOCE is inhibited by caly A. Moreover, intracellular stores are not affected by caly A, as only entry of calcium is of importance (Gratschev et al., 2004). Interestingly, activation of PLC through S1P2-receptors was crucial for the calcium entry (II, Figure 3, 4). As IP_3 is not important for caly A-evoked calcium influx, it suggests that DAG is activating the entry. Inhibition of the ABCC1 pump attenuated the calcium entry, which implies that this transporter shuttles S1P to the extracellular space where it activates the S1P2-receptor. ABCC1 has also been shown to transport S1P in other cell types (Mitra et al., 2006; Bergelin et al., 2009). In line with the hypothesis, exogenous S1P rescued the calcium entry when ABCC1 was inhibited. Overexpression of SK1, but not SK2, enhanced the calcium entry. Taken together, this shows that both removal of inhibitory sphingosine and the production of S1P is of importance for regulating caly A-evoked calcium entry. At the time when the study was published, we had no knowledge which channel was involved. As already discussed, knockdown of TRPC2 reduced caly A-evoked calcium entry in FRTL-5 cells; this strongly suggests the involvement of TRPC2.

5.3.3 Which phosphatase is involved? (II)

Caly A-treatment increased the S1P concentration in FRTL-5 cells, but the mechanism is still unknown (II, Supplementary Table 1). There is evidence for involvement of protein phosphatases in sphingolipid metabolism. One of the phosphatases inhibited by caly A is protein phosphatase 2A (PP2A). This phosphatase has been shown to inhibit SK1 activity by dephosphorylating SK1 at serine 225 (Barr et al., 2008). However, the increase in S1P was not due to enhanced activity of SK1 or SK2 in FRTL-5 cells. This implies that S1P phosphatase, S1P lyase, or pathways leading to their activation are regulated by phosphorylation of serine/threonine residues. In a yeast model, the inactivation of PP2A circumvented the requirement for sphingolipid synthesis (Friant et al., 2000). This suggests that PP2A could affect sphingolipid metabolism. Furthermore, the pro-apoptotic sphingolipid ceramide activates PP2A (Dobrowsky et al., 1993). This is of importance in ceramide-induced apoptosis, as dephosphorylation and inactivation of anti-apoptotic Bcl2 is mediated by PP2A (Ruvolo et al., 1999). Moreover, ceramide-evoked activation of PP2A dephosphorylates and activates pro-apoptotic Bax (Xin and Deng, 2006). In addition to ceramide, sphingosine may also activate

PP2A, as the immunosuppressive sphingosine analog, FTY720, activates PP2A (Matsuoka et al., 2003). In contrast, S1P inhibits PP2A through activation of S1P₂ (Salas et al., 2011). Although there is no evidence for regulation of S1P phosphatase by PP2A or any other phosphatase, sphingolipid and PP2A signaling is tightly intertwined, making this scenario at least hypothetically feasible.

5.4 The importance of TRPC2 in thyroid cells (III, unpublished)

The study describing autocrine S1P signaling turned our focus on TRPC channels in FRTL-5 cells. A PCR screen revealed that only TRPC2 was expressed in these cells (III, Figure 1), which was surprising as several members of the TRPC family are regularly found in different cell lines. Transcripts for TRPC2 were detected both in primary thyroid cells and in freshly isolated rat thyroids. In contrast, TRPC2 was not expressed in mouse thyroid. There are no reports that TRPC2 knockout mice would have defective metabolism or thyroid hormone levels (Leybold et al., 2002; Stowers et al., 2002).

5.4.1 Characterization of TRPC2 (III)

To study the effects of TRPC2 in thyroid cells, cell lines stably expressing control RNA or small hairpin RNA directed against TRPC2 were produced. The resulting shC and shTRPC2 cell lines were then compared in an attempt to find new functions for TRPC2. The knockdown of TRPC2 was verified with quantitative PCR only as no specific commercial TRPC2 antibody is available.

That TRPC2 is functional in FRTL-5 cells was investigated by stimulation of purinergic receptors (III, Figure 1). A concentration-dependent increase in intracellular calcium was seen when stimulating the cells with ATP. In the presence of calcium, the increase in intracellular calcium was reduced in shTRPC2 cells at concentrations tested. In the absence of calcium, no differences were seen in calcium peak amplitudes. However, there seemed to be a small, but significant, decrease in the calcium released from the ER, apparently due to reduced SERCA activity (Sukumaran et al., submitted). Patch-clamp recordings supported the calcium imaging results. Dedifferentiation was not an issue in the shTRPC2 cells, as the expression of important thyroid-specific transcription factors, TTF1 and Pax8, was not affected (III, Supplementary Figure 2).

5.4.2 Regulation of cAMP production by TRPC2 (III)

The TSHR is the main regulator of thyroid cells and it activates mainly ACs and cAMP production through the G_s α -subunit. This prompted us to investigate whether cAMP production is regulated by TRPC2. Stimulation of the cells with TSH or forskolin increased the cAMP concentration more potently in shTRPC2 cells as compared to shC cells (III, Figure 2). As PDEs activity was inhibited in the experimental setup, it suggests that AC activity and/or TSHR signaling might be affected. Both scenarios were true. The TSHR was upregulated on both mRNA and protein levels. Moreover, when the AC transcripts were screened it revealed the presence of AC5 and AC6, which both are inhibited by calcium. To investigate whether the decreased calcium signals seen in shTRPC2 cells are influencing cAMP production by AC5 and

AC6, the cAMP production assay was performed in the presence of the intracellular calcium chelator BAPTA-AM. In line with our hypothesis, calcium chelation increased cAMP in shC cells to the same level as seen in shTRPC2 cells. An extracellular calcium chelator, EGTA, was also tested. However, this chelator did not affect forskolin-induced cAMP production, only basal cAMP production (results not shown). Other studies have reported that BAPTA is a more effective chelator than EGTA when studying the effects of calcium on ACs (Willoughby and Cooper, 2007). G_q-mediated calcium signals have been shown to inhibit AC5 and AC6 (von Hayn et al., 2010). Defective SOCE signaling has been linked to activation of AC6 (Spirli et al., 2012). Furthermore, it has been shown that SOCE mediated by STIM1 and Orai1 selectively activates AC8, while TRPC-mediated calcium influx is incapable of AC8 activation (Martin et al., 2009).

5.4.3 Connecting cAMP, ERK1/2 and TSHR expression (III, unpublished)

As cAMP is able to activate the ERK1/2 pathway in thyroid cells (Iacovelli et al., 2001), we next studied whether increased cAMP production leads to phosphorylation of ERK1/2. The basal phosphorylation status of ERK1/2 in shTRPC2 cells was strongly induced when grown in the presence of serum and hormones including TSH (III, Figure 3). Acute stimulation of serum and hormone-starved cells with TSH more potently activated ERK1/2 in the shTRPC2 cells as compared to shC cells. Interestingly, in serum and hormone-starved cells both ATP and serum induced a stronger phosphorylation of ERK1/2 in shTRPC2 cells as compared to shC cells (Figure 8). This indicates that reduction of TRPC2 induces a global change that favors phosphorylation of ERK1/2 in FRTL-5 cells.

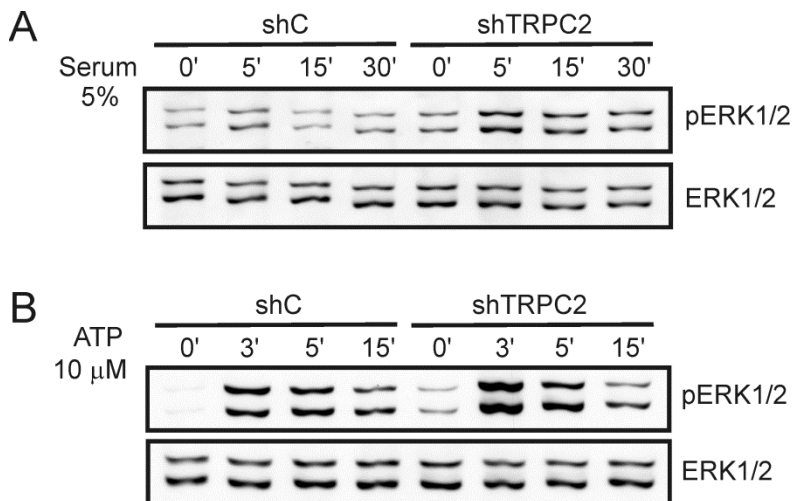


Figure 8. The phosphorylation of ERK1/2 is enhanced in shTRPC2 cells. In serum and hormone-starved cells, acute stimulation with serum (A) or ATP (B) more strongly phosphorylates ERK1/2 in shTRPC2 cells. Western blots are representative of three independent measurements.

Next we wanted to establish whether there is a link between reduced calcium entry and increased phosphorylation of ERK1/2 and the expression of the TSHR. In line with our hypothesis, chelation of extracellular calcium by EGTA or transient expression of a truncated non-conducting mutant of TRPC2 in native FRTL-5 cells resulted in increased phosphorylation of ERK1/2 and increased expression of the TSHR (III, Figure 4). Inhibition of MEK activity with a specific inhibitor (U0126) or expression of a constitutively active mutant of MEK1 in native FRTL-5 cells decreased and increased the expression of the TSHR, respectively. These results strongly suggest that the MEK/ERK1/2 pathway is involved in regulating the expression of the TSHR. Finally, we inhibited the cAMP pathway by overexpressing PDE4D3 to reduce cAMP in the cells. This decreased the acute TSH-induced phosphorylation of ERK1/2 and the expression of the TSHR (III, Figure 5). Furthermore, by expressing a dominant negative mutant of Rap1, ERK1/2 phosphorylation and TSHR expression were reduced to control levels. Together, these results show that increased cAMP production in shTRPC2 cells activates ERK1/2 via Rap1 and this leads to increased expression of TSHR.

5.4.4 Cross-communication between TRPC2 and cAMP (unpublished)

In a previous study investigating caly A-mediated entry, PKA activity was crucial for the influx of calcium (Gratshev et al., 2004). If cells were grown in the absence of TSH, calcium entry was severely reduced, whereas it was rescued if TSH was added together with calcium. These two experiments suggest that PKA is phosphorylating an effector in this pathway, perhaps even the channel protein itself. As mentioned above, there is evidence for TRPC2 mediating the caly A-evoked entry of calcium. Moreover, there are putative PKA phosphorylation sites in TRPC2, mutation of which could answer many questions regarding the involvement of TRPC2. A model where TSH is able to activate TRPC2 through PKA-mediated phosphorylation and TRPC2 subsequently inhibits ACs and cAMP-production fits nicely with a classical negative-feedback control mechanism.

An additional level of cross-communication might be mediated by EPAC. When inhibiting EPAC by transiently transfecting a dominant negative form of EPAC (N-EPAC) into shC and shTRPC2 cells, a second level of cross-communication may have been revealed. Inhibition of EPAC in shTRPC2 cells decreased the TSHR expression to control level (Figure 9). This is in line with a model where increased cAMP production in shTRPC2 cells activates a Rap1/ERK1/2 pathway, which increases the expression of the TSHR. In sharp contrast, when expressing N-EPAC in control cells the TSHR expression increased to the same level as seen in shTRPC2 cells. To provide a possible explanation for this, ATP-induced calcium transients were measured in the presence of N-EPAC. Interestingly, N-EPAC increased the calcium transient in control cells, whereas it was without effect in shTRPC2 cells. These results suggest that EPAC may inhibit TRPC2 activity through a still unknown mechanism. In addition, TRPC2 increases the expression of the TSHR through an alternative pathway when EPAC is inhibited.

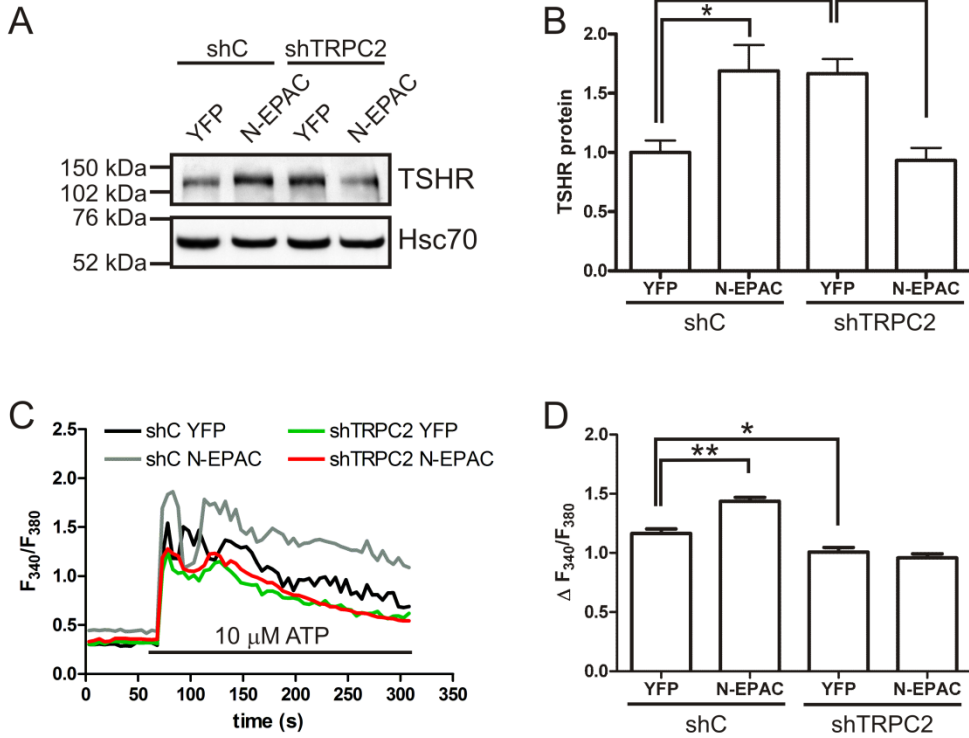


Figure 9. Inhibition of EPAC by expression of N-EPAC in shC and shTRPC2 cells. (A) TSHR expression is increased in shC and decreased in shTRPC2 cells by expressing a dominant negative mutant of EPAC (N-EPAC) as compared to control transfected cells (YFP). (B) Quantification of band densities in (A), bars indicates mean \pm SEM, $n=5$, * $P<0.05$, ** $P<0.01$. (C) ATP-induced calcium transients in shC and shTRPC2 cells expressing YFP and N-EPAC. (D) Quantification of result in (C). Bars indicates mean \pm SEM of $n\geq 60$ cells, * $P<0.05$, ** $P<0.01$.

5.4.5 TRPC2 and the secretion of thyroglobulin (III)

The precursor for thyroid hormones, Tg, was studied as FRTL-5 cells do not secrete detectable levels of thyroid hormones. Interestingly, in shTRPC2 cells an additional lower molecular weight band appeared on the western blot (III, Figure 6). We speculated that this was an immature form of Tg as the protein goes through post-translational modification before it is secreted. Total Tg mRNA (III, Supplementary figure 2) or protein (III, Figure 6) was not altered in the shTRPC2 cells. This shows that only part of the Tg produced in shTRPC2 cells is fully processed. Glycosylation of Tg takes place in the ER before it is transported to the Golgi for further processing. With Endo H treatment of cell lysates from shC and shTRPC2 cells, we concluded that the immature form of Tg never reaches the Golgi. We base this on the finding that the mature form of Tg did not exhibit a mobility shift on the western blot and thus its sugar residues have been processed by Golgi α -mannosidase II, whereas the immature form of Tg has not. If part of the Tg is not transported to the Golgi for processing, this implies that less Tg is secreted from shTRPC2 cells. Indeed, by immunoprecipitating secreted Tg from the growth media of the cells, we showed that less Tg is secreted from shTRPC2 cells. The scenario described above probably partly describes the lesser secretion of Tg. However, as calcium is also involved in vesicular transport, we do not rule out that the reduced calcium transients seen in shTRPC2 cells could influence other mechanisms involved in the secretion of proteins, e.g. vesicular transport (Hay, 2007).

5.4.6 The effects of TRPC2 knockdown are reversible (III)

To conclude that the effects seen by knockdown of TRPC2 are due to specific effects, a series of rescue experiments where exogenous TRPC2 was added back to the cells were performed (III, Figure 7). The ATP-induced calcium transients were rescued in shTRPC2 cells by transient transfection with GFP-TRPC2. However, both the phosphorylation of ERK1/2 and the expression of the TSHR needed co-transfection of RTP1 with GFP-TRPC2 to successfully reverse the effects. Co-transfection of RTP1 with GFP-TRPC2 had no additional beneficial effect on acute calcium signaling. The effect of RTP1 is therefore presumably on more long-term signaling. The immature form of Tg was decreased when RTP1 and GFP-TRPC2 were co-transfected to shTRPC2 cells. Taken together, the results seen with knockdown of TRPC2 are reversible, although RTP1 is required in concert with TRPC2 to restore downstream signaling.

6 CONCLUSIONS

The following conclusions are made based on the results obtained in the three original publications presented herein.

- 1) When overexpressed in HEK-293 cells, TRPC3 does not to any greater extent influence calcium entry. In contrast, overexpression of TRPC3 reduces the intracellular calcium stores, which reduces IP₃-mediated calcium responses. Our results indicate that TRPC3 is partly located in intracellular membranes, where it might form functional calcium release channels thus reducing the calcium stores of the ER.
- 2) Caly A-evoked calcium entry is mediated by TRPC2 in FRTL-5 cells.
- 3) The caly A-mediated calcium entry is regulated by SK1. Both decreasing the amount of the substrate, sphingosine, and increasing the amount of the product, S1P, is crucial for the calcium entry. Sphingosine potently blocks the influx of calcium, while S1P is transported out of the cells and activates S1P₂-receptors in an autocrine manner. The S1P₂-receptors activate PLC, which subsequently produces DAG that stimulates calcium entry. Caly A increases the net S1P concentration in the cells by a mechanism that is not dependent on SK activity.
- 4) TRPC2 is expressed in rat thyroid gland and TRPC2 is the only TRPC channel expressed in FRTL-5 cells derived from rat thyroid. TRPC2 is activated downstream of GPCR-activation and mediates calcium entry in FRTL-5 cells.
- 5) The activity of calcium-inhibited ACs, presumably AC5 and AC6, is increased in cells where TRPC2 expression is reduced by shRNA (shTRPC2). The increased cAMP in these 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.
- 6) Glycosylation of Tg is perturbed in shTRPC2 cells, which leads to retention of immature Tg in the ER. Due to this, less Tg is secreted from shTRPC2 cells compared to FRTL-5 cells with intact TRPC2 function.

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