TRPM7, Calcium and the cytoskeleton

Langeslag, Michiel

Chapter I

Introduction

Introduction

Every cell responds to external stimuli. Many of these stimuli act through receptors situated at the plasma-membrane that transduce the signal in the cytosol. After receptor activation, the signal propagates via so-called second messengers to cellular responses varying from regulation of gene expression to actomyosin contraction. These second messengers may be proteins, small molecules or ions. An example of the latter is calcium (Ca^{2+}), a universal second messenger in all cells. Other ionic species, e.g. Magnesium (Mg^{2+}) are also important because they regulate the activity of enzymes.

Because various cellular processes depend on the abundance of ions (be it as second messengers or as regulators of enzymes) the cell maintains gradients of ions over the plasma-membrane that are tightly regulated. Potassium (K^{+}) and Sodium (Na^{+}) are the main ions controlling the membrane potential of cells: the cell interior is usually negatively charged with regard to the extracellular side. Calcium (Ca^{2+}) and Magnesium (Mg^{2+}) play pivotal roles in the regulation of many cellular reactions and enzymatic activities. The lipid bilayer of the plasma-membrane itself cannot let ions pass through due to its lipophylic nature. Therefore, cells have adapted various mechanisms to control the distribution of ions over the plasma-membrane such as ionic channels and ion exchangers.

Ca^{2+} Homeostasis

The concentration of free cytosolic Ca^{2+} is maintained at extremely low concentrations of 60-100 nM, whereas the concentrations extracellularly and in the endoplasmatic reticulum are higher (~1 and ~3 mM, respectively). Combined with the electrical gradient, there is thus a steep gradient that tends to drive extracellular Ca^{2+} into the cells. Under these conditions, any increase in Ca^{2+} permeability of the membranes (either through release from the ER or through influx via ion channels at the plasma-membrane) causes a sharp rise in intracellular Ca^{2+} (Berridge et al., 2000) Thus, Ca^{2+} is ideally suited to act as a second messenger and many proteins and processes are triggered by local increases in Ca^{2+} concentrations. For these reasons, a strict regulation of intracellular Ca^{2+} is necessary. Cellular Ca^{2+} regulation or Ca^{2+} homeostasis is achieved by various mechanisms that act at the level of the plasma-membrane as well as at cellular organelles such as the endoplasmatic reticulum (ER) and mitochondria (See Figure 1).

Raising Cytosolic Ca^{2+} Levels

Since the lipid bilayer itself functions as a barrier for ions, passive transmembrane transport is handled by various ion channels with widely different properties. Based on the mode of activation, Ca^{2+} entry through channels can be divided into 4 major classes: receptor-operated (ionotropic) (Shuttleworth, 2004), second-messenger-operated (metabotropic) (Kaupp and Seifert, 2002), voltage-operated (Felix, 2005) and store-operated Ca^{2+} channels (Bolotina, 2004) (Figure 1). Voltage-operated Ca^{2+} channels in excitable cells are best characterized. These channels are capable of generating very fast Ca^{2+} elevations upon membrane depolarization and control fast cellular responses such as exocytosis and muscle contraction. Ionotropic channels are also fast because they are directly controlled through binding of extracellular ligands that open the channels, while gating of metabotropic channels is constrained by cytosolic second-messengers generated upon receptor activation. Yet other Ca^{2+} channels respond to a diverse array of stimuli including emptying of Ca^{2+} stores, temperature and mechanical stress (Voets et al., 2005; Pedersen et al., 2005). Most of these channels belong to the large family of transient receptor potential (TRP) ion channels that will be described in more detail in the sections on TRP ion channels and mammalian TRP channels.

Next to Ca^{2+} entry from the extracellular medium (influx), Ca^{2+} can also be raised by release from intracellular stores (Figure 1), which are primarily located at the ER or in muscle cells at the sarcoplasmic reticulum (SR). Both the ER and SR posses second messenger activated Ca^{2+} channels, the inostol-1,4,5-triphosphate receptors (IP_3-R) and ryanodine receptors (RYR) respectively. These channels are activated by IP_3 and cADPribose/ NAADP respectively and are controlled by intracellular Ca^{2+} ($[Ca^{2+}]_i$) (Yoshida and Imai, 1997; Guerrero-Hernandez et al., 2002).

Whenever IP_3 binds to the IP_3-R, it increases the sensitivity of the receptor for Ca^{2+}, which has a biphasic characteristic. At slightly elevated levels, Ca^{2+} acts synergistically with IP_3 to open the IP_3-R channel, whereas at high concentrations, for example after full Ca^{2+} release, it inhibits the IP_3-

R, either directly or indirectly via calmodulin (CaM) (Taylor, 2002).

Cytosolic Ca^{2+} Removal Mechanisms

Cells prevent Ca^{2+} overload and terminate cytosolic Ca^{2+} signals in several ways. First of all, free Ca^{2+} is effectively buffered by different Ca^{2+} binding proteins like parvalbumin, calbindin D-28, calretinin and, to a lesser extent, by Ca^{2+}-effector proteins such as CaM, protein kinase C etc. During Ca^{2+} elevations, these proteins will buffer Ca^{2+}, and subsequently, after termination of the Ca^{2+} signals, the buffers will be regenerated. In this way, the binding kinetics of these buffering proteins helps shaping Ca^{2+} transients in their amplitude and recovery time (John et al., 2001). Secondly, activated Ca^{2+} channels, at the plasma-membrane or internal stores, are closed by various regulatory mechanisms, such as phosphorylation, ionic inhibition, (de-)polarization of the plasma-membrane, or via inhibitory regulators of these channels (Hering et al., 2000). Alternatively, Ca^{2+}-pumps and exchangers located at the plasma-membrane and internal organelles lower elevated cytosolic Ca^{2+} levels to resting levels and ensure that internal stores are refilled with Ca^{2+} (Belkacemi et al., 2005).

Ca^{2+}-pumps and exchangers can be categorized into 4 classes: plasma-membrane Ca^{2+}-ATPases (PMCA), Na^{+}/Ca^{2+} exchangers (NCX), endo(sarco)plasmatic Ca^{2+} ATPases (SERCA) and the mitochondrial uniporter. Since PMCAs and SERCAs have a high affinity for Ca^{2+}, they detect and respond to even modest changes in cytosolic Ca^{2+}. These proteins have rather low transport rates but are most important to set basal Ca^{2+} levels. In contrast, NCX and the mitochondrial uniporter have a higher transport rate and a wider dynamic range, but they only act optimally at μM Ca^{2+} concentrations ("high capacity, low affinity" pumps).

Kinetics and Spatial Distribution of Ca^{2+} Signals

Ca^{2+} is a very versatile second messenger. The shape and duration of the Ca^{2+} elevation is tailor-suited to fit the spatial and temporal requirements of specific downstream Ca^{2+}-dependent signaling complexes. For example, proteins involved in rapid responses, such as exocytosis, are highly organized and all the downstream effectors are closely associated into a signal complex. These

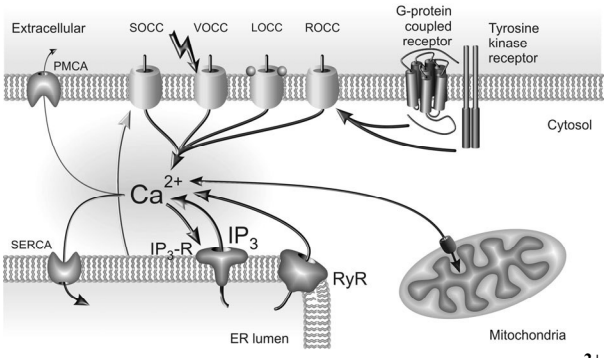

Figure 1: Schematic representation of cellular Ca^{2+} homeostasis.

After receptor activation intracellular Ca^{2+} can be raised from IP_3-sensitive and/or Ryanodine-sensitive stores. Also mitochondria may release Ca^{2+} into the cytosol via the uniporter. At the plasma-membrane various ion channels, non-selective cation or Ca^{2+}-specific, can elevate cellular Ca^{2+} levels after activation by receptors (ROCC), ligand binding (LOCC) or by membrane depolarization (VOCC). Elevated cytosolic Ca^{2+} levels are lowered to resting conditions by Ca^{2+} ATPases located at the plasma-membrane (PMCA) and the endoplasmic reticulum (SERCA). Furthermore, at very high Ca^{2+}_i elevations mitochondria will accumulate cytosolic Ca^{2+} and Na^{+}/Ca^{2+} exchangers at the plasma-membrane are activated (not shown).

fast responses rely on rapid voltage operated Ca^{2+} channel gating and are spatially restricted to tens of nanometers (Sorensen, 2004). In contrast, muscular actomyosin contractions, as well as other processes, require Ca^{2+} elevations that last from seconds to many minutes (Ebashi and Ogawa, 1988; Fields et al., 2005). Ca^{2+} signals often propagate as waves or oscillations throughout the cell. During prolonged stimulation, the Ca^{2+} concentration may also show repeated oscillations and this phenomenon has been implicated in physiological processes such as growth cone migration and turning, axonal outgrowth and oocyte activation (Berridge et al., 2000). These Ca^{2+}-regulated processes do not require the highly organized, preexisting signaling complex that controls e.g. exocytosis. Cells can interpret Ca^{2+} oscillations using highly sophisticated mechanisms. To date, a number of Ca^{2+} effectors that can decode Ca^{2+} oscillations have been identified, such as Nuclear Factor of Activated T cells (NFAT) (Tomida et al., 2003), Protein Kinase C (PKC) (Oancea and Meyer, 1998) and Calmodulin Kinase II (De Koninck and Schulman, 1998). These decoder proteins use frequency-encoded Ca^{2+} signals to regulate gene transcription, exocytosis and the redox state of mitochondria (Dolmetsch et al., 1997; Li et al., 1998; Dolmetsch et al., 1998).

Replenishment of Intracellular Stores through Store-Operated Calcium Entry

Responses to stimulation of GPCR or tyrosine receptors often are characterized by an initial, rapid Ca^{2+} release from IP_3- or ryanodine-sensitive intracellular stores that is not dependent on the presence of extracellular Ca^{2+}, followed by a second, smaller and more sustained phase that does rely on extracellular Ca^{2+}. Emptying of the intracellular stores is the trigger for this Ca^{2+} influx that is known as Store-Operated Calcium Entry (SOCE). The SOCE conductance is not purely selective for Ca^{2+}, for example Mn^{2+} can also enter the cytosol, as is evident from Fura-2 quenching essays (Jacob, 1990). Thus, depletion of intracellular stores somehow causes opening of a Ca^{2+} entry pathway in the plasma-membrane that is thought to be important for replenishment of intracellular stores. SOCE is proposed to continue as long as the IP_3-sensitive stores are not adequately refilled (Putney, Jr., 1986; Clapham, 1995). This event is observed in many different cell-types but the underlying molecular mechanism is still not understood and under scientific debate.

A major tool to investigate regulation of SOCE is thapsigargin, a naturally occurring sesquiterpene lactone isolated from the umbelliferous plant *Thapsia garganica* (Rasmussen et al., 1978). Thapsigargin specifically binds to and inhibits Ca^{2+} pumps (SERCA) located at the membranes of intracellular stores and thereby prevents Ca^{2+} to be pumped back into the lumen of the store (Thastrup et al., 1990). Under these conditions, basic leakage of Ca^{2+} through the intrinsic IP_3 receptor channels causes depletion of the stores. The rate of store depletion and consequently Ca^{2+} influx via SOCE is highly variable between cell-types, but it may be quite rapid (within 5 s for rat mast cells (Hoth and Penner, 1993)). This indicates that there is high basal Ca^{2+} turnover across the membranes of the Ca^{2+} stores.

The first indications for SOCE were obtained by Fura-2 measurements (Jacob, 1990). Hoth and Penner used electrophysiological recordings to identify ionic currents responsible for SOCE. Using mast cells in whole-cell recordings, they observed ionic currents induced by depletion of intracellular stores as a result of inclusion of IP_3 in the pipette solution (Hoth and Penner, 1992). These store-depletion-mediated currents were described as Calcium Release Activated Current (I_{CRAC}). In most cells, the current amplitude of I_{CRAC} is much smaller than background currents during patch-clamp, but it can be revealed by increasing extracellular Ca^{2+} to 10mM while intracellular Ca^{2+} is strongly buffered with EGTA or BAPTA. I_{CRAC} selectively permeates Ca^{2+} over monovalent ions, but surprisingly almost no Mn^{2+} entry through CRAC channels was observed (Hoth and Penner, 1992), notwithstanding the fact that Mn^{2+} can efficiently replace Ca^{2+} in SOCE assays with Fura-2 (Jacob, 1990). The amplitude of I_{CRAC} flowing through one channel is very low, a single channel conductance of only ~ 24 fS, which is far below the conductance of any ion channel identified to date (Zweifach and Lewis, 1993). I_{CRAC} can only be measured in a limited number of cells, due to the low current amplitude and the incapability of discriminating between background currents for technical reasons (Zitt et al., 2002). It has not been convincingly demonstrated that SOCE and I_{CRAC} reflect the same process. Until now, the ion channel(s) responsible for conducting I_{CRAC} has remained illusive and scientists all over the world are eager to identify the channel.

Mg^{2+} Homeostasis

Magnesium ions have an undisputable role in regulation of tissue and cell functions. Mg^{2+} is indispensable for the activity of numerous enzymes, e.g. kinases, DNA/RNA polymerases, various ATPases, small and heteromeric G-proteins. In addition, Mg^{2+} might alter transcription by binding to the transcription factor DREAM. Furthermore, regulation of several ion channels, like the IP_3 receptor, TRPM6 and TRPM7, L-type Ca^{2+} channels and ATP-sensitive K^+ channels is mediated by Mg^{2+}.

Given all of these functions, it is not surprising that Mg^{2+} is found in abundance intracellularly. The Mg^{2+} content in cells varies from 17-20 mM (Romani et al., 1995) and it is more or less homogenously distributed over the nucleus, mitochondria, endo(sarco)plasmatic reticulum and cytosol (Griswold and Pace, 1956; Gunther, 1986). Approximately 95% of the cellular Mg^{2+} ions is bound or sequestered. A considerable amount of Mg^{2+} (4-5 mM) found in the cytosol is bound to ATP or other phosphometabolites (Garfinkel et al., 1986) and as a result, in the cytosol only 0.5-1.0 mM ionized Mg^{2+} is free (see table I in Romani and Scarpa, 1992). In mitochondria the free Mg^{2+} levels are estimated to be between 0.5-1.0 mM (Jung et al., 1997) and in

the endo(sarco)plasmatic reticulum ~1mM (Sugiyama and Goldman, 1995).

Because both in the cytosol and the extracellular fluid Mg^{2+} is kept at (sub)millimolar concentrations, the transmembrane Mg^{2+} gradient is small, usually a factor of 2 or less. Therefore, influx or efflux of Mg^{2+} usually results in only small changes in free $[Mg^{2+}]_i$. Despite this small gradient, Mg^{2+} levels in the cells are under control of hormonal signaling: Mg^{2+} can be actively removed and accumulated into the cytosol after hormonal stimulation with e.g. epinephrine, phenylephrine and vasopressin. For details I refer to the box regulation of cellular Mg^{2+} by hormonal signaling.

Plasma-membrane Transport of Mg^{2+}

Data from Mg^{2+} flux-studies indicate the presence of several membrane transporters for Mg^{2+}. An electroneutral, extracellular Na^+-dependent Mg^{2+}/Na^+ exchanger in the plasma-membrane of chicken and turkey erythrocytes was the first characterized Mg^{2+} extruder (Gunther et al., 1984). Over the years, this Mg^{2+}/Na^+ exchanger has been identified in many other cell types (Gunther and Vormann, 1990; Handy et al., 1996; Tashiro and Konishi, 1997; Gunther et al., 1997). This exchanger can be stimulated by receptor-mediated increases in cyclic AMP (cAMP) and is dependent on extracellular Na^+. Removing Na^+ from the extracellular fluid (Romani et al., 1993b) or blocking Na^+ transport (Gunther et al., 1984; Feray and Garay, 1986) inhibits extrusion of Mg^{2+}. However, the data indicate that the mode of activation differs between cell types. Apart from its activation by cAMP (Gunther and Vormann, 1992), the Na^+/Mg^{2+} exchanger can also be turned on by increasing amounts of free cytosolic Mg^{2+} (Gaussin et al., 1997) and Ca^{2+} (Romani et al., 2000). Further more, the exchange stoichiometry varies between cell-types, e.g. in human red blood cells the exchange of Mg^{2+} for Na^+ has a stoichiometry of 1:3 (Ludi and Schatzmann, 1987; Schatzmann, 1993).

In addition, several laboratories have reported a Na^+-independent Mg^{2+} extrusion pathway that exchanges extracellular Ca^{2+} (Romani et al., 1993a), Mn^{2+} (Feray 1987), Cl^- (Gunther et al., 1990) or HCO_3^- (Gunther and Hollriegl, 1993) for Mg^{2+}. Extrusion appears to occur at a one-to-one ratio when Mn^{2+} is used as counter-ion and the exchanger can operate in reverse mode. In line with the presence of a Ca^{2+}/Mg^{2+} exchanger, inhibition of Ca^{2+} channels with nifedipine or verapamil prevents Mg^{2+} extrusion (Romani et al., 1993a).

Both Mg^{2+} extrusion mechanisms may coexist in a single cell. For example, results from the lab of Romani and Scarpa indicate that phenylephrine extrudes Mg^{2+} via a Ca^{2+}-dependent mechanism (accounting for 10-15% of the total Mg^{2+} extrusion) and via a Ca^{2+}-activated Na^+-dependent mechanism that accounts for the majority of extrusion (Romani et al., 2000).

Recently, two channels implicated in Mg^{2+} homeostasis have been cloned and characterized (Runnels et al., 2001; Nadler et al., 2001; Schlingmann et al., 2002; Walder et al., 2002). These highly related channels belong to the TRP family of ion channels and are known as TRPM6 and TRPM7 respectively. Both channels conduct Mg^{2+} and permeability appears to be regulated by the intracellular Mg^{2+} concentration: lowering of intracellular $[Mg^{2+}]$ by washout with Mg^{2+}-free patch pipette solution causes the channels to open, leading to Mg^{2+} influx (Nadler et al., 2001; Voets et al., 2004b). Whereas TRPM6 expression is restricted to the distal tubules of kidney and the intestinal tract (Schlingmann et al., 2002; Walder et al., 2002), TRPM7 is ubiquitously expressed (Runnels et al., 2001; Nadler et al., 2001). Therefore it has been proposed that TRPM7 is the first identified ion channel responsible for cellular Mg^{2+} handling in all cell types (Schmitz et al., 2003). Several mutations in TRPM6 are associated with hypomagnesia with secondary hypocalcaemia, an autosomal-recessive Mg^{2+} handling disorder (Schlingmann et al., 2002; Walder et al., 2002). A more detailed description of both channels can be found in sections on TRPM channels and TRPM7.

BOX 1: Regulation of Cellular Magnesium by Hormonal Signaling

Mg^{2+} Extrusion by Hormone Signaling and ATP Depletion

Stimulation of cardiac (Vormann and Gunther, 1987; Romani et al., 1993b; Howarth et al., 1994) and liver cells (Romani and Scarpa, 1990; Gunther and Vormann, 1991; Keenan et al., 1996) with β-adrenergic receptor agonists results in a marked extrusion of Mg^{2+} evident within 1 minute after application and reaching its maximum after 5-6 minutes. Subsequently, Mg^{2+} levels return towards basal levels, independent of the persistence of the agonist (Keenan et al., 1996; Romani et al., 2000). β-adrenergic receptor agonists act via adenylyl cyclases to increase cAMP levels, which in turn activates Protein Kinase A (PKA) (Huang et al., 1982; Wolf et al., 1997; Rothermel and Parker Botelho, 1988). Besides β-adrenergic receptor mediated extrusion, Cittadini's lab observed Mg^{2+} extrusion in spleen lymphocytes and Ehrlich cells after prostaglandin (PGE1 or PGE2) or arachidonic acid stimulation that is mediated by intracellular cAMP increase (Wolf et al., 1994; Wolf et al., 1996). Mg^{2+} extrusion over the plasma-membrane requires the presence of physiological levels of extracellular Na^{+} and Ca^{2+}.

Stimulation with α-adrenergic agonists, such as phenylephrine revealed a second pathway involved in cellular Mg^{2+} regulation (Jakob et al., 1989). Pretreatment of cells with insulin allows discrimination between α- and β-adrenergic receptor-activated Mg^{2+} extrusion. Insulin inactivates β-adrenergic receptors through tyrosine phosphorylation and interferes with intracellular cAMP levels through inhibition of the adenylyl cyclase (Karoor et al., 1995) or activation of phospho-diesterases (Smoake et al., 1995), and thereby prevents Mg^{2+} efflux (Keenan et al., 1996; Romani et al., 2000). In contrast, α-adrenergic receptors are coupled to small G-proteins that activate PhosphoLipase C (PLC) and result in release of Ca^{2+} from IP_3-sensitive stores (Minneman, 1988). Therefore insulin pretreatment does not prevent Mg^{2+} extrusion after α-adrenergic receptor stimulation (Keenan et al., 1996). The release of intracellular Ca^{2+} could directly activate a Ca^{2+}-dependent Mg^{2+} transporter or act as a counter-ion for Mg^{2+} extrusion.

Cellular ATP is the most abundant chelator of Mg^{2+} present in the cytosol. Chemicals that decrease the cellular ATP content, like cyanide, mitocondrial uncouplers (Wolf et al., 1994; Romani and Scarpa, 2000), fructose (Gaussin et al., 1997) or ethanol (Tessman and Romani, 1998), increase the amount of free cytosolic Mg^{2+} that is extruded in a Na^{+}-dependent fashion. Since there is no evidence that cAMP mediates this extrusion (Tessman and Romani, 1998), it is possible that the elevation of free cytosolic Mg^{2+} due to decreased cellular ATP content is sufficient to activate the Mg^{2+} transporter.

Accumulation of Cellular Mg^{2+}

The Mg^{2+} content of blood plasma and the body is mainly controlled by cells in the renal apparatus and in the intestine. Mg^{2+} uptake from the intestine and reabsorption in the kidney is under hormonal control, although many details of this are still unclear. For a more exhaustive description I refer to reviews of Quamme (Quamme and de Rouffignac, 2000) and Hoenderop (Hoenderop and Bindels, 2005). Here I will focus on Mg^{2+} handling by other cells in the body.

As mentioned before, in liver and cardiac cells adrenergic receptors stimulate Mg^{2+} extrusion, a process that is sensitive to insulin. In contrast, in 3T3 fibroblasts and other cell types insulin causes accumulation of Mg^{2+} (Gylfe, 1990; Ishijima and Tatibana, 1994). Agonists such as vasopressin and angiotensin II also cause accumulation of Mg^{2+} (Okada et al., 1992; Touyz and Schiffrin, 1996; Dai et al., 1998). In hepatocytes, vasopressin-induced Mg^{2+} accumulation is Na^{+}-dependent and mediated by PKC and Ca^{2+} (Romani et al., 1993a). However the precise mode of action is still obscure.

TRP Ion Channels

TRP channels were identified in 1970 in the phototransduction cascade of *Drosophila melanogaster*. In wildtype fruit flies, continuous illumination of the compound eye produces a long lasting depolarization of photoreceptor cells (Pak et al., 1970). In the *Drosophila trp* mutant, the initial onset of the response to prolonged illumination is identical to wildtype but the depolarization is transiently decaying towards baseline within seconds despite the continuous illumination. This results in a functional loss of sight in bright light (Minke et al., 1975).

Members of the mammalian TRP channel family are involved in a variety of functions, e.g. cation homeostasis and detection of sensory stimuli. I will briefly introduce the activation pathway of *Drosophila* TRP channels and its multiprotein signal complex as it has been extensively studied and is likely to be relevant for regulation of mammalian TRP channels.

TRP Channels in the Drosophila's Eye and Light Perception

Phototransduction in the compound eye of *Drosophila* is a complex signaling cascade that involves G-Protein Coupled Receptors (GPCR), G-proteins, Phospholipase C (PLC), and at least 2 types of channels, TRP and TRPL (Minke and Cook, 2002). Incoming photons isomerize *rhodopsin*, the light-sensitive GPCR, to the active form *metarhodopsin*. *Metarhodopsin* subsequently transduces the signal to a heterotrimeric G-protein (*transducin*) that activates PLC (*norpA*). Consequently, PLC activation leads to TRP and TRPL channel opening resulting in a light-induced current (LIC) via an as yet unknown pathway. Resolving this pathway is difficult and results are controversial, but the key role for PLC is undisputed: in a temperature-sensitive allele of PLC, ts-*norpA*, flies can be rendered fully blind by rapidly switching to the non-responsive temperature, and vice versa (Deland and Pak, 1973). Interestingly, gating of TRP and TRPL channels has long been associated with various PLC-related second messengers, including IP_3, DAG and PIP_2 (Figure 2). As gating of TRPM7 channels by these messengers is the subject of Chapters 2 and 3, I will here review the literature in some detail.

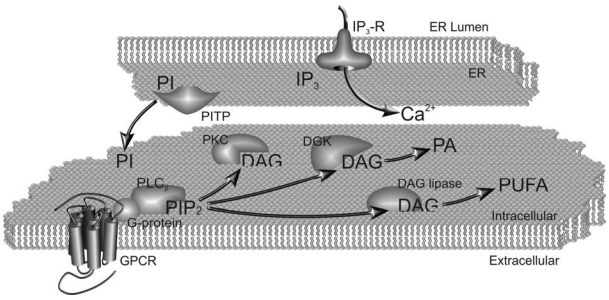

Figure 2: Schematic overview of phosphoinositide signaling cascade.

Upon binding of ligand to G-protein receptors (GPCR), the receptor is activated which in turn activates a heteromeric G-protein. Specific heteromeric G-proteins will activate phospholipase C at the plasma-membrane that catalyzes the hydrolysis of phosphatidylinositol 4,5-biphosphate (PIP_2) into the soluble second messenger inositol 1,4,5-triphosphate (IP_3) and the membrane bound diacylglycerol (DAG). The IP_3 binds to IP_3 receptors (IP_3-R) at the endoplasmasmatic reticulum, which has an internal Ca^{2+} channel that subsequently opens and releases Ca^{2+} into the cytosol. At the plasma-membrane, DAG activates protein kinase C (PKC) which initiates a phosphorylation cascade. DAG can be converted into 2 other potential second messengers: DAG lipases convert DAG to polyunsaturated fatty acids (PUFA) or phosphorylation of DAG by DAG kinases (DGK) it is transformed to phosphatidic acid (PA). Resynthesis of PIP_2 is dependent on the formation of PA that is further processed to CDP-DAG via CD-synthase (not shown). After conversion to PI at the endoplasmatic reticulum, PI is presumably transferred to plasma-membrane by a PI transfer protein (PITP) where 2 sequential phosphorylation steps by PI-kinases and PIP-kinases respectively is converted to PIP_2.

Phototransduction: Roles for IP_3 & Ca^{2+}

In the horseshoe crab *Limulus polyphemus* there is strong evidence that light induces release of Ca^{2+} from intracellular stores via IP_3 receptors localized in the submicrovillar cisternae (Payne et al., 1986). The release of Ca^{2+} precedes LIC by a few milliseconds. Not only PLC activation is required to evoke TRP opening but also the presence of extracellular Ca^{2+} is crucial. When photoreceptor cells are deprived from extracellular Ca^{2+} for prolonged time, this reversibly eliminates excitation (Hardie and Minke, 1992; Cook and Minke, 1999). Nevertheless, photolysis of caged Ca^{2+} , raising intracellular Ca^{2+} from the patch pipette or application of Ca^{2+} ionophores such as ionomycin all fail to activate TRP channels (Hardie, 1996). It has to be emphasized that these

experimental procedures do not mimic the locally very high Ca^{2+} increases found near the pore of IP_3 channels or plasma-membrane channels. This suggests that for example Calmodulin (CaM) may not be activated under these conditions. In Ca^{2+} free conditions, Ca^{2+} release from intracellular stores upon exposure to light was similar in *trp* mutant as in wildtype *Drosophila*. However, genetic elimination of the IP_3 receptor in *Drosophila* surprisingly abolished neither the light responses nor the Ca^{2+} release (Acharya et al., 1997). Furthermore, both caged IP_3 (Raghu et al., 2000) and caged GTPγS (Hardie, 1995) failed to activate phototransduction in *Drosophila*. This may reflect a diffusion barrier that prevents the chemicals from reaching the signaling membrane in the ommatidia. On the other hand, biochemical labeling studies with [^{3}H]-inositol convincingly showed that illumination caused accumulation of IP_3 and IP_2 resulting from PLC activation (Devary et al., 1987). Furthermore, addition of 2,3-diphosphoglycerate (DPG), which prevents hydrolysis of IP_3, prolonged the light response in *Lucillia cuprina* and *Musca domestica* (Devary et al., 1987; Suss et al., 1989). Thus, IP_3 itself can also not be excluded as a second messenger.

Activation by DAG Signaling

Diacylglycerol (DAG) forms the other signal generated by PLC. It is well established that formation of DAG inactivates TRP channels by phosphorylation through PKC (*Drosophila* homolog is called *inaC*). Application of phorbolesters to activate PKC suppresses the light-induced Ca^{2+} release and photon response in *Limulus* (Dabdoub and Payne, 1999). To make things more complicated, a signaling pathway downstream of DAG may lead to TRP and TRPL activation. DAG is a precursor for the generation of polyunsaturated fatty acids (PUFA), although no activity of DAG lipases has been demonstrated in *Drosophila*. However, impaired downstream DAG signaling caused by a mutation in *rdgA* (a *Drosophila* homolog of diacylglycerol kinase) causes light-independent degeneration (Masai et al., 1993; Masai et al., 1997). Thus, it was proposed that accumulation of DAG leads to increased formation of PUFAs that triggers opening of TRP and TRPL channels, leading to toxic intracellular Ca^{2+} levels and retinal degeneration. Exogenous application of PUFA to ommatidia in inside-out patches caused TRP and TRPL channel opening, making PUFA a potential candidate for TRP channel activation in *Drosophila* phototransduction. In line with this notion, a *rdgA/trp* double mutant prevented to a large extend the retinal degeneration (Chyb et al., 1999; Raghu et al., 2000).

PIP_2 Regulating TRP Channel Activity

Finally, it has been suggested that PIP_2 itself could have a role as second messenger in TRP channel activation. Two independent mutations (*rdgB*, a Phospho-Inositide (PI) transfer protein, and *cds*, an enzyme involved in PI resynthesis) in the PIP_2 recycling pathway prevent recovery of TRP channel activity from inactivation (Wu et al., 1995; Hardie et al., 2001). Furthermore, Ca^{2+} influx after illumination is required to maintain PIP_2 levels possibly through termination of PLC activity and/or facilitation of PIP_2 recycling. Hence, removal of extracellular Ca^{2+} results in sustained TRP channel opening after termination of the light exposure (Hardie et al., 2001). Recombinant expressed TRPL channels are activated by application of exogenous PLCβ and are suppressed by PIP_2 application in inside-out patches (Estacion et al., 2001). On the other hand, in *in vivo* experiments in *Drosophila trp* mutants prolonged illumination leads to PIP_2 depletion and closure of TRPL channels, which remain inactivated until PIP_2 is resynthesized (Hardie et al., 2001).

Since DAG/PUFA can activate TRP and PIP_2 regulates these channels, the interesting hypothesis arises that simultaneous generation of DAG and depletion of PIP_2 might trigger TRP channel opening. This suggests that TRP channels may possess domains for DAG/PUFA binding, stabilizing the open state of the channels, as well as for PIP_2 binding to retain the channel in a closed state.

Mammalian TRP Channels

Following the identification of TRP and TRPL channels as Ca^{2+} conducting channels in Drosophila, 28 mammalian genes encoding TRP channels have been cloned and their functions are now beginning to be understood. TRP channels are involved in many different responses such as perception of temperature, touch and pain, smelling odorants and pheromones, but also in regulation of cellular ion homeostasis and uptake of ions in the kidney and intestine. Similarly,

regulation of TRP channels shows a perplexing variety of different mechanisms.

The mammalian TRP channel family can be grouped into 6 subfamilies (Figure 3, Clapham et al., 2003): canonical (TRPC), vanilloid receptor (TRPV) and melastatin-related (TRPM) channels, and the smaller subfamilies mucolipins (TRPML), polycystins (TRPP) and TRPA (an anchorin-repeat containing channel). Whereas the focus of this thesis is on TRPM7, I will here present a brief overview of the current literature on the major subfamilies to provide a background for understanding the regulation and biophysical properties of TRPM7. Emphasis will be on the modes of activation, regulation and the proposed gating mechanisms of TRPC, TRPV and TRPM channels. Information on TRPA, and its Drosophila homologue *NompC*, which have both been implicated in mechanosensation, will be presented in the paragraph on mechanosensation by ion channels.

TRPC Subfamily

The mammalian TRPC subfamily consists of 7 channels, named TRPC1-7. Of all mammalian TRP channels, this subfamily shares the highest homology with *Drosophila* TRP and TRPL channels. TRPC channels can be divided in 3 groups based on phylogeny: first, TRPC1, TRPC4 and TRPC5, second, TRPC3, TRPC6 and TRPC7, and third TRPC2 (Clapham, 2003). All these channels share a structural feature, a so-called TRP box consisting of an invariant amino acid sequence EWKFAR juxtamembrane to the 6^{th} transmembrane domain. Furthermore, these channels possess N-terminal ankyrin repeats and are non-selective cation channels, which selectivity ratio P_{Ca}^{2+}/P_{Na}^{+} varies from 1.1 for TRPC4 to 9 for TRPC5 (Schaefer et al., 2000). Gating of all TRPC channels is downstream of GPCR- or tyrosine-kinase receptor-mediated PLC activation. In all cases, the exact gating mechanism is still unclear and subject to debate: both store-dependent and -independent mechanisms have been proposed. For example, both TRPC1 and TRPC7 were shown to be activated in a store-dependent manner (Zitt et al., 1996), whereas others have suggested a store-independent mechanism (Lintschinger et al., 2000). Adding to the confusion, a recent paper shows that expression of TRPC1 did not induce any measurable currents at all (Strubing et al., 2001).

Physiological Functions of TRPC Channels

Channels of the first TRPC group regulate a variety of cellular responses. TRPC1, which is expressed in various tissues, is involved in regulating vascular permeability (Bergdahl et al., 2003; Kunichika et al., 2004) and axonal turning upon chemotropic stimulation (Wang and Poo, 2005). The first TRP channel knocked out in mice was TRPC4. These TRPC4-deficient mice revealed that this channel is involved in agonist-induced relaxation of blood vessels and lung microvascular permeability (Freichel et al., 2001; Tiruppathi et al., 2002). TRPC5 is predominantly expressed in the central nervous system and is abundantly present in hippocampal neurons, where it might be an important determinant of axonal growth and growth cone morphology (Greka et al., 2003).

Members of the second group are relatively highly expressed in cardiac and smooth muscle cells. TRPC3 and TRPC6 are shown to be involved in vasoregulation and regulation of tracheal contractility. TRPC3 and TRPC7 are proposed candidates for non-selective cation channels that may regulate Ca^{2+}-dependent contractility. TRPC2 was reported to be a pseudogene in humans, but not in other mammal species (Vannier et al., 1999). This channel is expressed in the vomeronasal organ of the rat (Liman et al., 1999), where it most likely involves pheromone signaling, since TRPC2 deficient mice display abnormal mating behavior (Stowers et al., 2002). Besides, expression of TRPC2 in the head of mouse sperm is involved in the release of hydrolytic enzymes upon egg fertilization (Jungnickel et al., 2001).

Signaling Complex of TRPC Channels

The signaling complex associated with *Drosophila* TRP channels is known as the transducisome or signalplex (Li and Montell, 2000). This protein complex, which consists of TRP channels, the *InaD* scaffold protein and regulatory proteins, has served as a model for possible signaling complexes of TRPC channels. By analogy to this signaling complex, a human homologue of *InaD* protein was cloned that has at least 5 protein-interacting PDZ domains (Philipp and Flockerzi, 1997). Unfortunately, no binding partners have yet been identified. However, several TRPC channels are linked to possible regulatory proteins. For example, TRPC4 and PLCβ_1 form a protein complex with the protein NHERF

(regulatory factor of the Na^+/H^+ exchanger) that contains 2 PDZ domains and is also linked to the cytoskeleton. To date, still no experimental data are present on the impact of the cytoskeletal interaction on channel functioning (Tang et al., 2000). Like the *Drosophila* TRP channels, all TRPC family members can bind directly to both calmodulin and the IP_3-receptor, suggesting that both these proteins are involved in regulation of the channels. As the binding sites of CaM and the IP_3-R in TRP channels partially overlap, competition between both proteins may occur (Tang et al., 2001).

Interestingly, PLCs are also involved in trafficking of TRPC channels. TRPC3 contains a partial PH-domain that is complemented by the C-terminal split PH-domain of PLCγ_1. The interaction of both domains forms a complete PH-domain capable of interacting with PIP_2 and is required for proper trafficking of TRPC3 to the plasma-membrane (Van Rossum et al., 2005)

Hetero-tetramerization of TRPC Channels

TRPC channels are expressed in virtually all tissues, cell types and cell lines and it seems likely that they can co-assemble in hetero-tetrameric complex (Garcia and Schilling, 1997; Hofmann et al., 2000; Riccio et al., 2002). However, to date most data on multimerization of TRPC channels are from heterologous expression studies performed in cancer cell lines. A major drawback of this approach is that overexpression favors formation of TRPC homo-tetramers and cell may lack (sufficient) additional factors that may be involved in channel functioning and regulation. Do these channels exist and function as homo-tetramers *in vivo*?

Increasing evidence suggests that the TRPC channel functioning is far more complex than thought before. Several TRPC family members can bind to each other and form hetero-tetrameric channels with properties different from the individually expressed TRPC channels. Co-immunoprecipitation revealed that TRPC1 can form a hetero-tetrameric channel with TRPC3 (Lintschinger et al., 2000), TRPC4 (Strubing et al., 2003) and TRPC5 (Strubing et al., 2001). Co-expression of TRPC1 with TRPC3 (Lintschinger et al., 2000), TRPC4 or TRPC5 (Strubing et al., 2001) resulted in currents with properties different of those of individually expressed channels. From studies that use various different approaches such as electrophysiological recording, co-immunoprecipitation and FRET assays, the picture emerges that TRPC1, TRPC4 and TRPC5 can co-assemble together, and that TRPC3, TRPC6 and TRPC7 can interact with each other in both overexpression studies (Hofmann et al., 2002) as well as in native tissues (Goel et al., 2002). In contrast, in both studies, members of one group were unable to cross interact with members of the other group.

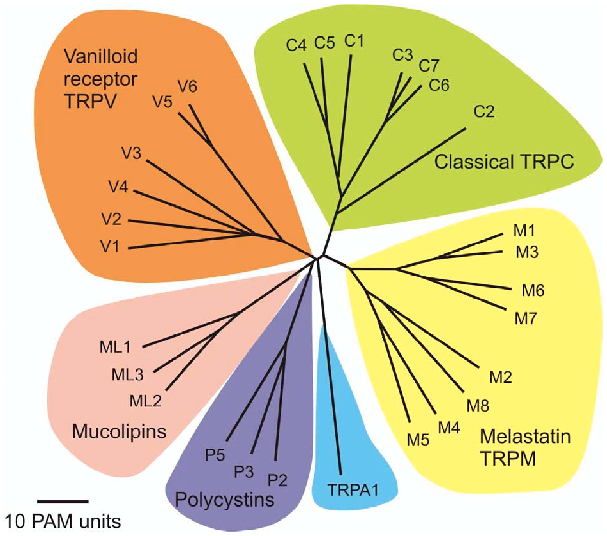

Figure 3: Phylogenetic tree of the mammalian TRP family.

The evolutionary distance is shown by the total branch lengths in point accepted mutations (PAM) units, which is the mean number of substitutions per 100 residues. Adapted by permission from Macmillan Publishers Ltd: Nature, (Clapham 2003) 2003

The domain responsible for interaction between TRPC proteins has not yet been defined, although recent studies suggest that a conserved coiled-coil domain at the C-terminus of all TRPC channels may be involved (Engelke et al., 2002). Strikingly, alignment of this conserved coiled-coil region reveals that it displays an offset in TRPC3, TRPC6 and TRPC7 compared to TRPC1, TRPC4 and TRPC5, which is consistent with the selective association of the TRPC subunits.

BOX 2: Structure, Ion Selectivity and Gating of TRP Channels

TRP Channels as Tetramers

TRP ion channels possess 6 transmembrane domains and cytosolic N- and C-terminus reside in the cytosol. In analogy to the pore-forming subunits of voltage-gated Ca^{2+}, Na^{+} and K^{+} channels, which consist of either 4 subunits (Shaker K^{+} and IRK channels) or internal 4-fold repeats (Ca^{2+} and Na^{+} channels), it is reasonable to assume that TRP channels also consist of 4 subunits.

The TRP Pore Region and Ion Selectivity

A short hydrophobic stretch of amino acids between transmembrane domain 5 and 6 is predicted to be the pore-forming region in analogy to the K^{+} channel KscA (Yool and Schwarz, 1991; Yellen et al., 1991). In a tetramer, 4 pore-forming regions together form the channel pore that is a often negatively charged ring that determines the ion selectivity of the channel (Doyle et al., 1998). Overall, the architecture of the selectivity filter of TRP channels is poorly defined. Mutational studies showed that replacing aspartate residues by neutral amino acids in the pore region alters the selectivity for Ca^{2+}, but also the sensitivity for intracellular Mg^{2+}, voltage-dependent gating and sensitivity to channel blockers (Owsianik et al., 2006). The aspartate residues in the proposed selectivity filter of TRPV4, TRPV5 and TRPV6 form a negatively charged ring that to some extent determines Ca^{2+} selectivity of the channel, analogous to voltage-gated Ca^{2+} channels (Voets et al., 2003). The amino acids involved in cation selectivity in the pore of the other monovalent-selective and non-specific TRP channels have not been identified yet. Alignment of the pore region shows that TRPC and TRPM channels have a relatively high degree of conservation within the subfamilies (Owsianik et al., 2006). There is only marginal homology within the TRPV pore region, and in the absence of further mutational studies, identification of amino acids involved in the selectivity of these channel pores are merely an educated guess.

Proposed Gating Mechanism of TRP Channels

All these channels are involved in sensing and responding to a variety of stimuli. The cytosolic end of the 6^{th} transmembrane domain is suggested to form the gating lever that is situated in line with the selectivity filter of the channel. Upon stimulation, opening and closure of the channel is probably managed by movement of the 4^{th} transmembrane domain. This assumption is based on voltage-gated channels, where this region is positively charged and moves in the extracellular direction in response to cell depolarization, probably pulling the gating lever open.

TRPV Subfamily

TRPV channels, which are also widely expressed, share a high homology with the Osm9 channel in *Caenorhabditis elegans*. Behavioral studies with Osm9-deficient *C. elegans* showed its involvement in response to odorants, osmotic strength and mechanical stimulation (Colbert et al., 1997). The founding member TRPV1 was identified by expression cloning with the hot pepper-derived vanilloid compound capsaicin (Caterina et al., 1997). Like TRPC channels, TRPV family members have a TRP box after the 6^{th} transmembrane domain and N-terminal ankyrin repeats. TRPV channels non-specifically conduct cations and the Ca^{2+} selectively is set by a single aspartic acid residue in the channel pore (Garcia-Martinez et al., 2000; Voets et al., 2003). The TRPV protein family can be divided in 2 groups based on phylogeny, biophysical properties and cellular function: first, TRPV1-4 and second TRPV5 and TRPV6. Typically, the first group of TRPV channels can be activated and regulated by temperature whereas TRPV5 and TRPV6 are constitutively active.

Temperature Regulated TRPV channels

TRPV1 is activated by temperatures above 43°C and in addition by the chemical compounds 2-APB (Hu et al., 2004), capsaicin and endogenous cannabinoid receptor ligands like anandamide. The binding domain for both ligands is located to an intracellular domain adjacent to the 3rd transmembrane domain (Jordt and Julius, 2002). Heterologous expression of TRPV1 channels displays an outward rectifying current-voltage relationship and reveals anomalous mole fraction behavior as apparent from linearization of the I/V in divalent free medium. TRPV1 currents are activated as well as potentiated by a low pH (< 5.9) (Caterina et al., 1997) and inhibited by PIP_2 (Chuang et al., 2001). Release from PIP_2 inhibition by receptor mediated PLC activation increases heat-activated TRPV1 currents (Chuang et al., 2001). Furthermore, TRPV1 current are sensitized by PKC and PKA activity, however the mechanisms of action remains to be clarified. A TRPV1 knock-out mouse implicated the involvement of this channel in nociception, inflammation and in the hypothermic effects of vanilloid compounds (Caterina et al., 2000). Moreover this channel is implicated in pancreatitis (Nathan et al., 2002) and asthma (Hwang and Oh, 2002).

TRPV2 shares 50% homology with TRPV1 and is activated by noxious heat (> 52°C) and 2-APB, rather than capsaicin or pH (Caterina et al., 1999; Jordt and Julius, 2002; Hu et al., 2004). Activated TRPV2 channels display moderately outward rectifying I/V characteristics. Activation by growth factors like Insulin Growth Factor-1 (IGF-1) translocates functional TRPV2 channels to the plasma-membrane by incorporation of intracellular vesicles that contain preassembled channels (Kanzaki et al., 1999). Stretch forces can similarly lead to incorporation of the channel in the plasma-membrane in vascular smooth muscle cells (Muraki et al., 2003).

TRPV3 and TRPV4 channels are also activated by heat, but in a more moderate temperature range: raising temperature above 25°C will activate TRPV4 channels (Guler et al., 2002) whereas above 31°C TRPV3 currents are activated (Peier et al., 2002; Smith et al., 2002; Xu et al., 2002). Moreover, TRPV3 channels can be activated by 2-APB while TRPV4 channels are unaffected (Hu et al., 2004). TRPV3 displays an outward rectifying I/V-relationship (Xu et al., 2002) and the I/V-plot of TRPV4 is linear (Watanabe et al., 2002). Unlike TRPV3, TRPV4 currents are enlarged upon osmotic cell swelling cells (Liedtke et al., 2000; Strotmann et al., 2000). This effect is mediated by phosphorylation of TRPV4 channels, reportedly downstream of the tyrosine kinase Src (Xu et al., 2003). Subsequent cell shrinkage reverses TRPV4 currents to basal values.

Ca^{2+} Gatekeepers TRPV5 and TRPV6

In contrast to the temperature regulated TRPV channels, TRPV5 and TRPV6 can not be activated by temperature changes. Heterologous expression in HEK293 cells showed that both channels are constitutive active (Vennekens et al., 2001) and are highly selective for Ca^{2+} ($P_{Ca}^{2+}/P_{Na}^{+} > 100$). The I/V-relationships of both channels are inward rectifying, with hardly any outward currents at positive voltages (Vennekens et al., 2000; Vennekens et al., 2001). The aspartic residue in the pore defining Ca^{2+} selectivity also influences the Mg^{2+} sensitivity (Nilius et al., 2001). Mg^{2+} ions cause a voltage-dependent block of TRPV5 and TRPV6 currents. TRPV5 channels function as the main gatekeeper of apical Ca^{2+} influx pathway in kidney and TRPV6 fulfils this role in intestine (den Dekker et al., 2003; Nijenhuis et al., 2003)

TRPV5 as well as TRPV6 are controlled by intracellular Ca^{2+} levels through a negative feedback loop with apparent affinity of ~100 nM (Vennekens et al., 2001). The precise mechanism of this feedback loop is still unclear. Interestingly, CaM binds Ca^{2+}-dependently to human TRPV6 and this interaction is regulated by PKC-mediated phosphorylation of the TRPV6 CaM binding domain. Phosphorylation of a threonine residue inhibits CaM binding and thereby attenuates inactivation of human TRPV6 channels (Niemeyer et al., 2001). The CaM-binding domain is poorly conserved between mouse and human, and this mode of regulation thus seems to be restricted to human TRPV6 channels. The human TRPV5 amino-acid sequence also shows poor homology in this binding domain and therefore it is doubtful whether the same Ca^{2+} dependent regulation of TRPV5 channels exists.

Heteromultimeric TRPV Complexes

As covered in the previous paragraph, based on phylogeny, functional and biophysical properties, the TRPV channel family can be divided into 2 subgroups. All TRPV channels do form homo-tetramers (Hellwig et al., 2005) but are they also capable to form hetero-tetramers?

Data from FRET and co-immunoprecipitation assays show that TRPV1 and TRPV2 can interact with each other, although, TRPV2 preferably

associates with other TRPV2 subunits and only to a minor extent with TRPV1 (Hellwig et al., 2005). Smith et. al found that TRPV1 can also co-assemble with TRPV3, based on responses to capsaicin and on immunoprecipitation assays (Smith et al., 2002). In contrast, a very recent study failed to detect functional or physical interactions between these TRPV family members (Hellwig et al., 2005). For this reason, it still remains unclear whether TRPV1 and TRPV3 can form hetero-tetrameric channels. The remaining member of this subgroup, TRPV4 is not able to form hetero-tetrameric channels upon co-expression with all other TRPV family members (Hellwig et al., 2005).

TRPV5 and TRPV6 are endogenously expressed in various epithelial tissues and their co-expression levels affect renal and intestinal Ca^{2+} absorption (van Abel et al., 2005). By various biochemical assays it was shown that TRPV5 and TRPV6 can co-assemble in hetero-tetramers (Hoenderop et al., 2003). In summary, formation of heteromultimeric TRPV channels may occur with some specificity, although the complete picture has not yet emerged.

TRPM Subfamily

TRPM channels show a large variety in cell-biological and biophysical properties. For convenience, members of this subfamily will here be loosely subdivided in 3 groups: TRPM4 and TRPM5, which share significant similarities in both functional and biophysical properties, TRPM6, TRPM7 and TRPM2, which are remarkable combinations of an ion channel and an enzyme, and the remaining channels.

TRPM1, TRPM3 and TRPM8

Melastatin (TRPM1), the founder of the TRPM family, was identified in a screen of human melanoma-correlated mRNAs. In melanocytes, short and full-length TRPM1 mRNA transcripts are present. Decreased expression of the short transcript of TRPM1 correlates with increased invasiveness of malignant melanomas and it is therefore used as a diagnostic marker (Duncan et al., 1998). TRPM1 is widely expressed in different tissues, but functional and electrophysiological properties have not been studied extensively so far. Untill now, the only ion known to be conducted by full-length TRPM1 is Ca^{2+} (Xu et al., 2001). While the precise function of TRPM1 remains unclear, a putative role in cellular differentiation and proliferation was suggested by Fang et al. (Fang and Setaluri, 2000). The short, cytosolic isoform of TRPM1 may be responsible for correct trafficking of the full length TRPM1 protein to the plasma-membrane (Xu et al., 2001)

The closest relative of TRPM1 is TRPM3, which is primarily expressed in human kidney and brain but not in mouse kidney (Grimm et al., 2003; Lee et al., 2003). Heterologous expression of TRPM3 results in formation of constitutive active, non-selective cation channels. Recently, 5 alternatively spliced TRPM3 variants have been characterized (α1-5). Interestingly, 2 isoforms, TRPM3α1 and TRPM3α2, differ only in their pore region (Oberwinkler et al., 2005). Whereas the TRPM3 was originally reported to have linear I/V characteristics (Grimm et al., 2003), both α1 and α2 splice variants revealed outward rectifying ion currents regulated by intracellular Mg^{2+} (Oberwinkler et al., 2005). However, their ion selectivity is remarkably different: TRPM3α1 channels are poorly permeable for divalent ions whereas TRPM3α2 conduct Ca^{2+} and Mg^{2+} rather well. Furthermore, TRPM3α2 channels are inhibited by extracellular monovalent ions whereas TRPM3α1 mediated currents are unaffected.

TRPM3 is reported to be regulated by osmolarity: in a hypotonic medium, the conductance of TRPM3 increased in amplitude (Grimm et al., 2003). In addition, activation of TRPM3 by store depletion (Lee et al., 2003) and by D-erythro-sphingosin (Grimm et al., 2005) was also reported.

The TRPM8 gene was identified as a prostate-specific gene and it appears to be upregulated in prostate as well as other cancers (Tsavaler et al., 2001). Heterologously expressed TRPM8 channels are activated by low temperatures (8 - 24°C) and by cooling agents such as menthol and icillin (McKemy et al., 2002; Peier et al., 2002; Nealen et al., 2003), which is consistent with a role in thermosensation and nociception. TRPM8 channels permeate both monovalent and divalent ions and they are responsible for the intracellular Ca^{2+} elevations induced by menthol and icillin. TRPM8 currents display strong outward rectification and reverse around 0 mV.

Reponses to cold temperature and icillin depend on a physiological intracellular H^{+} concentration: lowering the pH inhibits TRPM8 opening by these stimuli, but menthol-induced activation is unaffected (Andersson et al., 2004). The gating is caused by temperature-dependent

BOX 3: Imaging PIP_2 Kinetics in Living Cells

Confocal Imaging of PIP_2 Kinetics

The PH-domain of PLCδ1 fused to Green Fluorescent Protein (GFP) detects PIP_2 at the plasma-membrane and is widely used to study PIP_2 kinetics in living cells by confocal laser scanning microscopy (Figure box 3A, (Stauffer et al., 1998; Varnai and Balla, 1998). Unstimulated cells have high PIP_2 levels and GFP-PHd1 is bound to the membrane (Figure box 3A, inset 2). Receptor-activated PLCs hydrolyse PIP_2 causing translocation of the PIP_2 probe into the cytosol (Figure box 3A, inset 2). During resynthesis of PIP_2, the fluorescence recovers at the plasma-membrane.

PIP_2 Kinetics Measured by FRET

The principle of measuring PIP_2 kinetics by Frequency Resonance Energy Transfer (FRET) is similar as described above. In stead of GFP, the color variants cyan (CFP) or yellow (YFP) are fused to PHδ1 and simultaneously expressed in living cells (Figure box 3B, (van der Wal et al., 2001)). Because the emission spectrum of CFP (donor) overlaps with the excitation spectrum of YFP (acceptor) radiationless transfer of energy occurs. Because of the high plasma-membrane PIP_2 levels, the donor and acceptor are in close proximity (< 10 nM) and FRET appears as quenching of the donor and as gain of acceptor fluorescence (Figure box 3B). Upon hydrolysis of PIP_2, CFP- and YFP-PHδ1 translocate to the cytosol. Consequently the distance increases between both fluorophores (> 10 nM) and FRET decreases. This leads to a decline in YFP emission and an increased CFP fluorescence (Figure box 3B, graph). When PIP_2 levels are recovering, the FRET values increases.

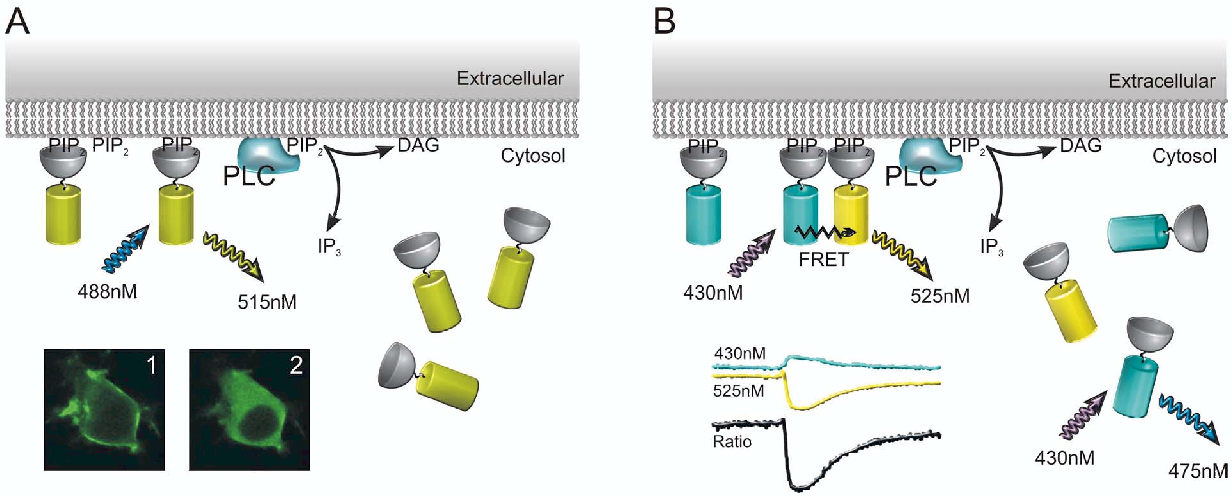

Figure box 3: Principles of imaging PIP2 kinetics in living cells

Cells transfected with FP-PHd1 and PIP2 dynamics are followed in time by either confocal laser scanning or bright field microscopy. During confocal experiments GFP is excited at 488 nM and GFP fluorescence detected at 525 nM. In FRET experiments, excitation occurs at 430 nM and fluorescence CFP and YFP are simultaneously recorded.

shift in voltage-dependence: low temperatures and cooling agents shift the voltage-dependence of TRPM8 negatively towards the physiological range (Voets et al., 2004a). Activity of TRPM8 channels depends on the presence of PIP_2 at the plasma-membrane that binds to the TRP-domain (Rohacs et al., 2005). Whole-cell and inside-out patch experiments produce rundown of TRPM8 channels because of PIP_2 depletion at the plasma-membrane (Rohacs et al., 2005; Liu and Qin, 2005).

TRPM4 and TRPM5: Channels Specific for Monovalent Ions

TRPM4 and TRPM5 are the only TRP channels impermeable to divalent ions: these channels selectively conduct monovalent ions and thereby depolarize the plasma-membrane (Launay et al., 2002; Hofmann et al., 2003; Prawitt et al., 2003). Signaling of GPCRs via PLC and release of Ca^{2+} from the ER will activate these channels, although the precise mechanism is unclear. For activation of the channels relatively high $Ca^{2+}{}_i$ levels are necessary, > 0.3μM for TRPM4 (Launay et al., 2002) and TRPM5 requires over 1μM of $Ca^{2+}{}_i$ (Hofmann et al., 2003), suggesting that both channels may be in close proximity to Ca^{2+} release sites of the ER. The I/V plots of both TRPM4 and TRPM5 are non-linear as a result of an intrinsic voltage-sensing mechanism that is independent of divalent cation binding (Launay et al., 2002; Hofmann et al., 2003). Sustained exposure to Ca^{2+} desensitizes both channels, but administration of PIP_2 reverses desensitization partially for TRPM5 (Liu and Liman, 2003) and restores TRPM4 currents fully (Zhang et al., 2005)

TRPM4 is ubiquitously expressed and possibly serves as a negative feedback regulator of Ca^{2+} oscillations (Launay et al., 2002): high Ca^{2+} levels during Ca^{2+} oscillations activate TRPM4, which subsequently depolarizes the plasma-membrane and decreases the driving force for Ca^{2+} entry in non-excitable cells. In addition, in excitable cells TRPM4-mediated depolarization may be important in regulation of Ca^{2+} entry through voltage-gated Ca^{2+} channels, consequently shaping action potential duration and frequency. The TRPM5 gene was originally identified in a chromosomal region that is associated with several tumors (Enklaar et al., 2000), but a later study showed that TRPM5 is primarily found in taste receptor cells (Perez et al., 2002). The taste receptors T1R and T2R both signal via $PLC\beta_2$ to TRPM5. This signaling pathway mediates taste sensation of sweet, bitter and amino acid (Perez et al., 2002; Zhang et al., 2003).

TRPM Channels with Intrinsic Enzymatic Activity

A separate group of TRPM channels is formed by TRPM2, TRPM6 and TRPM7. These three channels are characterized by a C-terminal enzyme moiety. TRPM2 channels posses a NUDT9 Nudix hydrolase motif (Perraud et al., 2001), whereas TRPM6 (Schlingmann et al., 2002; Walder et al., 2002) and TRPM7 (Runnels et al., 2001) both have a serine/threonine kinase at their C-terminus. Physiological and biophysical properties of TRPM7 will be discussed in detail in the following section.

TRPM2 forms a Ca^{2+} permeant non-selective ion channel that also conducts K^+, Na^+ and Cs^+. It has linear I/V properties and reversal potential around 0 mV (Perraud et al., 2001). The channel is activated by binding of ADP-ribose (~100 μM), cADP-ribose (> 100μM) or NAD (> 1mM) to the Nudix motif, which is an inefficient hydrolase (Perraud et al., 2001; Sano et al., 2001; Kolisek et al., 2005). Interestingly, cADP-ribose (< 10μM) potentiates ADP-ribose-mediated TRPM2 activation to nM concentrations (Kolisek et al., 2005). Opening of TRPM2 channels is facilitated by intracellular Ca^{2+}, but by itself Ca^{2+} can not activate the channel (Perraud et al., 2001; McHugh et al., 2003).

Furthermore, oxidative stress (hydrogen-peroxide) and tumor necrosis factor α also regulate TRPM2 channel opening. Therefore, Hara and collaborators suggest that TRPM2 may act as an intracellular oxidation-reduction sensor (Hara et al., 2002). Prolonged exposure to oxidative stress concurrently results in increased Ca^{2+} levels and apoptosis of cardiac myocytes and hematopoietic cells (Zhang et al., 2005; Yang et al., 2005).

TRPM6 (also known as ChaK2) channel currents are carried by mono- and divalent cations. TRPM6 currents reverse around 0 mV and their I/V-plot is outward rectifying. Moreover, the current is strongly regulated by internal Mg^{2+} ions (Voets et al., 2004b). TRPM6 expression is predominantly expressed in intestinal epithelia and kidney tubules, and this has lead to the hypothesis that it is involved in uptake of Mg^{2+} in the gut and renal reabsorption of Mg^{2+} and Ca^{2+}. Indeed, several mutations in TRPM6 are linked to a disorder in renal and intestinal Mg^{2+} and Ca^{2+} absorption that is known as familial hypomagnesemia with secondary hypocalcemia (FHSH, Schlingmann et al., 2002; Walder et al., 2002). Transport of TRPM6 to the plasma-

membrane and subsequent incorporation is dependent on hetero-tetramerization with TRPM7. A missense mutation in TRPM6 abrogated oligomerization with TRPM7, providing a possible explanation for the impaired epithelial Mg^{2+} reabsorption in patients diagnosed with FHSH (Chubanov et al., 2004).

TRPM7

Physiological Properties

TRPM7 (also kown as ChaK1, LTRPC7 and TRP-PLIK) shares many properties with TRPM6 but it has been studied much more extensively. Like TRPM6, the TRPM7 currents are carried by cations and, at least in whole-cells, they display an outward rectifying I/V-plot (Runnels et al., 2001; Nadler et al., 2001). Monovalent TRPM7 currents are inhibited by internal Mg^{2+} ions, i.e. the channels display an anomalous mole fraction behavior that has also been termed 'divalent permeation block'. In accordance, the I/V relationship linearizes in divalent-free solutions.

In addition, it was reported that depletion of intracellular Mg^{2+} (Prakriya and Lewis, 2002; Kozak and Cahalan, 2003) or Mg-nucleotides (Nadler et al., 2001; Demeuse et al., 2006) causes full activation of TRPM7 by a mechanism independent of Mg^{2+} permeation block (Kozak and Cahalan, 2003). A current with similar properties had been previously described as Mg-Nucleotide-regulated Metal (MagNuM) ion current (Nadler et al., 2001) or Magnesium Inhibited Current (MIC) (Kerschbaum and Cahalan, 1998). Subsequent inhibitor studies demonstrated that TRPM7 is the carrier of this current (Kerschbaum et al., 2003).

Besides Ca^{2+} and Mg^{2+}, TRPM7 provides a mechanism for entry of trace metal ions as Ni^{2+}, Co^{2+}, Mn^{2+} and Zn^{2+} (Monteilh-Zoller et al., 2003).

Potential Functions of TRPM7 Channels

Whereas it was originally suggested that TRPM7 would be a good candidate to conduct ICRAC (Cahalan, 2001), detailed follow-up studies have now dismissed this idea. It was shown that biophysical and pharmacological properties of TRPM7 differ from those of ICRAC. For example, ICRAC is strongly selective for Ca^{2+} while TRPM7 conducts both Mg^{2+} and Ca^{2+} (Runnels et al., 2001; Nadler et al., 2001). Unlike TRPM7, ICRAC is also impermeant to Mn^{2+} ions and has low Cs^{+} permeability. Furthermore, ICRAC displays a rapid desensitization in divalent-free media that is not seen in TRPM7. Inhibitor profiles of ICRAC and TRPM7 show distinct differences as well. In divalent free media, the aspecific Ca^{2+} entry blocker SKF 96365 fully inhibits ICRAC whereas TRPM7 currents are unaffected. Moreover, TRPM7 currents are insensitive to low 2-APB concentrations, while ICRAC is potentiated by this compound (Prakriya and Lewis, 2002).

The TRPM7 protein is ubiquitously expressed and TRPM7 knockout cells are not viable due to Mg^{2+} deficiencies. Therefore this channel was the first protein hypothesized to be directly involved in Mg^{2+} homeostasis (Schmitz et al., 2003). Knocking out the TRPM7 gene in mice is embryonically lethal at a very early stage (E6.5), showing the importance of the channel during development (Kim et al., 2005). A rare point mutation in TRPM7 was identified that increases the sensitivity of the channel to Mg^{2+} inhibition and that may contribute to the pathogenesis of 2 types of neurodegenerative disorders (Hermosura et al., 2005). However, alternative functions for TRPM7 have also been proposed, e.g. in proliferation of retinoblastoma cells (Hanano et al., 2004), anoxic cell death (Aarts et al., 2003) and in zebrafish skeletogenesis (Elizondo et al., 2005).

Regulation of TRPM7 Channel Activity

The α-Kinase Domain and TRPM7 Channel Gating

Since the cloning of TRPM7 it has been debated whether the kinase domain is involved in regulation of the channel gating. Initially it was reported that TRPM7 channel gating requires a functional kinase domain (Runnels et al., 2001). However, subsequent studies and our own data show that the channel gating can be dissociated from kinase activity and TRPM7 autophosphorylation. Whole-cell currents and Ca^{2+} influx through kinase-dead point mutants were indistinguishable from those of wild-type TRPM7 (Schmitz et al., 2003; Matsushita et al., 2005; Clark et al., 2006). Complete deletion of the kinase domain caused a significant, but not complete, decrease in both Ca^{2+} influx and whole-cell currents. According to Matsushita, impaired trafficking to the plasma membrane or assembly of tetramers can not be excluded, since the expression levels of the deletion mutant were similar to the full-length construct (Matsushita et al., 2005). In contrast with observations made by Matsushita,

Schmitz and coworkers found that kinase-dead TRPM7 channels are less sensitive to inhibition by intracellular Mg^{2+}. In their hands, the kinase-deletion mutant did not affect channel conductance but it was more potently inhibited by Mg^{2+}_i (Schmitz et al., 2003). Overall, these reports support the view that the kinase domain is neither required for activation of the TRPM7 channel nor that it contains the internal Mg^{2+} sensor.

TRPM7 Interacts with PLC Isozymes

The available evidence suggests that TRPM7 contains a number of domains that are involved in PLC-mediated signaling. The channel was originally cloned in the lab of Dr. D. Clapham in a yeast-two-hybrid screen for interactors with PLCβ1. Analysis showed that this interaction involves the kinase domain of TRPM7 and the C2-domain of $PLC\beta_1$ (Runnels et al., 2001). Later, it was shown that the kinase domain can also bind to the PLC isoforms β_2, β_3 and γ_1 (Runnels et al., 2002). As $PLC\gamma_1$ was shown to contain a split PH domain that interacts with a complementary split PH domain in TRPC3 (Van Rossum et al., 2005),

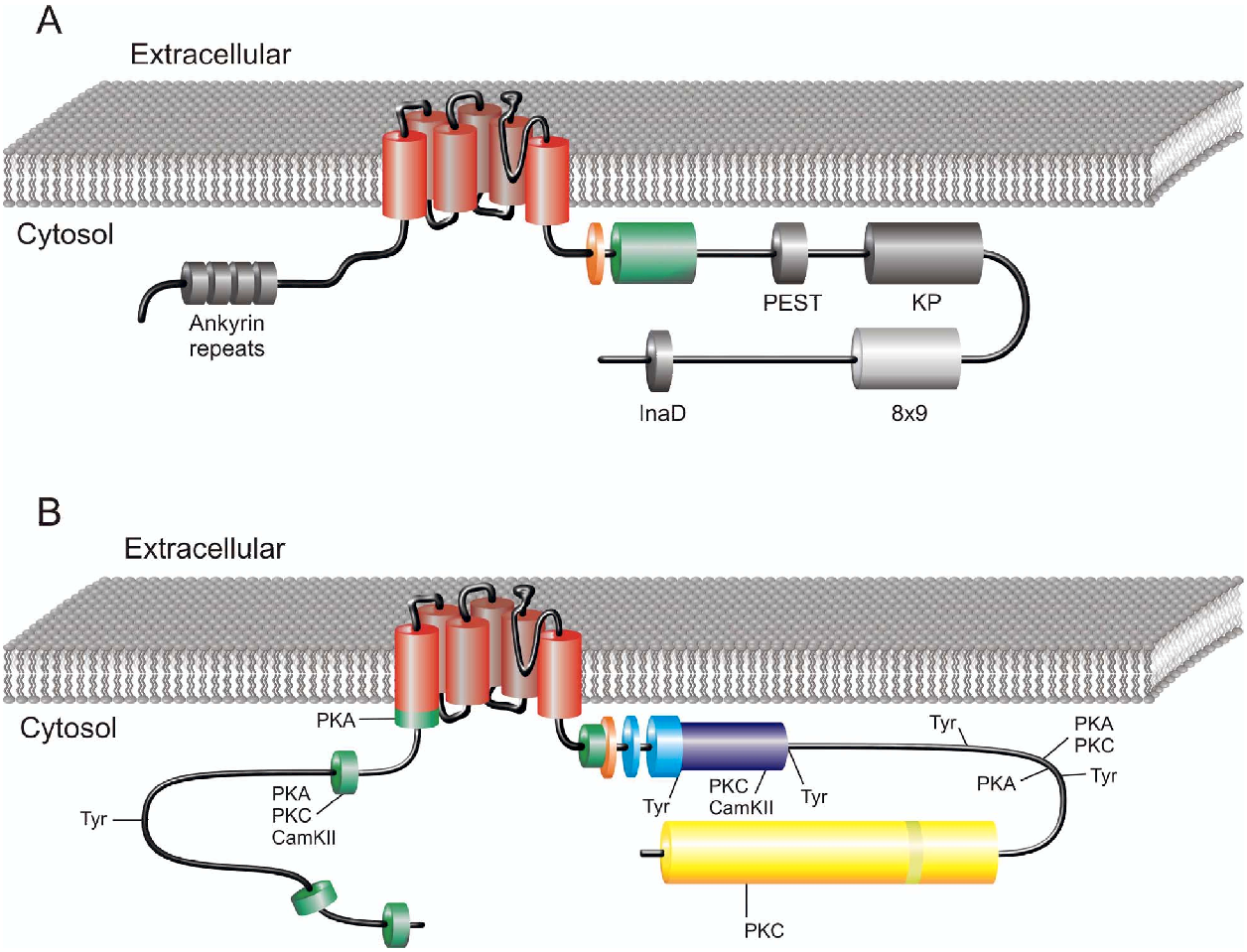

Figure 3: Putative domain structures of *Drosophila* TRP channels and the mammalian TRPM7 channel.

The protein sequence for both TRP (A) and TRPM7 (B) channels encodes for 6 hydrophobic transmembrane domains (red) and a pore region between the 5th and 6th domain. Downstream of the 6th transmembrane helix the TRP domain is located (orange), characteristic for all TRP channels. In TRP there is one putative Calmodulin-binding motif (green) directly downstream of the TRP domain. *In silico* analysis of the TRPM7 sequences revealed 5 putative CaM motifs and one is topological similar to the one located in TRP. At the end of C-terminus, the TRP channel has an InaD binding and other features of TRP are a PEST sequence, proline-rich region with the dipeptide lysine-proline (KP) repeating 27 times. The TRP domain has a unique and curious hydrophylic sequence (8x9) near the C-terminus. The TRPM7 gene at the C-terminus encodes a unique α-kinase (yellow) with embedded a split PH domain (light green). TRPM7 contains 2 putative PIP_2 binding sites at the C-terminal side of the TRP domain (blue, see text for details), which is followed by a coiled-coil region (purple). Amino acid analysis revealed several potential phosphorylation sites for PKA, PKC, CaM kinase II and tyrosine kinases.

we analyzed TRPM7 for the presence of such a split PH domain (van der.Krogt, unpublished). We used a search algorithm that was programmed in our lab to identify split PH domains similar to that of TRPC3. Interestingly, a partial PH-domain complementing the C-terminal half of the PLCγ_1 split PH-domain is located in the TRPM7 kinase (see Figure 3). Therefore we anticipate that the PLCγ_1 interaction with TRPM7 mediates trafficking to the plasma-membrane

TRPM7 Functioning is Dependent on PIP_2

The function of many TRP channels, including TRPM4, TRPM5 and TRPM8, depend on the presence of PIP_2 at the plasma-membrane. A role for PIP_2 in regulating TRPM7 has also been claimed (Runnels et al., 2002). Runnels observed that when TRPM7 channels were pre-activated by depletion of intracellular Mg^{2+} in whole-cells or inside-out patches, agonist-induced PIP_2 hydrolysis potently closed the channels. Subsequent addition of PIP_2 to the intracellular leaflet of the plasma membrane restored the conductance (Runnels et al., 2002).

This report has been the source of much confusion. First, an inhibitory role for PLC signaling contradicts observations that we had obtained earlier (Chapter 2 & 3, Clark et al., 2006). We found that activation of PLC activates TRPM7 channels, as detected by morphological and electrophysiological methods as well as using Ca^{2+} fluorometry. In Chapter 2 and 3, this issue is addressed in detail.

Second, the results of Runnels were also challenged by Takezawa and colleagues (Takezawa et al., 2004). They observed that upon pretreatment with the "specific" PLC inhibitor U73122 endogenous PLC-activating receptors were still able to inhibit TRPM7 currents and concluded that PLC signaling is not involved. However, Takezawas' data do not justify such as strong conclusion (see Chapter 3) because U73122 is NOT a specific inhibitor for PLC. Rather, it interferes with several GPCR-effector interactions (Balla, 2001) thereby additionally blocking e.g. phospholipase A_2 and phospholipase D signaling pathways. U73122 is quite unstable and it rapidly looses its inhibitory potential in solution. Furthermore, in our lab we observed that application of freshly prepared U73122 solution to cells caused a rapid drop in membrane [PIP_2], thereby inactivating Mg^{2+} depletion-induced TRPM7 currents (Chapter 3). Horowitz and Hille recently reached a similar conclusion for the effects of U73122 on M-type K^+ channels (Horowitz et al., 2005).

A further interesting twist was recently added by Kozak and coauthors (Kozak et al., 2005). These authors reported that cytosolic acidification inhibits both TRPM7 currents and endogenous MIC currents, and they showed that both can be rescued by application of PIP_2 in inside-out patches (Kozak et al., 2005). As a mechanism, they proposed that protons might exert this regulatory action by charge-screening of the negatively charged PIP_2 moieties. Taken together, most results point out that PIP_2 at the plasma-membrane is essential for keeping TRPM7 channels in an open conformation.

Putative Mechanisms of TRPM7 Regulation by PIP_2

PIP_2 may be important for TRPM7 currents through an interaction with the intermolecular (split) PH-domain, or via interactions independent of this domain. Indeed, sequence analysis identified 2 other putative PIP_2 interaction sites in TRPM7 (Dr. T. Balla, NIH, Bethesda, personal communication). Downstream of the TRP domain a short stretch of positively charged amino acids (1147 to 1153) is likely to interact with negatively charged inositol headgroups of PIP_2, and at amino acids 1196 to 1218, a stretch including positive residues may form a modular PIP_2 binding pocket that is analogous to a PIP_2-binding stretch in TRPV1 (Prescott and Julius, 2003). In several other PIP_2-regulated TRP channels including TRPM8, TRPM5 and TRPV5, the TRP-domain was reported to be responsible for PIP_2 binding and for maintaining the channel in an activated state (Rohacs et al., 2005); this TRP consensus sequence is an additional candidate site of PIP_2 interactions in TRPM7. The interaction between PIP_2 and TRPM7 at the plasma-membrane most likely reflects an electrostatic interaction (Kozak et al., 2005) rather than the 'key-and-lock' binding observed in PH domains. In conclusion, loss of PIP_2 binding to TRPM7 may underlay both cation interferences and PLC-mediated closure of TRPM7 channels.

Regulation of TRPM7 by cAMP Levels

As mentioned above, Takezawa and colleagues challenged a role for PIP_2 in regulating TRPM7 based predominantly on inhibitor studies using U73122. Instead, they observed that a decrease in intracellular cAMP levels after muscarinic receptor activation is responsible for TRPM7 current inhibition and that this effect depends on a functional TRPM7 kinase domain.

On the other hand, a raise in intracellular cAMP levels increased Mg^{2+}-depletion-activated TRPM7 currents. Inhibition of PKA prevented carbachol-mediated inhibition of TRPM7 currents (Takezawa 2004). Hence, alterations in cytosolic cAMP levels effects PKA activity and by this means regulate TRPM7 currents. Our *in silico* analysis of the TRPM7 amino acid sequence revealed that it contains several putative PKA phosphorylation sites that may be involved in regulation of TRPM7 function (Figure 3, www.phosite.com, Koenig and Grabe 2004).

Insertion of TRPM7 Containing Vesicles in the Plasma-membrane

A final possible mechanism of TRPM7 current/channel activation must also be mentioned here. By analogy to TRPC3 (Singh et al., 2004) and TRPC5 (Bezzerides et al., 2004), it is conceivable that TRPM7 currents may be activated by fusion of vesicles that contain pre-assembled TRPM7 channels with the plasma-membrane. Whereas the precise signaling steps involved in such regulation are unclear, it is possible that PIP_2-recognizing sequences (including the split PH domain) are involved in this process. Interestingly, Oancea et al (Oancea 2006) recently showed that shear force induced by fluid flow gave rise to some incorporation of native TRPM7 channels in the plasma membrane in vascular smooth mucle cells. How significant the increase is, and whether it is a general mechanism that is also effective in other cells remains to be determined.

Characteristics of the TRPM7 α-Kinase Domain

If the TRPM7 kinase domain does not regulate channel gating, what role does it fulfill in living cells? The majority of the eukaryotic protein kinases belong either the serine/threonine kinase family or to the tyrosine kinase family that together comprise the so-called "conventional protein kinases" or CPKs (Taylor et al., 1992). The human genome further encodes kinases that share sequence homology to this group and are distantly related to CPKs, for example the phosphoinositol-3-kinases (Hunter, 1995). In addition there are kinase known that have no sequence homology to CPKs, although they are structurally similar. The TRPM7 kinase is an example of this group (Yamaguchi et al., 2001). These kinases are known as α-kinases and the name refers to the capability of these kinases to phosphorylate amino acids located within an α-helix (Ryazanov et al., 1999). The TRPM7 kinase domain shares 28% homology to the myosin heavy chain kinase A of *Dictyostelium*, a member of the α-kinase family involved in regulation of myosin stability (Kolman et al., 1996).

Substrates of the TRPM7-Kinase

In vitro characterization of the catalytic domain of TRPM7 showed that it is subject to autophosphorylation and shows kinase activity towards myelin basic protein (MBP), a promiscuous substrate used as a control in many *in vitro* kinase assays. The kinase domain specifically uses ATP as substrate and is unable to use GTP, and it depends on the presence of Mg^{2+} (optimum at 4-10 mM). Importantly, increased Ca^{2+} concentrations up to 1 mM did not alter kinase activity (Ryazanova et al., 2004).

A follow-up study by the group of Ryazanov identified an intriguing substrate for TRPM7-kinase, namely annexin-1, an anti-inflammatory protein that is regulated by Ca^{2+} and can bind actin filaments (Dorovkov and Ryazanov, 2004). An evolutionary conserved serine located in α-helix at the N-terminus of annexin-1 is phosphorylated by the TRPM7 kinase. Phosphorylation of annexin-1 depends on the presence of Ca^{2+} (500μM) and EGTA almost completely prevents annexin-1 phosphorylation.

We have identified non-muscle myosin IIA heavy chain (MHC IIA) as an additional substrate for TRPM7 kinase (chapter 4, Clark et al., 2006). Like all myosin II isoforms, MHC IIA organizes into homo-dimers consisting of a long α-helical domain and a short head domain that interacts with actin filaments. Starting point for this study was the observation of F. van Leeuwen that bradykinin causes MHC IIA phosphorylation in N1E-115 cells, which leads to dissociation of actin filaments from MHC IIA, and consequent loss of contractility that is apparent as a marked cell flattening (van Leeuwen et al., 1999).

TRPM7-Kinase Activity Affects the Actomyosin Organization

The actin cytoskeleton controls many cellular functions, such as intracellular transport, and phagocytosis but also cell adhesion and migration. Actin is organized into filaments, held together by a number of actin bunding proteins. Membrane protrusive activity (force) can be generated by

actin polymerization, which occurs at the leading edge of migrating cells, for instance in response to a chemotactic stimulus (Small, 1981; Burridge et al., 1988; Small et al., 1995). In addition, actin filaments can generate intracellular forces (tension) by associating with myosins. Similar to muscle, also in non-muscle cells myosin II isoforms are the major motor proteins responsible for force generation (Redowicz, 2001). However, important differences exist between muscle and non-muscle myosin II isforms. Whereas in muscle cells, myosin II is assembled in stable, regularly-patterned myofibrils, in non-muscle cells myosin II is part of the dynamic actomyosin cytoskeleton that undergoes continuous remodeling in order to accommodate changes in cell adhesion and cell shape, for instance during cell migration (Burridge and Chrzanowska-Wodnicka, 1996).

As mentioned previously, we find that TRPM7 affects actomyosin function by Ca^{2+}- and kinase-dependent phosphorylation of MHC IIA. Activation of TRPM7 by GPCRs results in cytoskeletal relaxation, leading to increased cell spreading. Simultaneously, cells increase their adhesion to the extracellular matrix accompanied by the formation of large integrin-containing adhesions (Chapter 4, Clark et al., 2006).

We have proposed that TRPM7 may, at least in part, affect actomyosin function by phosphorylation of the myosin II heavy chain. However, additional (kinase-dependent and -independent mechanisms) may contribute to TRPM7-induced cytoskeletal remodeling. For instance, it was shown by Dorokov et al that TRPM7 can phosphorylate annexin-1 (Dorovkov and Ryazanov, 2004), a protein known to bundle actin filaments independent of Ca^{2+} (Campos-Gonzalez et al., 1990; Kusumawati et al., 2000). Furthermore, annexin-1 can interact with profilin and plasma-membrane lipids PIP_2 and phosphatidylserine (Alvarez-Martinez et al., 1996). This suggests that TRPM7-mediated phosphorylation of annexin-1 may regulate cytoskeletal structures. Additionally, we find that some of the effects of TRPM7 on cell spreading appear to be independent of kinase activity (Chapter 4, Clark et al., 2006).

Altogether, the picture emerges that TRPM7 plays a pivotal role in the regulation of the cytoskeleton and that regulation occurs at multiple levels (Chapter 4, Clark et al., 2006).

Actomyosin Regulation by Rho and Rac Proteins

Actomyosin regulation in response to cell surface receptors is controlled by GTP binding proteins of the Rho family. Of particular relevance to this thesis are the small GTPases RhoA and Rac1. Activation of RhoA increases actomyosin contractility by increasing phosphorylation of the myosin II regulatory light chain (MLC) (Chrzanowska-Wodnicka and Burridge, 1996). Increased phosphorylation of the MLC can be achieved via 2 separate Rho signaling pathways; first, activated Rho stimulates MLC kinase and Rho-kinases (ROCK, ROK) to phosphorylate MLC (Amano et al., 1996), and secondly, Rho-like GTPases are also able to inhibit MLC phosphatases (Kimura et al., 1996).

The effects of Rac1 on actomyosin function are often opposite to that of Rho. Rac activation by stimulation of growth factor receptors causes lamellipod formation and membrane ruffling (Ridley et al., 1992). The driving force for lamellipodia formation and membrane ruffling is Rac-induced actin polymerization. Rac can accomplish this through activation of the Arp2/3 complex which associates with members of the conserved WASP family (Miki et al., 2000; Eden et al., 2002). While Rho activity is the major determinant of actomyosin contraction, the small GTPase Rac1 promotes actomyosin relaxation by antagonizing RhoA (van Leeuwen et al., 1997; Kozma et al., 1997). A similar Rho/Rac antagonism was shown to be important during the formation and maintenance of focal adhesions (Rottner et al., 1999). As all of these studies show, myosin II is the endpoint of pathways that control cellular tension.

Regulation of Rho and Rac Activity

In common with other small GTPases, Rho and Rac act as molecular switches. Inactive small GTPases are in a GDP-bound state, and receptor stimulation causes activation by stimulating exchange of GDP for GTP. Hydrolysis of GTP by the intrinsic, low GTPase activity of these proteins terminates the activated state. Three types of regulatory proteins influence this balance: Guanine Nucleotide exchange Factors (GEFs) that speed up the exchange of GDP for GTP, GTPase Activating Proteins (GAPs) that dramatically enhance the low intrinsic GTPase activity, and GTPase Dissociation Inhibitors (GDIs) that stabilize small GTPases in

their inactive conformation (Nobes and Hall, 1995). It is the active conformation of the small GTPases that is located primarily at membranes and that interacts with the target proteins.

The mode of activation of Rho and Rac is complex and involves several signaling pathways. $G\alpha_{12/13}$-coupled receptors such as those for LPA and thrombin can trigger Rho via GAPs (Jalink et al., 1994; Hart et al., 1998; Kozasa et al., 1998). In addition, we found a new PKC mediated signaling pathway that leads to increased Rac activity at the plasma-membrane: elevation of cytosolic Ca^{2+}, originating either from intracellular stores or extracellular Ca^{2+} entry, sufficed to activate a Ca^{2+}-sensitive PKC that in turn enhanced Rac translocation to the plasma-membrane (Chapter 5, (Price et al., 2003).

To add to the regulatory complexity, Rac can bind to and activate both PI(4,5)-kinase and PI(3)-kinase (Tolias et al., 1995). Inhibition of PI(3)-kinase, the kinase that produces PIP_3 from PIP_2 at the membrane, prevented growth factor induced membrane ruffling but could not block ruffling induced by expression of activated Rac (Nobes and Hall, 1995). Many RacGEFs are capable of binding to PIP_2 and PIP_3 with their PH-domain, and recruit Rac GTPases to the plasma-membrane to activate them (Schmidt and Hall, 2002; Mertens et al., 2003). In addition to phosphoinositide dependent activation, binding of the p85 subunit of PI(3)-kinase to RacGEFs also results in activation of Rac (Innocenti et al., 2003).

Rac and Rho Mediated Formation and Maturation of Adhesive Structures

Both Rho and Rac are involved in formation and maturation of focal adhesion structures. Focal adhesions are large multi-protein complexes that anchor cells to the extracellular matrix and function as attachment for stress fibers. Cells can sense mechanical forces through focal adhesions, and the cytoskeleton is strongly influenced by these mechanical forces. I will here review some of the literature on this topic to provide a background for the work presented in Chapter 4 & 5.

Focal complexes arise from nascent integrin containing adhesive contacts that form at the leading edge of migrating cells and can grow out to focal adhesions. The formation of focal complexes depends on Rac, but not Rho (Rottner et al., 1999), but what triggers their formation is not well understood. When cells attach to the extracellular matrix, translocation of Rac to the plasma-membrane (del Pozo et al., 2000) may trigger the formation of focal complexes by inducing actin polymerization and branching followed by integrin clustering (Kraynov et al., 2000). Different Rac effectors may mediate these effects. Activation of PI(5)P-kinases mediated by Rac increases PIP_2 levels, which results in uncapping of actin filaments (Tolias et al., 2000). Other effectors implicated in focal complex formation are the WAVE/Scar complex (Machesky et al., 1999) and the serine-threonine kinase PAK (Edwards et al., 1999).

The maturation of focal complexes to focal adhesions requires activation of RhoA, although again, the exact mechanism is still poorly understood. It has been shown that the combined action of 2 Rho effectors, Rho kinase ROCK and diaphanous protein Dia1, can substitute for active Rho in the process of focal adhesion assembly (Watanabe et al., 1999). It was suggested, on the based on rather indirect evidence, that Dia promotes actin polymerization by targeting profilin and possibly by enhancing its function (Watanabe et al., 1997). Furthermore, similar to WASP, Dia may directly activate Arp2/3 (Alberts, 2001). On the other hand, formation of focal adhesions relies on increased myosin II-mediated contractility which is due to increased myosin light chain phosphorylation by inactivation of MLC phosphatases and/or activation of MLC kinases (Amano et al., 2000).

Podosomes are a third type of cell-matrix adhesion relevant for this thesis. Podosomes are integrin containing adhesions, clearly distinct from focal adhesions, which are a characteristic feature of cells that have the capacity to cross tissue boundaries. Examples are monocyte-derived cells (Marchisio et al., 1984) but also carcinoma-derived epithelial cells (Spinardi et al., 2004). Podosomes are anchored to the extracellular matrix through integrins and can develop from existing focal adhesions (Moreau et al., 2006) or are *de novo* synthesized by attachment to the matrix (Gaidano et al., 1990). Podosomes have a distinct dense core of F-actin and each podosome is surrounded by ring structure enriched proteins such as vinculin, α-actinin (David-Pfeuty et al., 1980) and regulators of contractility, e.g. myosin II isoforms and TRPM7 (Clark et al., 2006). Podosome structures share similarities with focal adhesion but contrast on several points. First of all, the actin core of podosomes contains proteins that regulate

actin polymerization, such as WASP, Arp2/3 and gelsolin that are absent in focal adhesions (Marchisio et al., 1988). Second, formation of podosomes frequently occurs from rapid and dynamic turnover of pre-existing podosomes (Stickel and Wang, 1987), while focal adhesions are less dynamic and formation requires continuous protein synthesis. At last, podosomes may remodel and tubulate the plasma-membrane (Burgstaller and Gimona, 2005). Furthermore ECM-degrading metalloproteases localize to podosomes (Sato et al. 1997; Delaisse et al. 2000). These observations point out that podosomes are necessary for extracellular matrix degradation.

Cellular Mechanisms Detecting Mechanical Forces

Cells and their cytoskeleton are continuously subjected to mechanical forces due to both physical interactions with the extracellular matrix and their own contractile machinery. Sensing mechanical forces induced by the cells' environment is critical for processes such as motility, adhesion, proliferation and apoptosis (Geiger et al., 2001; Burridge and Wennerberg, 2004). Therefore, cells have developed "force receptors" for mechanosensing that are still poorly characterized. Two types of protein complexes have been proposed to sense these forces, namely focal adhesions and ion channels. As we speculate (Chapter 4) that TRPM7 may act as a mechanosensor, or is involved in mechano-sensation, I will here discuss this topic to provide a background.

Focal Adhesions and Sensing of Mechanical Forces

Several lines of evidence indicate that integrin-mediated adhesion is involved in sensing mechanical stress. Application of forces to cells causes strengthening of their anchorage to the extracellular matrix. This depends on both clustering and occupancy of the integrins that link the cytoskeleton to the extracellular matrix at the adhesion sites (Wang et al., 1993; Choquet et al., 1997; Geiger et al., 2001). Focal adhesions are capable of adjusting their size to the degree of mechanical force applied. Application of force to either the cell (Riveline et al., 2001) or to the (flexible) matrix (Wang et al., 2001) transforms the focal complexes into mature focal adhesions. The tension that focal adhesions "sense" regulates both their maintenance and growth. Relieve of this tension, by inhibition of myosin-driven contractility, causes rapid disassembly of focal adhesions (Geiger et al., 2001). Furthermore, applied tension also determines the vinculin content in focal adhesions (Opazo et al., 2004). These local effects of force-induced changes in integrin-mediated adhesion indicate that focal adhesions can act as sensors for detection of internal or external forces.

How exactly focal adhesions detect forces is still poorly understood. One possibility is that tension reorganizes the molecular structure of focal adhesions, thus allowing additional cytosolic components to be incorporated. Force-driven maturation of focal complexes to focal adhesions is accompanied by an increase in $\alpha_v\beta_3$ integrins (Ballestrem et al., 2001). Furthermore, β_3 integrins can move into individual focal adhesions in an energy- and myosin II-dependent manner (Tsuruta et al., 2002)). Another possible mechanism relies on intrinsic properties of some of the molecules of the focal adhesion complex. Focal adhesion associated proteins like vinculin, fibronectin and possibly $\alpha\beta$ integrin dimers exist in an active (open) or inactive (closed) conformation (Alberts, 2001; DeMali et al., 2002). Normally, the transition between active and inactive conformation results from signaling involving kinases, phosphatases and changes in membrane lipid composition, but Geiger hypothesized that mechanical forces may similarly expose previously hidden interaction sites (Geiger et al., 2001).

Mechanosensation by Ion Channels

Membrane tension caused by mechanical forces can also be sensed by specialized ion channels (Gustin et al., 1988; Martinac, 2004). A wide variety of mechanosensitive or stretch-activated channels (SAC) are found in all prokaryotic and eukaryotic cells. In eukaryotic cells, 4 different families of ion channels have been implicated in sensing mechanical forces: the mechano-sensitive K^+ channels TREK and TRAAK, Na^+-selective MEC/DEG (for mechanosensory abnormal/degenerins) channels, ClC Cl^- channels and TRP channels.

Other ion channels potentially involved in sensing mechanical forces are linked to the actin cytoskeleton and protein complexes within focal adhesions. In flies it has been demonstrated that the TRP family member TRPN1 (NOMPC), which

binds to actin via the channels N-terminal ankyrin repeats, contributes to bristle mechanosensation (Walker et al., 2000) and the zebrafish homologue mediates mechanoreception in hair cells (Sidi et al., 2003). Mammals do not have a TRPN1 homologue, but recently the TRPA1 channel was implicated in mammalian nociception and hair cell transduction (Nagata et al., 2005).

Two modes of force detection by ion channels have been suggested. First, changes in intrinsic forces (lateral pressure) generated by lipid bilayer composition may regulate channel opening and closure (Kung, 2005). Secondly, mechanical tension may be transduced via either intracellular linkers (mainly the actin cytoskeleton) or external anchors to the channel pore to modulate the intracellular ion concentration (Gillespie, 2002). In this view, Howard hypothesized that the repetitive ankyrin repeats of TRPN1 form a helical turn that can act as a gating spring for channel opening (Howard and Bechstedt, 2004). Mechanosensation is not limited to specialized cells in sensory organs, but all adherent cells can sense mechanical forces. The external environment is probed by cells by applying actomyosin-driven forces to cell-matrix or cell-cell adhesion sites. In turn, the adhesion sites respond to these forces by changes in size, dynamic behavior and by signaling events (Geiger et al., 2001).

In view of the above, it is tempting to speculate that TRPM7 may act as a mechanosensory channel. Interestingly, in endothelial cells it was recently shown that TRPM7 ion channels become embedded into the plasma-membrane in response to fluid flow shear forces (Oancea et al., 2006). This may cause local Ca^{2+} influx at the plasma-membrane through TRPM7 channels located around focal adhesions. Subsequent Ca^{2+}-dependent myosin II phosphorylation or other TRPM7-mediated effects on MHC IIA activity, may induce the transformation of focal adhesions into podosomes as well as contribute to *de novo* formation of podosomal-like adhesions (Clark et al., 2006). Similarly, a recent report of Su et. al. has also implicated TRPM7 in regulation of cell adhesion (Su et al., 2006)

Overview of This Thesis

In this thesis, we address the regulation of TRPM7 channels by receptor-mediated signals and the effects of the ensuing ionic signals on the cytoskeleton. In **Chapter 2** signaling pathways were investigated that activate TRPM7 channel opening. We show that PLC-activating receptors open TRPM7 channels, leading to influx of extracellular Ca^{2+}. The results presented in this chapter contrast markedly with a report by Runnels et al that appeared during our studies who showed inhibition of TRPM7, rather than activation, by PLC. In **Chapter 3** we present experiments that explain these discrepancies: we explored the differences in PIP_2-mediated regulation of TRPM7 measured in perforated-patch and whole-cell configuration after intracellular Mg^{2+} depletion. This leads us to propose that the effects of PLC activation on TRPM7 currents as detected in whole cells can best be viewed as 'accelerated rundown' rather than as bona fide signal transduction.

In **Chapter 4** we examine how agonist-induced TRPM7 activation and subsequent Ca^{2+} influx affect the cytoskeleton. It is shown that TRPM7 phosphorylates the myosin II heavy chain to promote cytoskeletal relaxation and the conversion of focal adhesions to podosomes. Ca^{2+} influx appears crucial for the *in vivo* function of the TRPM7-kinase by triggering the association between the kinase and its substrate. In **Chapter 5**, we report that receptor-mediated Ca^{2+} influx can affect the cytoskeleton by translocating Rac in a PKC-dependent manner to the plasma-membrane, where it induces extensive membrane ruffling.

In conclusion, intracellular Ca^{2+} elevations agonist-induced due to channel activation affects the actin cytoskeleton in multiple ways.

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