# 1. Introduction

## 1.1. $Ca^{2+}$ signalling

Calcium ions ($Ca^{2+}$) function as intracellular messengers that play an important role in regulating many cellular processes, such as muscle contraction (Ebashi, 1972), neurotransmitter release (Llinás, 1982), or gene expression (van Haasteren *et al*., 1999). $Ca^{2+}$ exerts its multiple effects on cell function by modulating the activity of a variety of intracellular proteins. In most cases, rather than directly interacting with its target proteins, $Ca^{2+}$ binds to specific $Ca^{2+}$-binding proteins, e.g. calmodulin (Chin & Means, 2000), that function as multipurpose intracellular $Ca^{2+}$ receptors. Upon binding of $Ca^{2+}$, these proteins undergo a conformational change that allows subsequent interaction with and, thus, modulation of downstream target proteins. Besides its role as a ubiquitous messenger, $Ca^{2+}$ can also have cytotoxic effects. Accordingly, prolonged elevations of the free cytosolic $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) may lead to the irreversible cell damage that occurs e.g. during cardiac or cerebral ischaemia (Trump & Berezesky, 1995). Hence, for $Ca^{2+}$ signalling to work, $[Ca^{2+}]_i$ must be tightly controlled.

The general principles of cytosolic $Ca^{2+}$ homeostasis and $Ca^{2+}$ signalling are virtually identical in all cells and will be outlined in the following section (for reviews see Tsien & Tsien, 1990; Clapham, 1995; Berridge *et al*., 2000).

### 1.1.1. Major elements of the $Ca^{2+}$ signalling machinery

$[Ca^{2+}]_i$ of resting cells is kept extremely low (~ $10^{-7}$ M). Upon stimulation by such diverse stimuli as the binding of extracellular ligands to surface receptors, depolarization, osmotic challenge, or mechanical forces, rapid and reversible increases in $[Ca^{2+}]_i$ occur. Elevations in $[Ca^{2+}]_i$ can arise from either $Ca^{2+}$ influx across the plasma membrane, or $Ca^{2+}$ discharge from internal stores, or both. Since the concentration of free $Ca^{2+}$ in the extracellular fluid (~ $10^{-3}$ M) and the intracellular $Ca^{2+}$ stores (estimates for free luminal $[Ca^{2+}]$ ranging from $10^{-6}$ to $10^{-3}$ M, reviewed by Meldolesi & Pozzan, 1998) are 1000 to 10,000-fold higher than that of the cytoplasm, $Ca^{2+}$ passively flows into the cytosol as soon as $Ca^{2+}$-permeable channels in the plasma membrane or the membrane of internal stores are opened.

*Calcium release from internal stores*

The major $Ca^{2+}$-storing, buffering and signalling compartment within cells is the endoplasmic (sarcoplasmic) reticulum (ER/SR), or derived organelles (Pozzan *et al*., 1994). In the lumen of the ER, $Ca^{2+}$ is buffered by proteins with high binding capacity and low affinity, such as e.g. calreticulin (Michalak *et al*., 1999; Johnson *et al*., 2001) and calsequestrin (Volpe *et al*., 1990) which may have additional signalling functions. Recent evidence suggests that the ER cisternae and tubules form a continuous network, although functional compartimentalization may occur (Petersen *et al*., 2001).

Stimulus-induced $Ca^{2+}$ release from the ER is thought to be mediated by two main types of $Ca^{2+}$ release channels (Furuichi *et al*., 1994; Striggow & Ehrlich, 1996), ryanodine receptors (RyR) and inositol-1,4,5-trisphosphate ($InsP_3$) receptors ($InsP_3R$). With the exception of skeletal muscle where activation of RyRs, at least initially, results from direct coupling of the $Ca^{2+}$ release channel to L-type voltage-gated $Ca^{2+}$ channels (also known as dihydropyridine receptors) in the plasma membrane (Franzini-Armstrong & Protasi, 1997), RyRs (for reviews see Coronado *et al*., 1994; Meissner, 1994) are generally thought to be activated by an increase in $[Ca^{2+}]_i$, a process known as ***c**alcium-**i**nduced **c**alcium **r**elease* (CICR). Moreover, cyclic ADP ribose (cADPR) can activate these channels or modulate the ability of $Ca^{2+}$ to stimulate them (Galione *et al*., 1991). $InsP_3Rs$ (for reviews see Taylor, 1998; Galvan *et al*., 1999) are opened upon binding of $InsP_3$ (see below), but also regulated by cytosolic $Ca^{2+}$, with a bell-shaped concentration-effect relation. The subcellular distribution of $InsP_3Rs$ and RyRs can vary considerably between different cell types. Recently, another putative $Ca^{2+}$-mobililizing messenger, nicotinic acid adenine dinucleotide phosphate (NAADP), has been identified in several cell types (for reviews see Patel *et al*., 2001; Genazzani & Billington, 2002). NAADP has been suggested to promote $Ca^{2+}$ release from intracellular stores that are insensitive to both $InsP_3$ and cADPR. The newly identified messenger molecule is thought to be important for shaping cytosolic $Ca^{2+}$ signals by functionally coupling the known release mechanisms. However, its exact role remains to be elucidated.

*Calcium influx across the plasma membrane*

Stimulus-induced $Ca^{2+}$ influx across the plasma membrane can be mediated by different types of $Ca^{2+}$-permeable channels. These can be classified according to their mechanism of activation into *voltage-operated $Ca^{2+}$ channels* (VOCs) that are opened upon depolarization, and voltage-independent channels that are insensitive to changes in the membrane potential (cf. Elliott, 2001). Two main types of voltage-independent channels can be distinguished: 1. Channels that are opened by direct binding of an extracellular ligand to the channel protein, e.g. nicotinic acetylcholine receptors, N-methyl-D-aspartate (NMDA) receptors, and which will be referred to as *ligand-operated channels* (LOCs) in this study. 2. Channels that are also activated upon binding of extracellular molecules, but for which receptor and channel are separate proteins. These channels will be referred to as *receptor-operated channels* (ROCs). It has to be pointed out that there is no unified nomenclature for the classification of voltage-independent $Ca^{2+}$-permeable channels. As a consequence, a confusing panoply of names exists and the same names are often used to designate divergent groups of channels (cf. Barritt, 1999 (ROC=RACC); Mori *et al*., 2001 (ROC=RMCC); Clementi & Meldolesi, 1996 (LOC=ROC, ROC=SMOCC)).

VOCs and LOCs are well characterized, both functionally and at the molecular level. In contrast, knowledge about the activation mechanisms and the molecular composition of most ROCs is lacking. ROCs appear to form a heterogeneous group of channels that are activated by different components of the signal transduction pathways downstream of receptor stimulation. $Ca^{2+}$ entry through ROCs plays an important role upon stimulation of receptors linked to phospholipases C (PLC) in both non-excitable and excitable cells. PLC-dependent $Ca^{2+}$ signalling will be elaborated in the following section.

*Calcium pumps and exchangers*

All cells possess mechanisms that actively extrude $Ca^{2+}$ from the cytosol. This is crucially important for maintaining low resting $Ca^{2+}$ levels as well as for terminating stimulus-induced increases in $[Ca^{2+}]_i$. Both extrusion of $Ca^{2+}$ across the plasma membrane and resequestration by internal $Ca^{2+}$ stores are involved in maintaining and restoring basal $Ca^{2+}$ levels. $Ca^{2+}$ extrusion across the plasma membrane results from the

activity of the plasma membrane $Ca^{2+}$ pump (PMCA; Carafoli, 1994; Penniston & Enyedi, 1998), which is expressed in all eukaryotic cells. The PMCA is a member of the P-type ATPase family and functions as a high-affinity, low capacity $Ca^{2+}$ pump. In addition, some cell types, in particular muscle cells and nerve cells, possess an additional plasmalemmal $Ca^{2+}$ transporter that couples the efflux of $Ca^{2+}$ to the influx of $Na^+$. This so-called $Na^+/Ca^{2+}$ exchanger (Blaustein & Lederer, 1999) has a low affinity but a high transport capacity for $Ca^{2+}$. $Ca^{2+}$ resequestration into the SR/ER is achieved predominantly by $Ca^{2+}$-$Mg^{2+}$-ATPases called SERCA pumps (**s**arco/**e**ndoplasmic **r**eticulum **C**$a^{2+}$ **A**TPases; Hussain & Inesi, 1999; Misquitta *et al*., 1999).

### 1.1.2. Phospholipase C-dependent $Ca^{2+}$ signalling

Stimulation of mammalian cells with a variety of hormones and growth factors results in $[Ca^{2+}]_i$ increases that are dependent on the activation of PLC. PLC-dependent $Ca^{2+}$ signalling is schematically outlined in Figure 1. Ligand-binding to G protein-coupled receptors or receptor tyrosine kinases results, respectively, in PLCβ- or PLCγ-dependent hydrolysis of membrane-bound phosphatidylinositol-4,5-bisphosphate ($PIP_2$) into the soluble messenger $InsP_3$ and membrane-bound diacylglycerol (DAG). $InsP_3$ mediates $Ca^{2+}$ release by opening $InsP_3R$ channels in the membrane of the ER $Ca^{2+}$ stores whereas DAG couples receptor stimulation to the activation of protein kinases C (PKC) (for reviews see Putney, 1987; Berridge, 1993).

In addition to $Ca^{2+}$ release from the ER, $Ca^{2+}$ entry (through ROCs) is generally activated following stimulation of PLC-coupled receptors. However, the mechanisms underlying the regulation of PLC-dependent $Ca^{2+}$ entry are not well understood. Multiple pathways seem to be involved and may operate in parallel (for reviews see Fasolato *et al*., 1994; Clementi & Meldolesi, 1996; Barritt, 1999; Elliott, 2001).

In many cell types, depletion of intracellular $Ca^{2+}$ stores stimulates $Ca^{2+}$ influx via a pathway that is commonly referred to as the '*capacitative*' or '*store-operated*' $Ca^{2+}$ entry pathway (for reviews see Putney, 1997; Putney *et al*., 2001). The term '*capacitative $Ca^{2+}$ entry (CCE)*' was coined by Putney (1986) and refers to the idea that the stores behave like a capacitor in an electrical circuit. Electrical capacitors do not

conduct any net current when they are fully charged. By analogy, when the $Ca^{2+}$ stores are replete no $Ca^{2+}$ influx occurs, but once the stores discharge their contents, $Ca^{2+}$ entry is activated. Important for the concept of store-operated $Ca^{2+}$ entry is that receptor activation is not an indispensable requirement. Any means of depleting the intracellular $Ca^{2+}$ pool provides a full and sufficient signal for the activation of $Ca^{2+}$ entry, even in the absence of receptor stimulation or generation of $InsP_3$. The best characterized current through store-operated channels (SOCs) is the highly $Ca^{2+}$-selective calcium release-activated current ($I_{CRAC}$, for review see Parekh & Penner, 1997). However, other less $Ca^{2+}$-selective channels have also been described (for reviews see Parekh & Penner, 1997; Barritt, 1999). A major drawback of the concept of CCE is the fact that, despite intensive research, the mechanisms that link store depletion to $Ca^{2+}$ influx remain elusive. Different models have been discussed including conformational coupling, channel insertion into the membrane by vesicle fusion and release of a diffusible messenger by the depleted stores (Putney, 1997; Putney *et al*., 2001). At present, the conformational-coupling theory is favoured although it may not account for store-

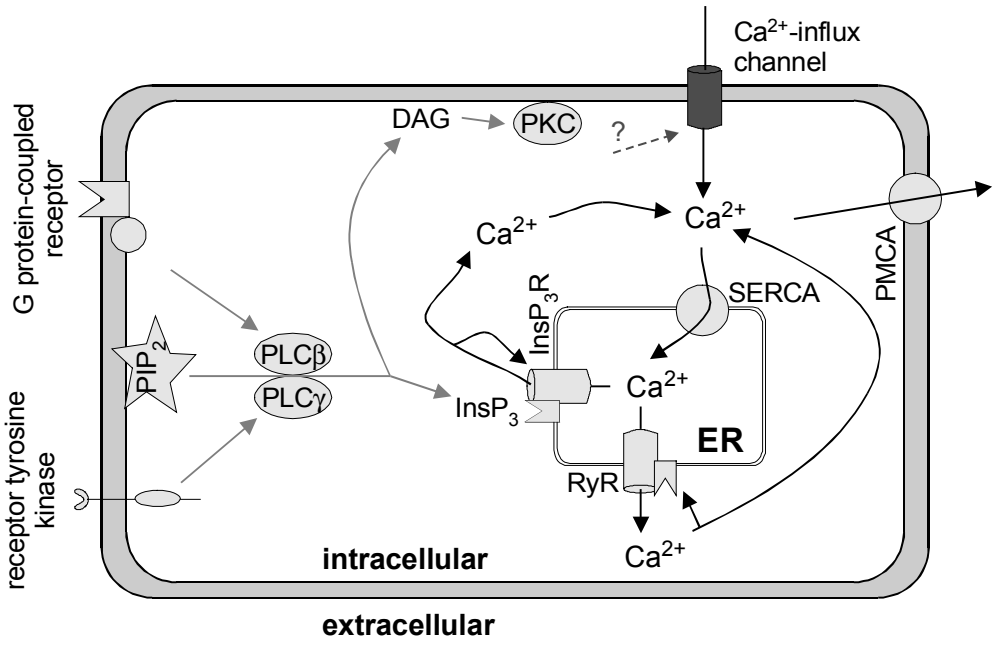


Fig. 1: **Phospholipase C-dependent $Ca^{2+}$-signalling in mammalian cells.** $Ca^{2+}$ influx channels are thought to form a heterogeneous group, with diverse gating mechanisms for different types of channels.

operated $Ca^{2+}$ entry in all cell types (for reviews see Mikoshiba & Hattori, 2000; Putney *et al*., 2001). The model, originally proposed by Irvine (1990), suggests that empty stores and $Ca^{2+}$ entry channels communicate via direct protein-protein interactions. Although current models of PLC-dependent $Ca^{2+}$ entry mechanisms focus on the capacitative concept, there is an increasing body of evidence that many PLC-dependent $Ca^{2+}$ entry pathways are activated independently of store depletion by various messengers of the signal transduction cascade such as $InsP_3$ (Vaca & Kunze, 1995), inositol-1,3,4,5-tetrakisphosphate ($InsP_4$, Lückhoff & Clapham, 1992), $Ca^{2+}$ (Loirand *et al*., 1991), DAG (Helliwell & Large, 1997; Tesfai *et al*., 2001), or arachidonic acid (Shuttleworth & Thompson, 1998; Broad *et al*., 1999; Mignen & Shuttleworth, 2000). Moreover, many stimuli have been reported to activate $Ca^{2+}$ entry independently of store depletion, but the exact activation mechanism is unknown (Clementi *et al*., 1992; Felder *et al*., 1992; Mathias *et al*., 1997). Like SOCs, receptor-stimulated channels activated independently of store-depletion do not appear to form a uniform group, but display a considerable variety of biophysical properties.

Because of the prevalence and potential importance of PLC-dependent $Ca^{2+}$ entry pathways in both non-excitable and excitable cells, there has been considerable interest in identifying the relevant channels at the molecular level. Despite intensive efforts, to date no clear picture has emerged. However, as will be discussed below, members of the transient receptor potential (TRP) channel family are promising candidates.

## 1.2. The transient receptor potential (TRP) channel family

The first clues as to which channels might be involved in PLC-dependent $Ca^{2+}$ entry in mammalian cells came from studies on *Drosophila* visual transduction. Unlike vertebrate visual transduction, phototransduction in *Drosophila* involves activation of PLC, rather than cGMP phosphodiesterase (for reviews see Hardie & Minke, 1995; Ranganathan *et al*., 1995). Light-induced activation of PLC is followed by $Ca^{2+}$ entry across the plasma membrane. Opening of the plasmalemmal $Ca^{2+}$-permeable channels results in cation influx into the photoreceptor cells and gives rise to a depolarizing

receptor potential. In an attempt to resolve the pathways involved in *Drosophila* phototransduction, mutants were generated and analyzed for changes in the pattern of their light induced currents. One mutant was found to be unable to sustain the depolarizing receptor potential upon prolonged illumination owing to a defect in the light-induced $Ca^{2+}$ entry pathway (Cosens & Manning, 1969; Minke *et al*., 1975). The name transient receptor potential (*trp*) mutant was coined. Further studies confirmed that TRP and related gene products such as TRPL (TRP-**l**ike; Phillips *et al*., 1992) form $Ca^{2+}$-permeable channels (Montell & Rubin, 1989; Hardie & Minke, 1992; Hu *et al*., 1994; Vaca *et al*., 1994; Niemeyer *et al*., 1996; Reuss *et al*., 1997).

Similarities in the signal transduction pathways involved in *Drosophila* phototransduction and PLC-dependent $Ca^{2+}$ entry into mammalian cells, led to the search for mammalian homologues of *Drosophila* TRP channels as candidate ROC proteins. 21 mammalian isoforms have been found on the basis of sequence homology or by expression cloning (Clapham *et al*., 2001; Montell, 2001; Montell *et al*., 2002a; Montell *et al*., 2002b) and TRP-related proteins have been identified in all eukaryotic organisms that have been subjected to genome-sequencing (Harteneck *et al*., 2000; Palmer *et al*., 2001).

The features that unify all members of the TRP channel family are summarized in recent reviews (Harteneck *et al*., 2000; Clapham *et al*., 2001; Montell, 2001; Mori *et al*., 2001; Montell *et al*., 2002a). All members of the TRP channel family display a membrane topology of six putative transmembrane segments (TM1-TM6), with a re-entrant pore loop between TM5 and TM6, and cytosolic amino and carboxy termini. As far as they have been studied in recombinant expression studies, all TRP channels form cation channels that are either non-selective or $Ca^{2+}$-selective. Their membrane topology and cation permeability place TRP channels in the superfamily of cation-permeable channels which also comprises voltage-gated $K^+$ channels, cyclic nucleotide-gated channels, and hyperpolarization-activated cyclic nucleotide-gated channels. By analogy with voltage-gated $Na^+$ and $Ca^{2+}$ channels, which have four linked domains of six transmembrane segments, TRP channels are thought to form tetramers.

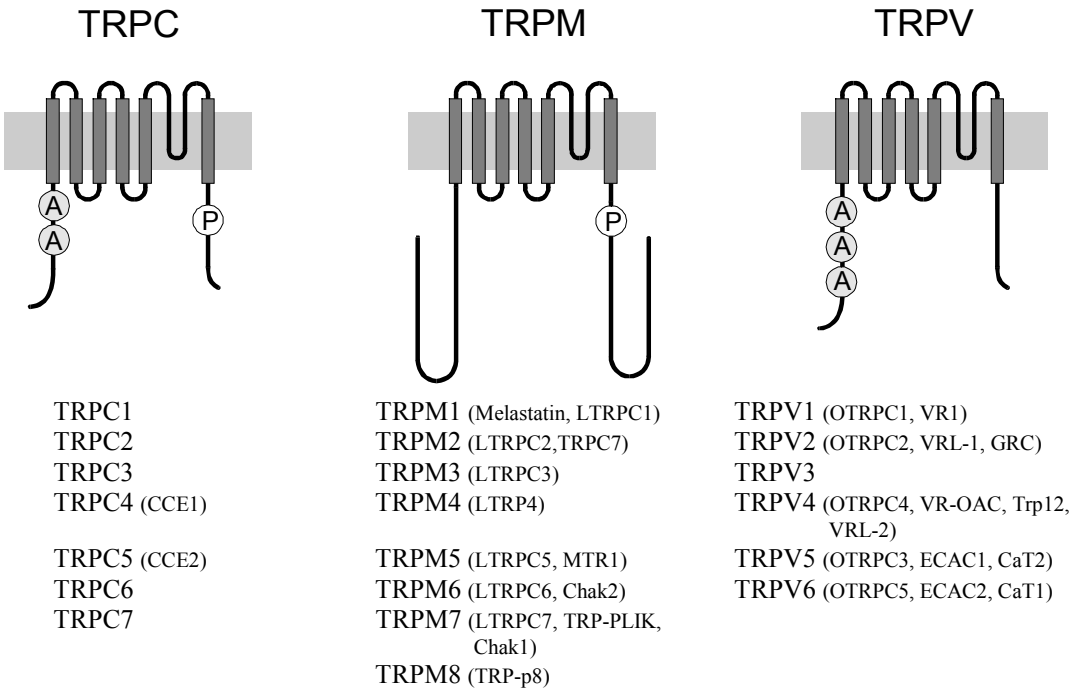


Fig. 2: **Classification, architecture and nomenclature of transient receptor potential (TRP) channels.** Conserved sequence motifs: N-terminal ankyrin repeats (A), C-terminal proline rich region (P). All known mammalian members for each subfamiliy are listed below, using the unified nomenclature (Montell *et al*., 2002b). Alternative former designations are given in parentheses.

As shown in Figure 2, TRP channels can be subdivided on the basis of sequence homology into three major subfamilies (TRPC, TRPM, and TRPV). Figure 2 also shows the predicted membrane topology of the three subfamilies as well as the grouping and nomenclature of the mammalian isoforms. The classification of TRP channels into three subfamilies was initially proposed by Harteneck *et al*. (2000) who suggested the designations short (S) for TRPC, long (L) for TRPM, and osm9-related (O) for TRPV. In a recent effort to unify TRP channel nomenclature, the new names TRPC, TRPM, and TRPV have been assigned to the subfamilies and their members (Montell *et al*., 2002b). The numbering system for the mammalian isoforms takes into account the order of their discovery and/ or the number that had formerly been assigned to that protein.

*TRPC subfamily*

The TRPC or TRP-**C**anonical subfamily is comprised of those proteins that are most closely related to *Drosophila* TRP. A total of seven mammalian isoforms have been identified, referred to as TRPC1 - 7 (Wes *et al*., 1995; Zhu, 1995; Philipp *et al*., 1996;

Zhu *et al*., 1996; Boulay *et al*., 1997; Okada *et al*., 1998; Philipp *et al*., 1998; Liman *et al*., 1999; Okada *et al*., 1999; Vannier *et al*., 1999). All TRPC channels contain two to four ankyrin repeats in their N-terminal cytosolic region and a highly conserved 25-amino acid segment of unknown function in the cytosolic C terminus close to TM6. This conserved motif (TRP domain) consists of an invariant sequence, called the TRP box (EWKFAR) and a proline-rich region that resembles the binding site for the scaffold protein Homer (Montell, 2001). Most TRPC channels have been shown to form cation channels in heterologous expression systems.

*TRPV subfamily*

The TRPV subfamily is named after the first mammalian member of this subfamily, the vanilloid receptor (VR-1, now TRPV1, Montell *et al*., 2002b). Like TRPC channels, members of the TRPV channels contain three to four ankyrin repeats in their N-terminal region (Gunthorpe *et al*., 2002). However, they lack the C-terminal TRP domain. To date, six mammalian isoforms have been cloned (TRPV1: Caterina *et al*., 1997; TRPV2: Caterina *et al*., 1999; Kanzaki *et al*., 1999; TRPV3: Peier *et al*., *in press;* and TRPV4-6: Hoenderop *et al*., 1999; Peng *et al*., 1999; Liedtke *et al*., 2000; Strotmann *et al*., 2000; Wissenbach *et al*., 2000). Mammalian TRPV have been shown to be activated by a wide variety of chemical and physical stimuli. The vanilloid receptor TRPV1, was identified in rat sensory neurones using an expression-cloning strategy (Caterina *et al*., 1997). TRPV1 has been extensively characterized and is thought to be involved in pain transduction (for review see Julius & Basbaum, 2001). Noxious stimuli such as heat (> 43°C) and protons (Tominaga *et al*., 1998) have been reported to activate TRPV1. The channel is also activated by the vanilloid compound capsaicin, the ‘hot’ component of chili peppers, and by other molecules (Sterner & Szallasi, 1999; Szallasi & Blumberg, 1999) that contain a vanilloid moiety. Moreover, non-vanilloids including several lipoxygenase products (Hwang *et al*., 2000) and the endocannabinoid anandamide (Zygmunt *et al*., 1999; Smart *et al*., 2000) activate TRPV1. All activators of TRPV1 are assumed to act directly on the channel protein (Tominaga *et al*., 1998; Jordt *et al*., 2000; Jordt & Julius, 2002). The vanilloid receptor-like protein TRPV2 is also activated by heat, albeit with a higher temperature threshold (Caterina *et al*., 1999).

However, TRPV2 is insensitive to vanilloids. TRPV3 (Peier *et al*., *in press*) is specifically expressed in keratinocytes and has an activation threshold around 33 – 35°C. Another vanilloid receptor-like protein TRPV4 is an osmotically-regulated channel activated by cell swelling (Liedtke *et al*., 2000; Strotmann *et al*., 2000). However, TRPV4 is not gated directly by membrane stretch suggesting the involvement of additional signalling molecules. A recent study demonstrated PKC-independent channel activation by phorbol derivatives (Watanabe *et al*., 2002). While TRPV1, TRPV2 and TRPV4 are non-selective or moderately $Ca^{2+}$-permeable channels, TRPV5 and TRPV6 are highly $Ca^{2+}$-selective (Hoenderop *et al*., 1999; Peng *et al*., 1999; Peng *et al*., 2000; Vennekens *et al*., 2000; Nilius *et al*., 2001; Vennekens *et al*., 2001). Evidence has been put forward that TRPV6 is gated by store depletion and might constitute part of, or the entire $I_{CRAC}$ pore (Yue *et al*., 2001). However, this view is not universally accepted (Voets *et al*., 2001).

*TRPM subfamily*

The TRPM subfamily receives its name from melastatin (now TRPM1, Montell *et al*., 2002b), the first mammalian isoform that was identified. To date, eight mammalian members of this subfamily are known (Duncan *et al*., 1998; Nagamine *et al*., 1998; Enklaar *et al*., 2000; Runnels *et al*., 2001). The proximal C-terminal region of TRPM channels contains the TRP domain, however their N-terminal region is devoid of ankyrin repeats and considerably longer than the corresponding regions of TRPC and TRPV. The total length of TRPM proteins varies considerably (~ 1000 to 2000 amino acids) owing to a variations in the cytosolic C terminus. Functional data for TRPM channels is only just emerging. TRPM1 has been suggested to be a putative tumor suppressor gene because it is down-regulated in metastatic cells (Duncan *et al*., 1998; Fang & Setaluri, 2000). In contrast, TRPM8 is upregulated in prostate tumors (Tsavaler *et al*., 2001). TRPM2 was shown to be activated by changes in the redox status and involved in apoptosis (Hara *et al*., 2002). Information available on *C. elegans* isoforms also suggests that these channels may be important for in cell growth, cell differentiation and cell death (Harteneck *et al*., 2000), but it is not clear if this holds true for all members of the subfamily. Recent evidence suggests that TRPM8 is activated by

thermal stimuli in the cool or cold range and by molecules, such as menthol, that produce a cooling sensation (McKemy *et al*., 2002; Peier *et al*., 2002). Three TRPM isoforms, TRPM2 (Perraud *et al*., 2001), TRPM6 (Ryazanov, 2002) and TRPM7 (Nadler *et al*., 2001; Runnels *et al*., 2001) were shown to contain an enzyme domain in their C-terminal region, which is a unique feature for ion channels. TRPM7 and TRPM2 have been characterized in recombinant expression studies (Nadler *et al*., 2001; Perraud *et al*., 2001; Runnels *et al*., 2001; Runnels *et al*., 2002). TRPM7 contains an atypical serine/threonine protein kinase domain, whereas TRPM2 includes an ADP-ribose pyrophosphatase. As yet, the exact role of these enzymatic activities for channel activation is unclear.

### 1.2.1. Properties of TRPC channels

Since all members of the TRPC subfamily are activated through signalling cascades that are coupled to PLC (for reviews see Harteneck *et al*., 2000; Clapham *et al*., 2001; Montell, 2001; Mori *et al*., 2001; Montell *et al*., 2002a), TRPC channels are currently thought to be the most promising molecular candidates for channels mediating $Ca^{2+}$ influx upon stimulation of PLC-coupled receptors in native cells, though neither the contribution of other TRP-related gene products, such as e.g. TRPV6 (Yue *et al*., 2001), nor the involvement of other unrelated proteins can be excluded. The properties and activation mechanisms of TRPC1 - 7 have been extensively studied in heterologous expression systems. However, the gating mechanisms of the recombinant mammalian TRPCs remain highly controversial. Although receptor-mediated stimulation of phospholipase C is undisputedly a key event in the regulation of TRPC channels, different studies suggest divergent activation mechanisms for the same TRPC isoforms, involving both store-operated (Zhu *et al*., 1996; Zitt *et al*., 1996; Kiselyov *et al*., 1998; Philipp *et al*., 1998; Vannier *et al*., 1999; Warnat *et al*., 1999; van Rossum *et al*., 2000; Riccio *et al*., 2002) and store depletion-independent mechanisms (Okada *et al*., 1998; Zhu *et al*., 1998; Hofmann *et al*., 1999; Liman *et al*., 1999; Okada *et al*., 1999; McKay *et al*., 2000; Schaefer *et al*., 2000; Kanki *et al*., 2001; Strübing *et al*., 2001; Schaefer *et al*., 2002). The reasons for these contradictory findings from different laboratories remain elusive, but could be due to the expression systems or species variants used.

Furthermore, it is conceivable that variations in the expression levels of the proteins may lead to divergent results. Accordingly, Kiselyov & Muallem (1999) propose that activation of TRPC3 and *Drosophila* TRP by lipid mediators might be an atypical form of channel activation that becomes evident when endogenous components of the 'true' signal transduction cascade are not unlimitedly available, making the spare channel proteins susceptible to modulation by other compounds. Alternatively, heteromultimers (see below) formed with endogenous TRPC channels may predominate in the presence of low concentrations of the recombinant protein, whereas at higher concentrations most channels are homomultimers.

Based on amino-acid sequence homology, the mammalian members of the TRPC subfamily can be subdivided into four groups (Fig. 3): TRPC1, TRPC2, TRPC3/6/7 and TRPC4/5 (Harteneck *et al*., 2000; Clapham *et al*., 2001; Montell, 2001; Mori *et al*., 2001; Montell *et al*., 2002a; Montell *et al*., 2002b). The abundance of functional data available on TRPC3 - 7 supports this classification. In several independent studies, currents mediated by TRPC3, TRPC6 or TRPC7 were shown to be activated by DAG independently of PKC (Hofmann *et al*., 1999; Okada *et al*., 1999; Ma *et al*., 2000; Venkatachalam *et al*., 2001; Zhang & Saffen, 2001), although there is some controversy about the physiological significance of DAG stimulation (Kiselyov & Muallem, 1999). By contrast, recent evidence suggests that unidentified components of the PLC pathway other than DAG or store depletion activate TRPC4 and TRPC5 (Okada *et al*., 1998; Schaefer *et al*., 2000; Strübing *et al*., 2001; Schaefer *et al*., 2002). TRPC3/6/7 and TRPC4/5 can be further discriminated by their biophysical properties and pharmacology. Accordingly, differences in channel open times (Zitt *et al*., 1997; Kiselyov *et al*., 1998; Hofmann *et al*., 1999; Kiselyov *et al*., 1999; Schaefer *et al*., 2000; Yamada *et al*., 2000; Schaefer *et al*., 2002) and in the regulation by external lanthanides have been reported (Halaszovich *et al*., 2000; Schaefer *et al*., 2000; Inoue *et al*., 2001; Strübing *et al*., 2001; Riccio *et al*., 2002; Schaefer *et al*., 2002).

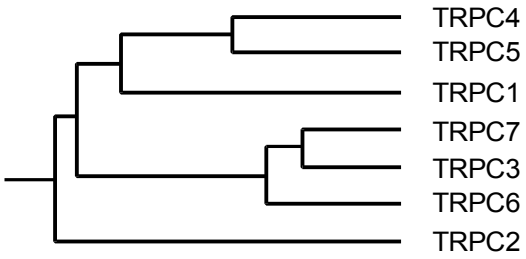


Fig. 3: **Phylogenetic tree of the TRPC subfamily.** The amino acid sequences of the mouse TRPC variants were aligned using the Clustal algorithm.

Much fewer functional data are available on TRPC1 (Zhu *et al*., 1996; Zitt *et al*., 1996; Lintschinger *et al*., 2000; Singh *et al*., 2000; Strübing *et al*., 2001; Delmas *et al*., 2002) and TRPC2 (Vannier *et al*., 1999; Hofmann *et al*., 2000). The activation mechanisms and regulation are even more controversial than for the other TRPC members. A general role for TRPC2 in receptor-activated calcium signalling is questionable. *trpc2* was found to be a pseudogene in humans (Wes *et al*., 1995). Although, full length transcripts were identified in mouse (Vannier *et al*., 1999) and rat (Liman *et al*., 1999), TRPC2 appears to be exert rather tissue-specific functions (Hofmann *et al*., 2000). Its expression was shown to be more or less confined to the vomeronasal organ (VNO) in rat (Liman *et al*., 1999), and to VNO and testis in mouse (Vannier *et al*., 1999; Hofmann *et al*., 2000).

Interestingly, for some TRPC channels, the existence of splice variants with differences in their activation and/or regulation have been reported (Hofmann *et al*., 2000; Mery *et al*., 2001; Zhang & Saffen, 2001; Schaefer *et al*., 2002)

Various studies report differential expression of mammalian TRPC proteins across tissues and cell lines (Garcia & Schilling, 1997; Otsuka *et al*., 1998; Mizuno *et al*., 1999; Sylvester *et al*., 2001; Walker *et al*., 2001). Accordingly, TRPC1 shows ubiquitous expression (Wes *et al*., 1995; Zhu, 1995; Funayama *et al*., 1996; Chang *et al*., 1997; Sinkins *et al*., 1998), whereas TRPC2 is tissue-specific (see above). Expression of TRPC3 and TRPC6 is highest in the brain, but they are also found in peripheral tissue (Garcia & Schilling, 1997; Zhu *et al*., 1998; Mizuno *et al*., 1999). By contrast, TRPC7 is mainly expressed in the heart, lung, and eye and at lower levels in the brain, spleen, and testes (Okada *et al*., 1999). While TRPC5 mRNA is

predominantly expressed in brain (Philipp *et al*., 1998), TRPC4 is expressed in the brain but also in a number of peripheral organs (Okada *et al*., 1998; Walker *et al*., 2001). In most cases, more than one TRPC channel isoforms is expressed in the same cell (Garcia & Schilling, 1997; Sylvester *et al*., 2001; Walker *et al*., 2001). Since, TRP channels are thought to be tetrameric in structure, it is possible that heteromeric interactions among the TRPC proteins occur. This has been shown to be the case for *Drosophila* TRP, TRPL and TRPγ (Gillo *et al*., 1996; Xu *et al*., 1997; Leung *et al*., 2000; Xu *et al*., 2000). Similarly, TRPC1 and TRPC3 (Xu *et al*., 1997; Lintschinger *et al*., 2000) as well as TRPC1 and TRPC4 or TRPC5 (Strübing *et al*., 2001) have been suggested to heteromultimerize when co-expressed recombinantly. Co-expression gave rise to channels with altered biophysical properties. A comprehensive study on the ability of the different members of the TRPC channel subfamily to form heteromultimers has been published recently (Hofmann *et al.*, 2002).

In contrast to the abundance of reports on TRPC channels in heterologous expression systems, relatively little information is available on their role in native tissues. At the time this work was started, virtually all reports on the role of TRPCs in native tissues, suggested a functional significance of these channels mainly for store-operated $Ca^{2+}$ entry (Groschner *et al*., 1998; Liu *et al*., 2000; Philipp *et al*., 2000; Wu *et al*., 2000). Only one study (Li *et al*., 1999) proposed a role for TRPC channels in receptor-mediated cation currents regulated independently of store depletion. This is intriguing because considerable evidence from recombinant studies points to a store-independent regulation for several, if not most, mammalian TRPC channels. Furthermore, a major drawback of the reports on the physiological role of TRPC channels was that none of the native channels studied could be convincingly shown to share properties with the TRPC isoform suggested to be involved.

## 1.3. Aims

The present study was designed primarily to clarify the physiological role of TRPC channels in PLC-dependent stimulation of $Ca^{2+}$-permeable channels in vascular smooth muscle cells. In vascular smooth muscle cells, $[Ca^{2+}]_i$ is the key regulator of contraction and relaxation. Most vasoconstrictors increase $[Ca^{2+}]_i$ following binding to PLC-coupled receptors (Kuriyama *et al*., 1998). Vasoconstrictor-induced $Ca^{2+}$ entry may occur via voltage-activated $Ca^{2+}$ channels, but $Ca^{2+}$-permeable channels stimulated downstream of PLC activation form an additional, important $Ca^{2+}$ entry pathway (van Breemen & Saida, 1989; Davis & Hill, 1999; Jackson, 2000). For the latter channels, both store-operated (Noguera *et al*., 1998; McDaniel *et al*., 2001; Trepakova *et al*., 2001; Albert & Large, 2002) and store depletion-independent (Byron & Taylor, 1995; Helliwell & Large, 1997; Broad *et al*., 1999) activation mechanisms have been described (reviewed by Sanders, 2001; McFadzean & Gibson, 2002). In the present study, A7r5 cells, a clonal cell line derived from embryonic rat thoracic aorta (Kimes & Brandt, 1976), were used as a model system for vascular smooth muscle cells. This cell line has been shown to have retained a number of characteristics of smooth muscle cells (Marks *et al*., 1990; Missiaen *et al*., 1990; Obejero-Paz *et al*., 1993; Byron & Taylor, 1995; Lapidot *et al*., 1996; Karaki *et al*., 1997). In A7r5 cells, $[Arg^8]$-vasopressin (AVP) is known to activate $Ca^{2+}$ influx through store depletion-dependent and store depletion-independent pathways (Van Renterghem *et al*., 1988; Byron & Taylor, 1995; Broad *et al*., 1999; Gardner & Benoît, 2000) and some electrophysiological properties of different non-selective cation conductances have been described (Van Renterghem *et al*., 1988; Krautwurst *et al*., 1994; Nakajima *et al*., 1996; Iwasawa *et al*., 1997; Iwamuro *et al*., 1998).

Because A7r5 cells display PLC-dependent $Ca^{2+}$ entry, the aim of the present study was to evaluate the role of TRPC channels in receptor-activated, PLC-dependent $Ca^{2+}$ entry in this cell line and to compare the properties of the native cation channels with those of TRPC channels in heterologous expression studies.