Review

The mammalian TRPC cation channels

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Abstract

Transient Receptor Potential-Canonical (TRPC) channels are mammalian homologs of Transient Receptor Potential (TRP), a Ca^{2+}-permeable channel involved in the phospholipase C-regulated photoreceptor activation mechanism in *Drosophila*. The seven mammalian TRPCs constitute a family of channels which have been proposed to function as store-operated as well as second messenger-operated channels in a variety of cell types. TRPC channels, together with other more distantly related channel families, make up the larger TRP channel superfamily. This review summarizes recent findings on the structure, regulation and function of the apparently ubiquitous TRPC cation channels.
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1. Origin, classification and nomenclature of TRPCs

The recognition that the protein product derived from the transient receptor potential (*trp*) gene from *Drosophila melanogaster* encoded a PLC-activated Ca^{2+} permeable channel eventually led to identification of seven TRP homologs in mammals [1,2]. Today, the TRP superfamily of channel forming proteins is comprised of these seven homologs of *Drosophila* TRP together with more distantly related channel genes identified through other searches for channels with specific functions [3–8]. TRP superfamily proteins fall into one of the three major families on a phylogenetic basis: the TRPC or canonical TRP family, with seven members (TRPC1 through TRPC7), which are the most closely related to the original *Drosophila* TRP channel; the TRPV family, with six members (TRPV1–6) named after the first group

member, the vanilloid receptor; and the TRPM family, with eight members (TRPM1–8), named after the original member, melastatin (Fig. 1). Other more distantly related members of the superfamily have been identified as genes whose dysfunction forms the bases for certain inherited diseases. The TRP superfamily and the corresponding family and subfamily designations follow a recent consensus nomenclature [9] that will be used throughout this review.

TRP superfamily members are cation channels involved in a continually growing number of cellular functions [6], and overall share 20–60% homology. Based on structural and functional similarities, the TRPC family can be further subdivided into four different subfamilies: TRPC1, TRPC2, TRPC3, 6 and 7 and TRPC4 and 5 (Fig. 1). TRPC2 is a pseudogene in humans, and in old world monkeys and apes [10], but TRPC2 apparently forms fully regulated channels in other mammalian species (see below). TRPC3, 6 and 7 form a closely related subfamily, sharing a high degree of amino acid identity (70–80%) and functional, regulatory and pharmacological similarities. A similarly close structural, and apparently func-
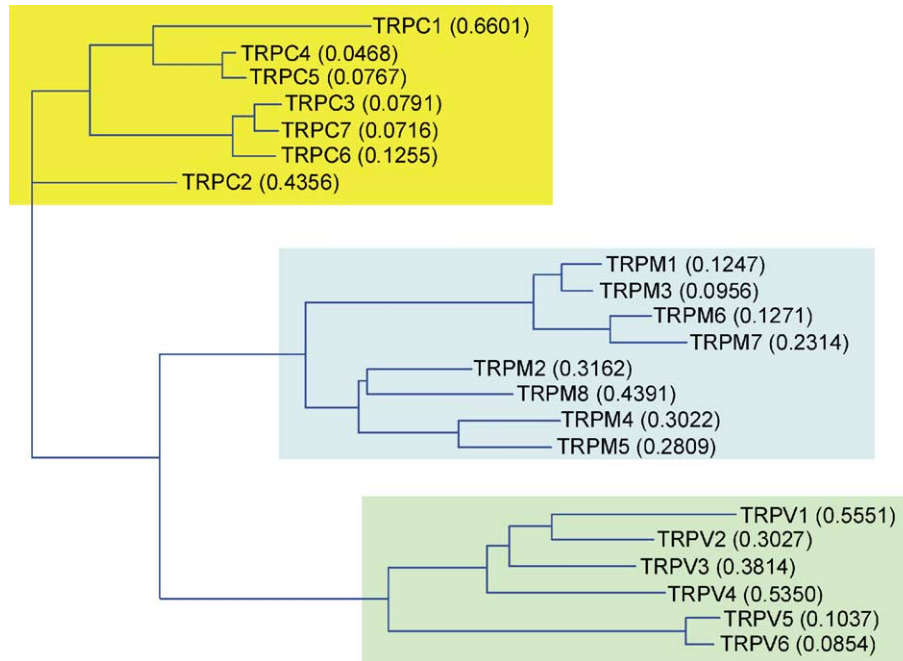
Fig. 1. Structural relatedness of the three major TRP channel families. A multiple alignment of the ion channel domains of TRPC, TRPM and TRPV family members was carried out with VectorNTI software (AlignX) which uses the Clustal W algorithm. Kimura's correction was applied [140]. The phylogenetic tree was built using the Neighbor Joining method of Saitou and Nei [141]. All sequences with the exception of TRPC2 (mouse, pseudogene in human) are human. Calculated distance values are displayed in parentheses.

tional, relationship also exists between TRPC4 and TRPC5.

2. Structure–function relationships of TRPC channels

All members of the TRPC family are believed to share a common topology, based on the original analysis by Vannier et al. [11]. The cytoplasmic N- and C-termini are separated by six predicted transmembrane domains (TM1–TM6), including a putative pore region between TM5 and TM6. However, there is uncertainty as to the exact assignment of the firsts two transmembrane segments [11,12]. The structure shown in Fig. 2 is based on the assignments given in Ref. [11]; alternative assignments are shown in the bottom half of Fig. 2, and the reader is referred to Ref. [12] for a discussion of this issue. The N-terminus is composed of three to four ankyrin repeats, a predicted coiled coil region and a putative caveolin binding region. The cytoplasmic C-terminus includes the TRP signature motif (EWKFAR), a highly conserved proline rich motif, the CIRB (calmodulin/IP$_3$ receptor binding) region and a predicted coiled-coil region. An extended C-terminus containing a PDZ binding motif is unique to TRPC4 and TRPC5. The following section summarizes insights gained from structure–function studies of different TRPC isoforms.

Ankyrin repeats fold into a helix-loop-helix-beta-hairpin/ loop structure with several repeats stacked against one another to form a single domain with a protein-binding interface (recently reviewed in Ref. [13]). Unlike other protein–protein interaction domains, ankyrin repeats do not recognize a conserved sequence or structure, but in contrast can accommodate a variety of binding partners mainly by variation of surface exposed residues. It is therefore hard to predict possible binding partners for the TRPC ankyrin repeats. Based on truncation studies, the ankyrin repeats appear to be required for correct targeting of TRPC3 to the plasma membrane [14]. While N-terminal deletion of TRPC3 up to the ankyrin repeats yields functional and plasma membrane targeted channels, further deletion of the ankyrin repeats results in accumulation of the protein in intracellular compartments. A similar trafficking defect has been described for a TRPC6 mutant lacking 131 N-terminal amino acid residues that include the first ankyrin repeat [14]. Not all ankyrin repeats seem to be crucial for functional expression of channels, as is the case for TRPC1, since a splice variant missing the third out of four ankyrin repeats forms channels that are activated in response to store depletion [15].

The coiled-coil (CC) is a ubiquitous protein motif that is commonly used to control oligomerisation. CC sequences consist of several heptad repeats—seven residue patterns— folding into an alpha-helix. Two (dimer), three (trimer) or more of these alpha-helices are wound around each other to form a supercoil. As mentioned above, there is one CC in each of the cytosolic N- and C-termini of TRPCs (CC-N and CC-C). It is therefore conceivable that CC domains contribute to homo- and heteromerization of TRPCs to form ion channel tetramers with distinct characteristics, or to link TRPC to other coiled domain containing proteins. A
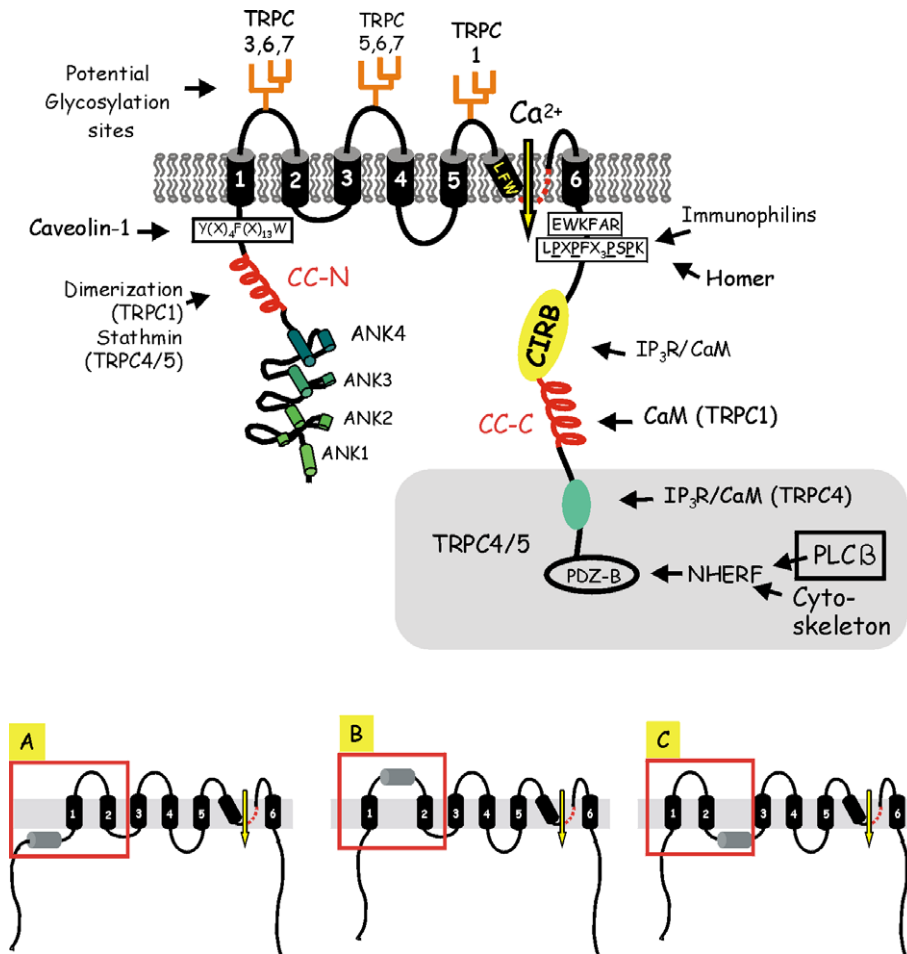
Fig. 2. Structure of TRPC channels. Top: Structural features of TRPC channels. See text for description of structural features. ANK, ankyrin-like repeats; CC-N and CC-C are N-terminal and C-terminal coiled-coil domains, respectively. The region shaded in gray at the extreme C-terminus is specific for TRPC 4 and 5. Bottom: The arrangement of the first three hydrophobic segments of TRPCs is uncertain; three alternatives are shown (A, B and C). See Refs. [11,12]. The acronyms are: PDZ-B, PDZ binding domain; CC-N, N-terminal coiled coil region; CC-C, C-terminal coiled coil region; ANK1–4, ankyrin repeats 1–4; CIRB, calmodulin/IP$_3$ receptor binding region; PLC, phospholipase C; IP$_3$R, IP$_3$ receptor; CaM, calmodulin; LFW, amino acid motif conserved in the putative pore region of all TRPCs.

recent study showed that CC-N of TRPC1 was able to homodimerize based on a yeast two-hybrid screen while the C-terminus including CC-C was unable to dimerize [16]. The microtubule destabilizing phosphoprotein stathmin has recently been pulled out of a yeast two-hybrid screen using the N-terminus of TRPC5 as a bait, and CC-N was found to be responsible for the interaction, presumably via the predicted CC region of stathmin [17]. This interaction seemed to be specific for TRPC4 and TRPC5 since the N-termini of TRPC4 and 5 but not TRPC1 or TRPC7 were able to interact with the stathmin helical domain. TRPC5 appears to regulate axon length and growth cone morphology in hippocampal neurons where its activation and associated Ca^{2+} influx may prevent random axonal outgrowth. The C-terminal CC region can be deleted in TRPC3 without loss of plasma membrane targeting and channel activation when compared with the wild type [14]. However, in TRPC1, calmodulin (CaM) binding to the predicted CC-C domain has been reported and its deletion resulted in diminished Ca^{2+}-dependent inactivation of store-operated Ca^{2+} entry [18].

Caveolins are enriched in plasma membrane microdomains called caveolae (for a recent review see Ref. [19]). Co-immunoprecipitation or co-localization with caveolins has been shown for TRPC1 and TRPC3 [20–22]. Caveolin-1 binding usually occurs at aromatic-rich (θ) regions that contain a specific spacing following the consensus sequences $\theta X\theta X_4\theta$, $\theta X_4\theta X_2\theta$, or $\theta X\theta X_4\theta X_2\theta$ [23]. A similar motif is conserved in all members of the TRPC family located in the cytosolic N-terminus adjacent to the first transmembrane domain [20]. Deletion of this region prevents TRPC1 from targeting to the plasma membrane and the respective mutant dominant-negatively affects store-operated Ca^{2+} entry [20].

A systematic analysis of the predicted pore region of TRPC family members has not yet been carried out. However, several studies have examined the effect of point mutations within the predicted pore region and extrac-

ellular loops bordering the pore. Change of a conserved LFW motif in the predicted pore helix of TRPC5 and TRPC6 to AAA resulted in dominant negative mutants [24,25]. A means to discriminate endogenous receptor operated/store operated channels from overexpressed TRPCs has been the fact that the latter are insensitive to inhibition by lower micromolar concentrations of lanthanides. In the case of TRPC4 and TRPC5, higher micromolar concentrations of lanthanides potentiate currents, while they inhibit overexpressed TRPC6 [26]. Jung et al. [26] identified charged residues in the pore loops of TRPC5 that when mutated from E to Q (E543Q and E595Q/E598Q) caused a loss of lanthanide potentiation. Likewise, E576K or D581K mutants of TRPC1 exhibited decreased store-mediated Ca^{2+} but not Na^+ current suggesting that these residues play an important role in calcium selectivity [27]. This is an interesting finding since conventional thought of the function of a nonselective cation channel would not predict that mutations would affect Ca^{2+} permeability selectively; in other words, the mutation was in reality a gain of function mutation converting a nonselective channel to one selective for monovalent cations. Interestingly, E576 is conserved between TRPC1, 4 and 5 and part of a CX_4CE motif unique to these isoforms. Since these cysteines likely face the extracellular space, they may form disulfide bridges thus potentially explaining preferred heteromer formation between TRPC1, 4 and 5 on one hand and the 'cysteineless' TRPC3, 6 and 7 on the other [25].

A proline-rich motif (L**P**X**P**FXXX**P**S**P**K) downstream of the EWKFAR motif is conserved in all members of the TRPC family and is thought to be responsible for interaction with Homer (for TRPC1) and/or immunophilins [28,29]. Homer proteins dimerize via a CC motif while the EVH domain of each monomer is able to bind to a consensus PPXXF motif in their target molecules thus linking, for example, cell surface receptors and intracellular Ca^{2+} release channels. In analogy to this scheme Homer binding to TRPC1 and the IP_3 receptor would make it a linker involved in the proposed conformational coupling model of TRP channel activation (discussed in a subsequent section). In support of this hypothesis, mutations within the proline-rich motif disrupted Homer binding and resulted in channels with high constitutive activity and greatly reduced agonist regulation [28]. A direct interaction of TRPC channels with IP_3 receptors will be discussed separately (Section 4.2). It is, however, important to note that a TRPC1 mutant lacking the entire cytoplasmic C-terminus was apparently able to function as a store-operated channel [30].

Recently an interaction of TRPC with the immunophilins FKBP12 and FKBP52 has been demonstrated [29]. Immunophilins are peptidyl-prolyl *cis–trans* isomerases binding to a XP motif in their binding partners. Pulldown experiments showed that FKBP12 preferentially interacted with TRPC3, 6 and 7, while FKBP52 preferred TRPC1, 4 and 5. Mutation of the first proline in the proline rich motif of

TRPCs disrupted that interaction. Furthermore, FK506, which displaces immunophilins from their targets, was able to inhibit agonist-induced currents through TRPC6 channels [29].

Evidence for functional roles of the CIRB (CaM/IP_3 *receptor binding*) region is discussed in Section 4.2.3. As mentioned above, TRPC4 and 5 share an extended C-terminus terminating in a PDZ binding motif. A part of this region is missing in a TRPC4β splice variant, and this variant has been proposed to have autoinhibitory function [31]. This region has also been shown to interact with the C-terminus of IP_3 receptors and CaM [32,33]. Little is known about the function of this region in TRPC5 and the degree of homology between the C-termini of TRPC4 and TRPC5 is very low. The PDZ binding motif TRL of TRPC4 and TRPC5 seems to be responsible for the interaction with the adaptor protein, NHERF, possibly linking the channels to PLCβ and the cytoskeleton [34]. Deletion of the PDZ binding motif of TRPC4 not only reduced the surface expression of TRPC4 but also changed it from a general membrane distribution to a predominant expression in cell outgrowths [35].

Recently, different patterns of N-glycosylation have been summoned to explain different levels of constitutive activity within members of the TRPC3, 6 and 7 family. TRPC3, which is supposedly monoglycosylated in the first extracellular loop, shows high constitutive activity, while TRPC6, dually glycosylated in the first and second extracellular loop is tightly regulated. By converting TRPC3 into the TRPC6-like dually glycosylated version and likewise converting TRPC6 into the monoglycosylated TRPC3-like version, Dietrich et al. [36] were able to alter constitutive activity as hypothesized. It is worth noting, however, that TRPC7 exhibits considerable basal activity despite the fact that it has a predicted N-glycosylation site in the first and second extracellular loop like TRPC6 [37].

The major structural features of TRPC channel subunits are summarized in Fig. 2.

3. Pharmacology and electrophysiological properties

3.1. TRPC1

TRPC1 was the first member of the TRPC family to be cloned [1,2], and the first shown by electrophysiological measurements to form a calcium-permeable cation channel [15]. In this initial study, expression of TRPC1 in Chinese hamster ovary cells (CHO) resulted in a linear nonselective cation current, activated by intracellular infusion (via the patch pipet) of either IP_3 or thapsigargin to deplete intracellular calcium stores. This current was shown to be sensitive to Gd^{3+} (20 μM). Noise analysis provided evidence that the current was passed through single channels with a conductance of 16 pS. The current–voltage relationship of these channels was shown to be predom-

inantly inward, with little outward current [38,39]. In this respect, the TRPC1 current was described as I_{crac}-like [40] (the properties of I_{crac} are discussed in a subsequent section dealing with regulation of TRPC channels, and the behavior of store-operated channels), although unlike I_{crac}, the current was not Ca^{2+}-selective.

3.2. TRPC2

There has been little direct work to date regarding the electrophysiological characteristics of TRPC2. Work on mice vomeronasal neural dendrites has shown that knocking out the TRPC2 gene resulted in the near loss of a diacylglycerol (DAG)-activated linear current, which was not activated by store depletion [41], suggesting that TRPC2 may be a component of a DAG-operated channel in situ. However, using fluorescence rather than electrophysiological methodology, Vannier et al. [42] reported that expressed TRPC2 appeared to behave as a store-operated channel.

3.3. TRPC3, 6 and 7

TRPC3, 6 and 7 appear to form nonselective cation channels that show both inward and outward rectification, at negative and positive voltages, respectively. The current–voltage relationships for these TRPCs demonstrate a far greater outward current (at positive voltages) than inward current (at negative voltages) with a reversal potential at around 0 mV.

Zhu et al. [43] first described TRPC3 as an agonist-activated channel when expressed in HEK293 cells and demonstrated that it was relatively insensitive to low concentrations of Gd^{3+}, but was blocked by concentrations in the 100 μM range. Hofmann et al. [44] later demonstrated that TRPC3 could also be activated directly by DAG analogues.

TRPC3 channels have also been shown to have two potential conductance states when expressed in HEK293 cells, one of 66pS and one of 17pS [45]. In this study it was shown that these channels were activated by calcium store depletion in intact cells, consistent with an earlier report by Preuβ et al. [46], or by addition of recombinant IP_3 receptors in excised patches. In a subsequent study, however, activation of TRPC3 was shown to be dependent solely upon DAG with no requirement for either IP_3 or the IP_3 receptor [47].

Since their discovery, these channels have been shown to be sensitive to a variety of agents with relative sensitivities varying dramatically depending upon expression system. In HEK293 cells, TRPC3 was inhibited by high concentrations of: SKF96365, verapamil, La^{3+}, Gd^{3+} and Ni^{2+} [43] and was partially inhibited by moderate (30 μM) concentrations of 2-aminoethoxydiphenyl borane (2APB) [48]; in DT40 cells by low concentrations of Gd^{3+} [48]; and in CHO cells they are reported to be sensitive to low concentrations of La^{3+}, Gd^{3+} and

SKF96365 [49]. However, in the latter case, it appeared that the lanthanides acted by first entering the cell and blocking from the inside. This requirement for penetration, which does not normally occur with lanthanides, may explain the relative insensitivity of TRPC3 to lanthanides in other cellular systems.

TRPC6 has also been shown to be a DAG-activated channel [44] and is very similar to TRPC3 and 7 with regard to its current–voltage relationship. The single channel conductance was 35pS, with no second conductance state observed [44]. TRPC6 does, however, possess a unique characteristic to this TRPC subfamily. When expressed in HEK293 cells, TRPC6 currents are enhanced by the cation channel blocker flufenamate while TRPC3 and 7 currents are not [50].

TRPC7, the final member of this subfamily, demonstrates properties very similar to TRPC3 and 6 with regard to its voltage–current relationship, and activation by DAG [37]. TRPC7 has demonstrable sensitivity to SKF96365 and, in a similar fashion to TRPC3 and 6, is relatively insensitive to lanthanides.

The differences between the three channel types may lie in their ion selectivity, in that TRPC6 is reported to be somewhat Ca^{2+}-selective, while TRPC3 and TRPC7 do not appear to be. Reported values for ($P_{Na} \backslash P_{Ca}$) are 1\1.5, 1\5 and 1\2 for TRPC3, 6 and 7, respectively (cf. [51]).

3.4. TRPC4 and 5

TRPC4 was originally cloned by Philipp et al. [52]. These investigators reported that, when expressed in mammalian cells, TRPC4 resulted in a novel, inwardly-rectifying Ca^{2+}-selective current that was activated by either IP_3 or thapsigargin-induced store depletion. This role of TRPC4 in store-operated entry has since been questioned with reports of TRPC4 being activated by G-protein coupled receptors [53]. Interestingly, TRPC4 currents have been shown to be potentiated by lanthanides [53], in contrast to their inhibitory effects on endogenous store-operated channels or other TRPCs. TRPC4 has a reported single channel conductance of 41 pS [53], with a current–voltage relationship similar to that of TRPC3, 6 and 7, having a greater current at positive voltages than at negative voltages.

TRPC5 was originally cloned by the same group who first cloned TRPC4 [54], and was shown to have properties expected of a store-operated channel. However, subsequent studies from other laboratories demonstrated that TRPC5 behaved as a receptor-operated cation channel [53,55]. The current–voltage relationship of this channel indicates that far more current is passed at negative voltages (inward current) than at positive. In a similar fashion to TRPC4, TRPC5 currents are also potentiated by lanthanides [53]. The single channel conductance of TRPC5 has been estimated at around 63 pS [53]. The reported ion selectivities of TRPC4 and 5 vary (see Ref. [6]).

4. Signaling mechanisms and regulation of TRPC channels

4.1. Store depletion

In most types of cells, membrane receptors coupled to phosphoinositide-specific phospholipase C (PLC) promote inositol 1,4,5-trisphosphate (IP_3)-mediated release of Ca^{2+} from endoplasmic reticulum (ER) and subsequent Ca^{2+} entry across the plasma membrane which occurs, predominantly, through a process known as capacitative calcium entry (CCE) or store-operated calcium entry [56]. Under physiological conditions, CCE is triggered when IP_3 discharges Ca^{2+} from ER stores; it is the subsequent reduction in ER Ca^{2+} content that signals to the plasma membrane for activation of store-operated channels (SOCs). When measured by electrophysiological techniques, in hematopoetic cells and perhaps some other cell types, CCE corresponds to a highly Ca^{2+}-selective current termed the calcium-release activated calcium current, or I_{crac} [40]. In other cell types, most notably smooth muscle, the currents underlying CCE appear to be less Ca^{2+}-selective [57,58], indicating that no one gene product is likely to be responsible for store-operated currents in all cell types. Neither the precise activation mechanism nor the molecular identity of the SOCs is known with certainty [59,60]. The fact that activation of *Drosophila* TRP channel was found to require activation of the PLC cascade (see Ref. [5] and references therein) originally suggested that the mammalian counterparts might be molecular candidates for SOCs. Consequently, a number of studies on the molecular nature of SOCs have focused primarily on mammalian TRP channels, particularly those from the TRPC family. As a result, most of the members of the TRPC family, either endogenous or ectopically expressed in cell lines, have been implicated in store-operated cation entry.

There is a considerable body of evidence supporting the idea that TRPC1 constitutes, or is a part of, SOCs (reviewed in Ref. [61]). Ectopic expression of TRPC1 in many cell lines has resulted in either nonselective [15] or Ca^{2+}-selective [62] cation channels activated by either agonist stimulation or pharmacological depletion of stores with thapsigargin. The most compelling evidence for TRPC1 as a subunit of SOCs is derived from studies using TRPC1 antisense constructs. This strategy proved to be efficient in reducing SOC entry in human submandibular gland cells [62], HEK293 cells [63], *Xenopus* oocytes [64], human lung epithelial A249 cells [65] and pulmonary artery smooth muscle cells [66]. Additionally, genetic disruption of the TRPC1 gene significantly reduced I_{crac} in chicken DT40 B lymphocytes [67]. Other studies provided evidence for TRPC1 as part of endogenous SOCs by using antibodies directed against extracellular loops of TRPC1. Extracellular application of these antibodies significantly reduced SOC entry in vascular smooth muscle cells [68] and human platelets [69].

TRPC2, which in humans appears to have degenerated to a pseudogene [2], has been shown to be functional in mouse, rat and other mammalian species. Ectopic expression of two splice variants of mouse TRPC2 (TRPC2a and TRPC2b) in COS-M6 cells increased the magnitude of SOC entry induced by muscarinic receptor stimulation [42], although the constitutive activity of the overexpressed proteins was not assayed. Importantly, an antibody targeting an extracellular site of mouse TRPC2b, near the pore region, blocked thapsigargin-evoked Ca^{2+} entry and the acrosome reaction in sperm [70], suggesting that TRPC2 might be a subunit of endogenous SOCs in sperm. Two additional splice variants of mouse TRPC2 (TRPC2α and TRPC2β) cloned by a different lab [71] were devoid of any measurable function upon heterologous expression, in principle due to a plasma membrane trafficking defect. Expression of a TRPC2 antisense construct in CHO cells, which mainly express TRPC1 and TRPC2, significantly reduced SOC entry induced by thapsigargin [72].

TRPC3 is the most extensively characterized of the TRPC family members, although its role in CCE has been controversial. While earlier observations suggested a role for TRPC3 in CCE when transiently expressed in HEK293 cells [46,73], subsequent studies showed that this likely reflected constitutive channel activity, rather than a regulated mode of Ca^{2+} entry [43]. TRPC3 constitutive activity has frequently caused misinterpretation of studies aimed to address the role of store depletion in channel regulation (see Ref. [48]). This is due to an apparent increase in thapsigargin-induced Ca^{2+} entry, which results from diminished ER Ca^{2+} buffering capacity, and the concomitant enhancement of constitutive channel activity. A considerable body of literature now supports the idea that TRPC3 commonly behaves as a receptor-activated channel that cannot be activated by store depletion (discussed in Refs. [44,74–77]). An interesting exception occurs with ectopic expression of TRPC3 in the avian B lymphocyte DT40 cell line. In these cells, TRPC3 is clearly activated by store depletion, but only when expressed at very low levels [48,78,79], as conditions that provide higher levels of channel protein expression give rise to the receptor-regulated mode of TRPC3 activation [79,80], indicating that the coupling mode of TRPC3 might be related to its expression level. Channel expression level was also shown to have a critical impact on the coupling mechanism for TRPV6 activation [81]. A thorough discussion of the potential relationship between channel expression level and channel regulation has recently been published [82].

Evidence for store-operated regulation of other members of the TRPC3, 6 and 7 subfamily is also controversial. Transient ectopic expression of mouse TRPC7 in HEK293 cells resulted in a constitutively active cation channel, whose activity was further stimulated by activation of the PLC-linked P2Y purinergic receptor, but not by store depletion alone [37]. In contrast, Riccio et al. [83] reported

that stable expression of human TRPC7 in HEK293 cells results in a cation channel activated by store depletion. The authors suggested that the different coupling modes of activation for mouse TRPC7 versus the human ortholog could be due to a difference in the amino acid at position 111 (proline in their human TRPC7 clone vs. leucine in mouse TRPC7). However, in the study by Riccio et al., TRPC7 constitutive activity was not assessed. Thus, based on prior experiences with other TRPCs, it must be said that for now there is no convincing evidence that TRPC7 behaves differently from its close relatives, TRPC3 and TRPC6.

Although conflicting results exist regarding the role of PLC-derived products, and more recently, phosphorylation (see below) in regulation of TRPC6, the literature is relatively consistent with regard to the role of store depletion on channel activity. Both mouse and human TRPC6, when ectopically expressed in cell lines, appear to behave as nonselective cation channels whose activation is independent of store depletion but linked to PLC-derived products [44,50,84] (for a review, see Ref. [74]).

TRPC4 and TRPC5 are structurally similar, and thus it is not surprising that, in a given study in a given laboratory, they appear to be regulated similarly. Ectopic expression of bovine TRPC4 in both HEK293 and CHO cells increased SOC entry in response to either IP$_3$ or thapsigargin [52,85]. In addition, bovine TRPC4 markedly augmented I_{crac} currents when expressed in RBL cells [85]. Rat TRPC4 increased thapsigargin-induced inward calcium-dependent chloride currents, an indirect reflection of SOC entry, when expressed in Xenopus oocytes [64,86]. A TRPC4 antisense construct markedly reduced an I_{crac}-like current in SBAC cells, an adrenal cortical cell line, suggesting TRPC4 is part of the endogenous CRAC channels in these cells [87]. Similarly, vascular endothelial cells derived from TRPC4 knock-out (TRPC4$^{-/-}$) mice showed impaired store operated calcium entry [88]. In a recent study of the properties of lung endothelial cells derived from the same TRPC4$^{-/-}$ mice, Tiruppathi et al. [89] expanded the observations of Freichel et al. [88], and found that absence of TRPC4 was correlated with a loss of endothelial cell responses to thrombin, suggesting a key involvement of TRPC4 in microvascular permeability. More recently, TRPC4 antisense oligonucleotides were shown to partially inhibit store-operated calcium entry in mouse mesangial cells, suggesting that TRPC4 might also form part of endogenous SOCs in that cell type [90].

In contrast with these findings, mouse TRPC4 expressed in HEK293 cells, adrenal chromaffin cells and PC12 cells was found to form agonist-PLC regulated cation channels. The channels could not be activated by IP$_3$, DAG or store depletion [53,91]. In another study, human TRPC4 was not regulated by either store depletion or PLC activation, and showed only constitutively active when expressed in HEK293 cells [77]. Stable expression of a TRPC4 antisense construct in the same cell line did not alter store-operated

entry [92]. Fewer reports have dealt with regulation of TRPC5 compared to TRPC4. Almost simultaneously, two different labs reported the cloning of mouse TRPC5, its transient expression in HEK293 cells rendering a receptor-activated channel, independent of store depletion for one group [55] and store-operated for the other [54]. Subsequently, in one instance in which one laboratory compared both TRPC4 and TRPC5 in the same study, the same mode of regulation was observed—PLC-dependent activation not related to store depletion [53]. These authors expressed mouse TRPC5 in CHO and HEK293 cells, and found that the channel was activated by either G-protein-coupled receptor or receptor tyrosine kinase stimulation but not by IP$_3$, DAG or store depletion. Upon ectopic expression in cell lines, TRPC5 showed significant constitutive activity, similar to that seen for other TRPC channels. Unlike TRPC4, expression of mouse TRPC5 in Xenopus oocytes significantly increased muscarinic receptor-induced calcium-dependent chloride currents, but not those stimulated by thapsigargin, indicating, again, that TRPC5 was receptor-activated rather than store-operated [93].

4.2. Interaction between IP$_3$ receptors and TRPC channels

The inositol 1,4,5 trisphosphate receptor (IP$_3$R) is a calcium release channel found predominantly in the membrane of the endoplasmic reticulum (ER). IP$_3$R releases calcium from the ER in response to the second messenger IP$_3$ generated by the enzymatic activity of phospholipase C (PLC). One of the hypotheses for the mechanism of activation of capacitative calcium entry was originally proposed by Irvine [94] and termed "conformational coupling". According to this model, further developed by Berridge [95], SOCs in the plasma membrane interact with IP$_3$ receptors in the endoplasmic reticulum resulting in physical coupling of Ca^{2+} release and Ca^{2+} entry. With the discovery of TRPC channels and their subsequent proposal as candidates for capacitative calcium entry channels, investigators assessed the possibilities of a direct interaction between TRPC channels and the IP$_3$ receptor (for review, see Ref. [96]). Although its physiological significance is still a matter of great debate, physical interactions between the IP$_3$R and virtually every member of the TRPC family have been reported. Tang et al. [97] described CaM and IP$_3$R binding sites in all TRPC proteins and suggested a reciprocal regulation of TRPC channels by IP$_3$R and CaM. The affinity of IP$_3$R peptide in displacing CaM was found to be lower in TRPC1,2,4,5 than in TRPC3,6,7 [97]. Specific findings relating to interactions between IP$_3$ receptors and the TRPC sub-families are discussed below.

4.2.1. TRPC1
Rosado and Sage [98] showed that in human platelets TRPC1 and type II IP$_3$R co-immunoprecipitate when internal calcium stores are depleted by thapsigargin and

that xestospongin, an IP_3R antagonist, blocks CCE. In a subsequent study, the same authors showed that xestospongin also blocks the thapsigargin-induced association of TRPC1 with type II IP_3R suggesting that TRPC1 is a component of CCE channels, and that the mechanism of activation involves interaction with IP_3 receptors [99]. Rosado and Sage showed that the association with TRPC1 and IP_3R is reversible; the TRPC1–IP_3R complex dissociates upon refilling of the stores. They treated platelets with jasplakinolide, a drug that increases peripheral actin accumulation, and found that this blocked CCE and prevented the association of TRPC1 and IP_3R (see also ref. [100]). In a subsequent study, again in platelets, Rosado et al. [69] showed that store depletion with an agonist (i.e., thrombin), as seen with thapsigargin, caused reversible coupling of the type II IP_3R with the Ca^{2+} entry channel, TRPC1. A more recent study from the same laboratory [101] reported a direct interaction between type II IP_3 receptor and TRPC1 within 1 s upon stimulation of human platelets with thrombin, consistent with a gating mechanism of the TRPC1 channels by IP_3R. Mehta et al. [102] reported a Rho-induced association of IP_3R with TRPC1 that was dependent on actin filament polymerization, and upon store depletion either with agonist or thapsigargin. These authors subsequently concluded that Rho activation signals interaction of IP_3R with TRPC1 at the plasma membrane of endothelial cells, and triggers Ca^{2+} entry following store depletion. Similarly, Yuan et al. [28] proposed that the adaptor protein Homer facilitates a physical association between TRPC1 and the IP_3R that is required for TRPC1 activation. Lockwich et al. [21] showed that endogenous TRPC1 in HSG cells is Triton X-100-insoluble, suggesting association with the cytoskeleton and lipid rafts; TRPC1 was found associated with caveolin, type III IP_3R and $G\alpha q$, suggesting the existence of a signalplex comprised of TRPC1 and several players of the PLC pathway.

4.2.2. TRPC2

Tang et al. [97] showed that TRPC2, like all members of the TRPC family, has the potential to interact with the IP_3R. The IP_3R-binding domain was also found to interact with CaM in a Ca^{2+}-dependent manner with an affinity of 10 nM and CaM inhibits the TRPC–IP_3R interaction. The authors then concluded that both CaM and IP_3R play reciprocal roles in controlling the activation of all TRPC channels. Brann et al. [103] showed an overlapping of immunoreactivity for the type III IP_3R with that of TRPC2 in the epithelium of the vomeronasal organ of the rat. This observation was further confirmed by co-immunoprecipitation of type III IP_3R and TRPC2 from vomeronasal organ lysates.

4.2.3. TRPC3, 6 and 7

Although evidence for interaction with IP_3R has been reported for all members of this subfamily [97], most of the evidence is derived from studies with TRPC3 channels. The

interaction between TRPC3 and IP_3R was reviewed earlier by Trebak et al. [74]. Presuming that TRPC proteins might function as store-operated channels, Kiselyov et al. [45] used a HEK293 cell line stably expressing TRPC3 (T3–9, from Zhu et al. [43]) to demonstrate "conformational coupling" of TRPC3 and the IP_3R. As pointed out by Trebak et al. [74], a large body of evidence, including data from Zhu et al. [43] on T3–9 cells, showed that TRPC3 overexpressed in HEK293 behaves as a non-store-operated, receptor-activated channel. It is therefore unclear why, in the electrophysiological studies by Kiselyov et al. [45], TRPC3 exhibited characteristics of store-operated channels. In the studies of Kiselyov et al. [45], T3–9 cells stimulated by carbachol or thapsigargin showed increased single channel activity that was absent in wild-type HEK293 cells. Channel activity declined after patch excision, but was reactivated by IP_3. After extensive washing of the patches, IP_3 could no longer activate TRPC3 channels. In this situation, channels could be activated by a combination of IP_3 and IP_3R. The ability of IP_3 to activate TRPC3 channels in excised patches was blocked by the IP_3R antagonists, heparin and xestospongin C. The authors concluded that TRPC3 channels are store-operated and that the mechanism of activation involves conformational coupling to the IP_3R in its IP_3 bound state [45]. However, in a series of studies Trebak et al. [47,48] evaluated the involvement of TRPC3 in store-operated Ca^{2+} entry and the role of IP_3 in TRPC3 activation. When expressed in HEK293 cells, TRPC3 was shown to form channels that are receptor-operated but not activated by store depletion [48] in agreement with Zhu et al. [43]. In a subsequent study Trebak et al. [47] demonstrated that DAG is sufficient to activate TRPC3 and that neither IP_3 nor G proteins are required for TRPC3 activation in HEK293 cells, including the T3–9 cell line used by Kiselyov et al. [45]. Furthermore, the IP_3R antagonist, heparin, was unable to block TRPC3 activation in response to agonist. In an earlier study, Inoue et al. [50] showed that TRPC6 could not be activated by IP_3 and that DAG was sufficient for TRPC6 activation. A report by Albert and Large [104] describing a native TRPC6-like calcium channel in rabbit portal vein myocytes showed that those TRPC6-like channels could not be activated by IP_3; however, they report that IP_3 appears to synergize with DAG to produce greater TRPC6-like channel activation.

Kiselyov et al. [105] further identified a TRPC3 binding domain in the C-terminus of the IP_3R. Transiently expressed TRPC3 increased the basal rate of Ba^{2+} entry in HEK293 cells, and this was further increased by co-expression of the IP_3R binding domain or an IP_3R construct lacking the transmembrane domain, but not by the full-length IP_3R protein. This indicated that forms of IP_3R that are uncoupled from intracellular stores activate TRPC3, while the full-length receptor may require store depletion or some additional signal.

Boulay et al. [106] also investigated the interaction between human type 3 IP_3R and human TRPC3. IP_3R

proteins could be recovered in TRPC3 and TRPC6 immunoprecipitates. The authors identified the minimal mutual binding domains in TRPC3 and IP$_3$R: two sequences in IP$_3$R that interact with the C-terminus of TRPC3, designated F2q and F2g, corresponding to amino acids 669–698 and 751–821, respectively. F2q is modestly conserved among human IP$_3$Rs, while F2g is well conserved. For TRPC3, a small fragment designated C8, corresponding to amino acids 777–797 in human TRPC3, interacted with the N-terminus of IP$_3$R. This sequence is well conserved among TRPC3, TRPC6 and TRPC7. Transient expression of the C7 fragment (54 amino acids containing the C8 peptide) significantly inhibited entry due to either agonist or thapsigargin in wild-type HEK293 cells. Transient expression of an IP$_3$R fragment, which contains F2q, inhibited both agonist and thapsigargin-induced entry. These experiments are suggestive of an involvement of TRPC3 and IP$_3$R in native store-operated Ca^{2+} entry in wild-type HEK293.

Zhang et al. [107] identified a sequence in the C-terminus of TRPC3 corresponding to amino acids 761–795 (termed CIRB domain for CaM/IP$_3$ receptor binding domain, discussed above) that binds CaM, and overlaps with the C8 region identified as the IP$_3$R binding region by Boulay et al. [106]. CaM was found to compete with the IP$_3$R peptide, F2q, for binding to CIRB and activation of TRPC3 channels was also observed with a CaM binding peptide, and with calmidazolium, a CaM antagonist [107].

A pharmacological study by Ma et al. [76] investigated the involvement of IP$_3$R in store-operated Ca^{2+} entry and TRPC3-mediated cation entry by examining the actions of 2APB, a purported membrane permeant IP$_3$R antagonist [108]. These authors observed ATP-stimulated entry of Sr^{2+} in a HEK293 cell line stably transfected with TRPC3, but not in control cells; 2APB blocked this entry. Furthermore, 2APB blocked capacitative Ca^{2+} entry in response to agonist or thapsigargin in control HEK293 cells. Ma et al. [76] suggested that the action of 2APB is consistent with the "conformational coupling" hypothesis, assuming that 2APB is acting by blocking IP$_3$ receptors. However, it is now clear that 2APB blocks the endogenous store-operated Ca^{2+} pathway by acting directly on SOCs [109–115]. It is currently unclear whether the effect of 2APB on TRPC3 channels is also due to a direct action on the channels, unrelated to IP$_3$R. A study by Li et al. [116] focused on the regulation of endogenous TRPC3 channels. They found that the neurotrophin BDNF activates a Ca^{2+}-dependent nonselective cation current in pontine neurons, reminiscent of that activated by TRPC3. The BDNF-induced current could be blocked by the PLC antagonist U73122 as well as by IP$_3$R antagonists, xestospongin C and heparin. While thapsigargin did not activate this current, including IP$_3$ in the patch pipette did. The data from Li et al. [116] are consistent with an endogenous TRPC3 that is activated by a PLC- and IP$_3$-dependent coupling mechanism, not related to store depletion.

In summary, the exact role of IP$_3$R in TRPC3 or SOC activation is still unclear. In a DT40 cell line lacking all forms of IP$_3$R, TRPC3 is activated by receptor agonists to the same extent as in wild-type cells [80] and this cell line also exhibits normal Ca^{2+} entry following passive depletion of the stores by thapsigargin [117,118]. Furthermore, the physiological significance of the biochemical interactions is uncertain. The effects of the TRPC3 and IP$_3$R peptides are small [106]. As shown by Trebak et al. [47], TRPC3 channels expressed in HEK293 cells are activated independently of IP$_3$ and G proteins; in addition, heparin could not block TRPC3 activation via G protein-coupled agonists. Nonetheless, Tang et al. [97] showed that all TRPC family members (1 through 7) associate with IP$_3$R (see also Ref. [32]). Therefore, interaction with the IP$_3$ receptor might play a role in assembling signaling complexes that involve phospholipase C, underlying IP$_3$ receptors, and TRPC channels.

4.2.4. TRPC4 and 5

The activation mechanism of the two members of this subfamily was reviewed recently by Plant and Schaefer [119]. Using a yeast two-hybrid assay and glutathione-*S*-transferase pulldown experiments, Mery et al. [32] reported that the C-terminus of an alternatively spliced variant of TRPC4, the alpha isoform, but not the beta, associates in vitro with the C-terminal domain of IP$_3$R receptors, types 1, 2 and 3. Subsequently, Tang et al. [97] confirmed these findings and discovered an additional CaM/IP$_3$R binding region common to all members of the TRPC family, as discussed above. In the presence of Ca^{2+}, the TRPC4–IP$_3$R interaction was inhibited by CaM. A synthetic peptide representing a TRPC-binding domain of IP$_3$R inhibited the binding of CaM to TRPC3, 6 and 7 more effectively than that to TRPC1, TRPC2 and TRPC4 and 5. In inside-out membrane patches, TRPC4 was activated strongly by calmidazolium, an antagonist of CaM. Kanki et al. [93] showed that mouse TRPC5 expressed in *Xenopus* oocytes was activated by agonist but not by depletion of stores with thapsigargin. The agonist activation of TRPC5 was inhibited by the PLC inhibitor, U73122 and by the IP$_3$R antagonist xestospongin C. The authors also showed that the IP$_3$R agonist, adenophostin A, activated TRPC5 in a dose-dependent manner. However, the effect of injection of IP$_3$ itself on TRPC5 activity was apparently not examined. TRPC5 activation by adenophostin A was blocked by a pre-injection of either xestospongin C or a peptide mimicking the IP$_3$ binding domain of *Xenopus* IP$_3$R [93]. These authors concluded that the IP$_3$R is essential for TRPC5 activation. This conclusion conflicts with the report that TRPC5 transfected into the DT40 cell line lacking all isoforms of the IP$_3$R showed normal TRPC5 activation [120].

4.3. Role of membrane lipids in TRPC activation

When expressed in different cell lines, only members of the TRPC3, 6 and 7 subfamily have been consistently shown to be activated by membrane permeant DAG analogs such as 1-oleoyl-2-acetyl-sn-glycerol (OAG). These channels can also be activated by DAG lipase and DAG kinase inhibitors, but not by monoacylglycerols. This provides a possible mechanism of activation of these channels by PLC-coupled receptors, independently of IP$_3$R or store depletion.

Hofmann et al. [44] reported that exogenously applied DAG analogues can activate TRPC6 and TRPC3. These authors demonstrated that both TRPC6 and TRPC3, expressed transiently in COS cells, are activated by DAG, most efficiently by the membrane permeant DAG analog, OAG. They also demonstrated that the effects of DAG on TRPC6 are not mediated by protein kinase C (PKC). Using whole-cell patch clamp recordings and single cell fluorimetry, Trebak et al. [47] showed that receptor-mediated activation of PLC activates TRPC3 via DAG production independently of IP$_3$. They also showed that phorbol esters inhibit OAG-mediated activation of TRPC3 suggesting a negative regulation of TRPC3 by PKC [47]. The activation of TRPC3-mediated cation entry by DAG has been confirmed by several groups using different expression systems [44,49,76–78,80].

Ma et al. showed that in TRPC3-transfected HEK293 cells, TRPC3 was activated after exogenous application of OAG or a DAG lipase inhibitor (RHC80267) to the cells, while control cells showed no response to either of these stimuli. In addition, OAG could activate TRPC3 after 2APB treatment whereas ATP failed to activate TRPC3 in the presence of 2APB. The authors concluded that DAG activates TRPC3 by a mechanism distinct from activation by IP$_3$R [76]. However, as discussed in the IP$_3$R section, it is likely that the effect of 2APB on TRPC3 channels is due to a direct action on the channels themselves, unrelated to IP$_3$R, as it is the case for native SOCs.

TRPC6 appeared to function as a receptor-regulated, but not a store-regulated cation channel. Hofmann et al. [44] showed that neither thapsigargin, ionomycin, PIP$_2$ nor IP$_3$ activated TRPC6. The PLC inhibitor, U73122, blocked agonist activation of TRPC6 leading the authors to conclude that TRPC6 is activated through PLC production of DAG, not by IP$_3$ or IP$_3$R, and not directly by G-proteins. Following TRPC6 transfection into superior cervical ganglion neurons by Delmas et al. [121], OAG, but not thapsigargin, activated a nonselective cation current, confirming that TRPC6 is not sensitive to store depletion. Inoue et al. [50] confirmed that TRPC6 is activated by DAG in a PKC-independent manner and is insensitive to activation by thapsigargin or IP$_3$.

Okada et al. [37] showed that OAG and the DAG lipase inhibitor RHC80267 activated TRPC7-mediated cation entry. The effect of OAG was PKC-independent but PMA blocked activation of TRPC7 by OAG suggesting a negative regulation of TRPC7 by PKC as described for TRPC3 and TRPC6 [47,50]. Reciprocally, a PKC inhibitor, BIM I, caused a slight potentiation of OAG effects in TRPC7-mediated cation entry [37].

The mechanisms of activation of TRPC1, TRPC2 and TRPC4 and 5 are much less certain. Ectopically expressed TRPC4 and 5 were shown to be insensitive to DAG although their activation clearly depended upon phospholipase C [53]. Lintschinter et al. [39] reported that TRPC1 was only activated by DAG when associated with TRPC3; however, subsequent work by Hofmann et al. [25] cast doubt on the likelihood of co-assembly of TRPC3 and TRPC1 subunits. The evidence that TRPC1 may be a component of store-operated channels, or regulated by IP$_3$R, has been discussed above. The mechanism of activation of TRPC2 is still largely unknown. The sensitivity of TRPC2 to DAG when ectopically expressed has apparently not been investigated. Recently, Lucas et al. [41] identified a native calcium-permeable channel in vomeronasal neuron dendrites that was activated by DAG, independently of Ca^{2+}, IP$_3$, arachidonic acid and PKC. When the TRPC2 gene was deleted in mice, a severe deficit in the native DAG-activated channel was observed suggesting the involvement of TRPC2 protein as a component of this DAG-activated channel. Based on antisense studies, TRPC4 has been implicated in arachidonic acid-mediated Ca^{2+} entry in mammalian cells [92]. This activation was PKC-independent.

4.4. Calcium and CaM

Calcium is known to influence TRPC activity, and there is evidence that CaM is involved in this regulation. Initial studies on TRPC3 channels concluded that TRPC3 could function as a calcium-activated channel [75]. However, stimulation by raising Ca^{2+} was only seen in whole cell mode, and not with single channels in excised patches. It is possible that this effect may reflect Ca^{2+} activation of PLC. Nonetheless, there is considerable evidence for Ca^{2+} and CaM regulation of TRPCs, more commonly in an inhibitory mode.

CaM, a soluble Ca^{2+} binding and Ca^{2+} regulatory protein, is known to be involved in the regulation of many cellular functions, including channel activity [122]. CaM has been shown to bind to *Drosophila* TRP [123,124] at two distinct sites, both of which are capable of regulating channel activity. Zhang et al. [107] demonstrated that TRPC3 bound calcium-CaM at a site that overlapped with the IP$_3$ binding domain. In this study it was shown that TRPC3 activity could be increased by protocols that displaced CaM from TRPC3 and also by calmidazolium. This CaM IP$_3$ binding domain (CIRB) was later shown to be present on other TRPC channels, with CaM binding in a calcium-dependent manner [97]. In the presence of calcium, this binding was inhibitory, although the apparent affinity of TRPC channels for binding differed for each TRPC channel

type. Interestingly, when the CIRB region was deleted from TRPC3 channels, a loss of function was observed that was due to incorrect trafficking of the channel [14].

Although most studies have implicated Ca^{2+} and CaM as inhibitory regulators of TRPC channels, in one study, TRPC6 appeared to require CaM function for positive regulation. Boulay [125] reported that CaM binding to TRPC6 was inhibited by the CaM inhibitors calmidazolium and trifluoperazine. These inhibitors also prevented agonist-activated entry through TRPC6 channels, but not the endogenous store-operated channels. However, in an earlier study, the closely related channel, TRPC3, was shown to be unaffected by the CaM inhibitor calmidazolium [126].

4.5. Phosphorylation of TRPC channels

Despite the existence of multiple potential phosphorylation sites in the primary sequence of all TRPCs, few studies have specifically addressed the role of kinases in TRPC channel activity. Phorbol 12-myristoyl 13-acetate (PMA)-induced activation of PKC has been shown to block OAG-induced activation of ectopically expressed mouse TRPC7 [37] and rat TRPC6 [127], but, curiously, reported to have no effect on human TRPC6 [128]. Another PKC activator, phorbol 12,13-dibutyrate, was reported to significantly reduce spontaneous inward currents mediated by mouse TRPC7 in HEK293 cells [37]. Recent work provided pharmacological evidence that PKC negatively modulates DAG-activation of the human isoform of TRPC3 [47]. The authors showed that in TRPC3-expressing HEK293 cells, activation of PKC with PMA completely abrogated the ability of OAG to activate TRPC3, whereas down-regulation of PMA-sensitive PKC isoforms prevented blockade by the phorbol ester. Similar findings were reported by Venkatachalam et al. [120], who showed that TRPC5 and TRPC4 were also inhibited by phorbol esters. In their studies, exogenous application of OAG, or pharmacological inhibition of DAG metabolism, prevented activation of human TRPC4 and TRPC5 when expressed in HEK293 cells or DT40 B lymphocytes.

Resolution of the temporal interrelation between DAG-induced activation of TRPCs and DAG-dependent activation of PKC upon receptor stimulation, as well as identification of the PKC isoform/s involved, would significantly improve understanding of the physiological relevance of such a mechanism in regulation of these channels. Of note, recent work from Minke's lab indicates that *Drosophila* TRP and TRPL channel activities are antagonized by PKC-dependent phosphorylation, but also phosphatase inhibition by calyculin A (calyA) abolishes channel activation, suggesting that a phosphorylation-dephosphorylation cycle regulates channel activity [129]. As of this writing, there appear to be no studies specifically addressing the role of protein phosphatases in the signaling mechanisms for the mammalian TRPC family.

In contrast to the findings reported for TRPC3, a positive modulatory role of PKC was proposed in TRPC1 regulation [130]. Pharmacological inhibition of PKC or expression of a kinase-defective mutant of PKCα markedly inhibited IP$_3$-induced TRPC1-mediated currents in human endothelial cells. Also, store depletion through either thrombin stimulation or thapsigargin induced a rapid and sustained phosphorylation of TRPC1. Channel phosphorylation required functional PKCα, as it was not observed in cells overexpressing the kinase-defective mutant of the enzyme. Although the data indicated that PKCα activity is required for signaling to TRPC1, the question remains whether the channel itself is the direct substrate for PKCα.

Protein kinase G (PKG) was also reported to have a role in negative regulation of human TRPC3 activity [131]. In vitro analysis of TRPC3 phosphorylation showed that the channel protein, either immunoprecipitated from HEK293 cells transiently expressing TRPC3 or synthesized in vitro, could be phosphorylated by active PKGα1, and that a specific PKG inhibitor abolished this phosphorylation. Point mutations at two of the three potential PKG phosphorylation sites within the TRPC3 sequence (Thr-11 and Ser-263) reduced the phosphorylation signal in the in vitro PKG assays. In the same study, fluorescence monitoring of TRPC3-mediated calcium entry indicated that cGMP inhibited TRPC3-mediated calcium entry in a PKG-dependent fashion, an effect not observed in the TRPC3 mutants. The nature of the assay used in this study makes it likely that only constitutive activity of the channels was assessed. In ^{32}P-labeled platelets, TRPC6 was significantly phosphorylated by PKA and PKG, and associated with other unidentified phosphoproteins; in this study, channel phosphorylation did not appear to affect either thrombin- or OAG-induced TRPC6 activity [132].

The possibility that tyrosine kinases might play a role in regulation of TRPCs has recently received some attention. Hassock et al. [132] reported that in platelets, under conditions that provide widespread tyrosine phosphorylation of cellular proteins, endogenous TRPC6 and TRPC1 did not undergo tyrosine phosphorylation. A more recent report implicated the Src family kinase Fyn in epidermal growth factor (EGF)-dependent regulation of human TRPC6 [133]. In COS-7 cells overexpressing both Fyn and TRPC6, the channel was tyrosine phosphorylated following EGF treatment, and Fyn phosphorylated TRPC6 in an in vitro kinase assay. Fyn and TRPC6 were shown to interact through the SH2 domain of Fyn and the channel N-terminal domain of TRPC6 in a manner independent of channel phosphorylation. In inside-out patches excised from TRPC6-expressing HEK293 cells, Fyn-dependent phosphorylation of TRPC6 increased OAG-induced channel activity. These findings contrast with those from Hassock et al. [132] who observed no tyrosine phosphorylation of endogenous TRPC6 in human platelets.

Thus, despite some contradictory findings, the currently available evidence suggests that protein phosphorylation may

play an important regulatory role in signaling to TRPC channels. A number of kinases have been recently implicated in regulation of TRP channels outside the TRPC family. PKC and PKA modulate TRPV1 activity by direct channel phosphorylation [134], whereas Src is involved in regulation of TRPM7 [135], TRPV1 [136] and TRPV4 [137]. Thus, protein phosphorylation is emerging as a potential critical regulatory element within the signaling mechanisms to TRP superfamily channels in general.

5. Understanding the physiological functions of TRPC channels: future directions

In non-excitable cells, plasma membrane receptors coupled to PLC drive mobilization of Ca^{2+} from ER stores through IP_3-mediated Ca^{2+} release, and Ca^{2+} entry across the plasma membrane through both store-operated (SOC) and/or non-store-operated (non-SOC) Ca^{2+} entry pathways. The multitude of cellular functions and responses that are dependent upon Ca^{2+} entry into the cell (reviewed in Refs. [56,138,139] requires a finely tuned regulation of the Ca^{2+} channels mediating the entry process. Currently, the molecular identity of SOCs and many other regulated channels, especially those regulated by PLC, remains uncertain. However, evidence continues to accrue indicating important roles for members of the TRPC family of cation channels in several different modes of Ca^{2+} entry. As discussed above, and despite the existence of controversial results, each member of the TRPC family has been implicated in both store-operated and store-independent Ca^{2+} entry in different cell lines. Importantly, TRPC channels share the common property of being activated by PLC-linked signals, an early and common event in G-protein-coupled receptor and receptor tyrosine kinase calcium signaling in a variety of physiological situations. Perhaps the most significant example of the potential physiological importance of these channels is as a component of the endogenous nonselective cation channels in smooth muscle cells. In smooth muscle cells, the biophysical properties of the endogenous channels as well as their sensitivity to DAG closely resemble ectopically expressed TRPC6 [50], pointing to TRPC6 as an important player in regulation of vascular tone.

The next era of research on TRPC channels should focus on several issues. First, it is important to resolve the many discrepancies in the literature that impede understanding of the true functions and modes of regulation of these channels. Understanding subtle effects of expression conditions and environment, for example differences in expression level, can help to resolve these inconsistencies. Second, it is important to resolve the current conundrum involving the role of TRPCs in store-operated channels. Although there are clearly examples of store-operated nonselective cation channels for which the TRPCs make excellent candidates, there is also evidence for a role of TRPCs in the more Ca^{2+}-selective SOCs, such as I_{crac}. How

can channels that consistently behave as nonselective cation channels when expressed ectopically form Ca^{2+}-selective channels in situ, or in fact do they? Finally we need to move from the logical first step of understanding the regulation of the channels in expression systems to investigations of their roles in real cells in real physiological systems. To this end, we will need to rely to a greater extent on knock-down strategies such as knock-out animals and RNAi gene silencing. Hopefully in the near future a clearer and more consistent picture of the true functions and regulatory mechanisms for TRPC channels will emerge.

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