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Mechanism and Functional Significance of TRPC Channel Multimerization

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Abstract

Ca^{2+} signaling regulates many important physiological events within a diverse set of living organisms. In particular, sustained Ca^{2+} signals play an important role in controlling cell proliferation, cell differentiation and the activation of immune cells. Two key elements for the generation of sustained Ca^{2+} signals are store-operated and receptor-operated Ca^{2+} channels that are activated downstream of phospholipase C (PLC) stimulation, in response to G-protein-coupled receptor or growth factor receptor stimulation. One goal of this review is to help clarify the role of canonical transient receptor potential (TRPC) proteins in the formation of native store-operated and native receptor-operated channels. Toward that end, data from studies of endogenous TRPC proteins will be reviewed in detail to highlight the strong case for the involvement of certain TRPC proteins in the formation of one subtype of store-operated channel, which exhibits a low Ca^{2+}-selectivity, in contrast to the high Ca^{2+}-selectivity exhibited by the CRAC subtype of store-operated channel. A second goal of this review is to highlight the growing body of evidence indicating that native store-operated and native receptor-operated channels are formed by the heteromultimerization of TRPC subunits. Furthermore, evidence will be provided to argue that some TRPC proteins are able to form multiple channel types.

Keywords

store-operated channels; receptor-operated channels; heteromeric channels; TRPC channels; capacitative calcium entry

1. Introduction

When at rest, mammalian cells maintain their cytosolic Ca^{2+} concentration at ~100 nM, thereby establishing a Ca^{2+} concentration gradient of approximately 10,000-fold across the plasma membrane. Thus, opening a small number of Ca^{2+} channels allows Ca^{2+} to move down a large electrochemical gradient, resulting in a rapid rise in cytosolic Ca^{2+}. The elevated Ca^{2+} can be used to signal a number of important biological events such as cell proliferation, cell differentiation, neurotransmitter release, activation of immune cells and initiation of apoptosis. In general, cells utilize two major classes of Ca^{2+} channels (voltage-gated and non-voltage gated) to initiate Ca^{2+} influx for signaling purposes. By the late 1980s, it was known that cells express multiple subtypes of voltage-gated Ca^{2+} channels (now known as T, L, N, P/Q and R channels), that can be distinguished on the basis of their pharmacology and their biophysical properties (reviewed in [1]). Also, it was known that cells can express at least two major

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subtypes of non-voltage gated Ca^{2+} channels that could be activated downstream of receptors coupled to the phospholipase C (PLC) signaling pathway [2]. One channel subtype, store-operated channels (SOCs), is activated by the depletion of intracellular Ca^{2+} stores [3], while the other channel subtype, receptor-operated channels (ROCs), is activated by second messengers (e.g. diacylglycerol) generated by receptor activation [4]. At a time when the subunit of the skeletal muscle L-type Ca^{2+} channel had been purified, sequenced and the gene cloned [5] [6], little progress had been made toward identifying subunits of the non-voltage gated Ca^{2+} channels. However, this changed dramatically in1989, when the gene for the transient receptor potential (TRP) channel was cloned from *Drosophila* photoreceptors [7], and the TRP proteins were demonstrated to form Ca^{2+} permeable channels [8], that are activated downstream from the G protein-coupled receptor/PLC signaling pathway (reviewed in [9]). This discovery allowed investigators to rapidly identify mammalian homologs of *Drosophila* TRP based on nucleotide sequence homology. This approach eventually identified 28 close and distant relatives of TRP in the mammalian gene data bank. These homologs have been divided into 7 subfamilies (TRPC, TRPV, TRPM, TRPN, TRPA, TRPP and TRPML) of TRP genes.

The TRP proteins that are thought to form channels regulated by PLC signaling are the 7 proteins belonging to the “canonical” or “classic” (TRPC) subfamily, although there are only six TRPC proteins expressed in humans, as human TRPC2 is a pseudogene. These TRPC channel subunits have 3 to 4 ankyrin repeats in the cytosolic N-terminal domain, 6 transmembrane domains, a putative pore region between transmembrane domains 5 and 6, and a conserved TRP domain at the beginning of the cytosolic C-terminal domain. Binding domains for various signaling molecules exist in the N-terminal and C-terminal domains of certain TRPC proteins (reviewed in [10]). TRPC channels are thought to be formed by the combination of 4 TRPC subunits in a similar fashion as observed for voltage-gated K^+ channels.

While there is general agreement within the field that TRPC proteins are regulated downstream of activation of the PLC signaling pathway, the early TRPC literature generated considerable controversy concerning whether individual TRPC proteins form SOCs or ROCs, with much of the conflicting data coming from studies where TRPC proteins were overexpressed. Since it is much easier to investigate overexpressed TRPC proteins, due to the higher Ca^{2+} entry, higher ion currents and more abundant protein for biochemical studies, overexpression studies dominated the early TRPC literature. However, when techniques allowed, a growing number of laboratories shifted to the investigation of whether endogenous TRPC proteins can form native SOCs and ROCs. One goal of this review is to summarize the recent body of evidence that now strongly supports the involvement of endogenous TRPC proteins in forming native SOCs and ROCs. In discussing this issue, it will be useful to divide the discussion of TRPC channel function into two parts, one covering results from overexpression studies and one covering results from studies of native TRPC channels. The other main goal of this review is to outline the body of evidence indicating that native SOCs and ROCs are formed by the heteromultimerization of TRPC subunits. In addition, heteromultimerization will be discussed as a possible explanation for why early TRPC overexpression studies created such confusion as to the role of TRPC proteins in forming native SOCs and ROCs.

2. Studies of overexpressed TRPC proteins

As there is insufficient room to discuss all 7 TRPC homologs, this review will focus on the 3 TRPC homologs (TRPC1, TRPC3 and TRPC4) most frequently mentioned as candidates for SOC subunits. References for studies of other TRPC homologs are provided in Table 1.

TRPC1

Early TRPC1 overexpression studies provided mixed results in terms of evaluating whether TRPC1 can form SOCs in recipient cells. Initial expression of TRPC1A in CHO cells resulted in a significant increase in the thapsigargin-stimulated current via a non-selective cation channel [11], while a subsequent expression of TRPC1 in Sf9 insect cells produced an elevated basal Ca^{2+} influx, but no effect on the thapsigargin-stimulated current [12]. However, in later studies, the ability of TRPC1 to form SOCs was demonstrated on a consistent basis. For example, overexpressing TRPC1 in a human submandibular gland cell line (HSG) [13], TRPC1 in intestinal epithelial cells [14] and TRPC1 in human glomerular mesangial cells [15] increased store-operated Ca^{2+} entry following depletion of intracellular Ca^{2+} stores. Furthermore, overexpressing TRPC1 in superior cervical ganglion neurons [16], mouse TRPC1 in HEK-293 cells [17], human TRPC1 in HEK-293 cells [18], and human TRPC1 in human microvascular endothelial cells [19] increased store-operated currents following Ca^{2+} store depletion. When I-V curves were reported, they indicated that the currents observed were via non-selective cation channels [16,18]. Thus, there now exist a significant number of overexpression studies that point to TRPC1 as a likely subunit of SOCs.

TRPC3

Numerous heterologous expressions studies with TRPC3 have been performed and the evidence from these studies clearly favors overexpressed TRPC3 forming ROCs, rather than SOCs. While the initial report on TRPC3 overexpression in HEK-293 cells suggested that TRPC3 formed a SOC [20], a subsequent paper by this group reported that TRPC3 expression enhanced basal Ca^{2+} entry, and when this was taken into account, there was no enhanced store-operated Ca^{2+} entry observed in cells overexpressing TRPC3 [21]. However, another early study supported a role for TRPC3 in the formation of SOCs by showing that the overexpression of rat TRPC3 in COS-1 cells increased the thapsigargin-stimulated Ca^{2+} entry [22]. Likewise, in HEK-293 cells stably expressing TRPC3, channel activity from cell attached patches could be stimulated by the addition of thapsigargin to the cells. Also, addition of $InsP_3$ to inside-out excised patches stimulated channel activity [23] leading the investigators to argue that TRPC3 channels are store operated via a mechanism involving direct interaction between the TRPC3 channels and the IP_3 receptors, a concept that was supported by data in a subsequent paper from this group [24].

In opposition to the two early studies supporting a role for TRPC3 in the formation of SOCs, a string of papers came out indicating that overexpressed TRPC3 formed ROCs, rather than SOCs [25-31]. In general, TRPC3 was observed to form a constitutively active channel that is further enhanced by agonist stimulation, or by the addition of 1-oleoyl-2-acetyl-sn-glycerol (OAG), a diacylglycerol analog, but not by treatment with thapsigargin.

A possible explanation for the inconsistencies in the TRPC3 overexpression data was provided by a study which demonstrated that the type of channel formed by TRPC3 depends on its level of overexpression. When expressed at a low level, TRPC3 formed SOCs in DT40 cells, while at a higher expression level TRPC3 formed ROCs [32]. These findings suggested that some protein in the recipient cell must combine with TRPC3 in order to make functional SOCs, and that the abundance of this protein must be the limiting factor in determining either the number of SOCs made, or the number of channels activated. This limiting factor could be a regulatory protein, as seen in recent studies for the highly Ca^{2+} selective I_{CRAC} subtype of SOCs, where the ER Ca^{2+} sensor STIM1 [33,34] must be co-expressed with Orai1, the CRAC channel protein [35,36], to see a significant increase in CRAC current [37-39]. On the other hand, TRPC3 may need to combine with other SOC channel subunits to form the subtype of SOC that exhibits low Ca^{2+} selectivity, and these subunits may be the limiting factor when TRPC3 is overexpressed at high levels. Evidence for the heteromeric combination of TRPC proteins

in forming a subtype of SOCs that has a much lower Ca^{2+}-selectivity than CRAC channels will be discussed in a later section.

TRPC4

When a bovine TRPC4 (initially called bCCE) was cloned and expressed in HEK-293 cells, store-operated currents were observed to increase dramatically [40]. The I-V curve was inwardly rectifying with significant outward current at membrane potentials above +10 mV. In a later paper, the expression of rat TRPC4 in Xenopus oocytes resulted in an increased chloride current, which is known to be induced by store-operated Ca^{2+} entry [41]. While these early findings were supported by later studies showing that endogenous TRPC4 can form native SOCs (discussed in the next section), a number of subsequent papers on overexpressed TRPC4 failed to show a link between TRPC4 and SOC activity, but rather demonstrated that overexpressed TRPC4 formed basally active, or receptor-stimulated, channels [29,42-44].

3. Studies of endogenous TRPC proteins

Evidence for endogenous TRPC protein participation in the formation of native SOCs is reviewed here, as well as some evidence that certain TRPC proteins can participate in the formation of more than one channel type.

TRPC1

Two early studies pointed to the participation of endogenous TRPC1 in the formation of native SOCs. In both human submandibular gland cells (HSG) [13] and in HEK-293 cells [45], the expression of antisense to TRPC1 inhibited the native thapsigargin-induced Ca^{2+} entry. A subsequent paper from a third laboratory reported that addition of an antibody specific for an external domain of TRPC1 could inhibit thapsigargin-stimulated Ca^{2+} entry in arterial smooth muscle cells [46]. It was later demonstrated that suppression of TRPC1 levels also could inhibit a store-operated current in a lung epithelial carcinoma cell line (A549). In control cells, thapsigargin-stimulated a current that was slightly inward rectifying and had a reversal potential of +40 mV (in a bath solution containing 10 mM Ca^{2+} and 120 mM TEA Asp and a pipette solution containing 130 mM NMDG Methylsulfonate) and this current was reduced by 50% when antisense to TRPC1 was expressed [47]. A series of subsequent papers supported these early findings by reporting that inhibition of SOC activity could be observed following suppression of endogenous TRPC1 levels, using antisense or siRNA methods, or by adding antibodies targeting external domains of TRPC1 [14,15,48-58].

The fact that 14 different laboratories have produced evidence for a role of endogenous TRPC1 in forming native SOCs in submandibular gland ductal cells, arterial smooth muscle, lung epithelial, DT-40 cells, pulmonary artery smooth muscle, human platelets, cultured hippocampal neurons, parotid gland ductal cells, mouse astrocytes, keratinocytes, glomerular mesangial cells, and intestinal epithelial cells presents a very strong case that endogenous TRPC1 is a subunit of at least one subtype of native SOCs. In those studies where store-operated currents were measured, the data generally agree that TRPC1 participates in forming a store-operated cation channel, with low Ca^{2+} selectivity, that is distinctly different from the highly Ca^{2+}-selective CRAC channel. The one exception comes from the studies in A549 cells [47], in which the +40 mV reversal potential suggests a high Ca^{2+} selectivity. However, those studies were performed under conditions where Ca^{2+} was the only relatively permeant cation in the bath and pipette solutions.

TRPC1 also has been implicated in the formation of a channel linked to stretch activation, as TRPC1 was identified as the protein responsible for the mechanosensitive cation channel (MscCa) in frog oocytes. A protein purification and liposome reconstitution screen identified

an 80 kDa protein that could reconstitute the channel activity, and this protein was identified by immunological techniques as TRPC1 [59]. Overexpression of human TRPC1 in CHO-K1 cells greatly increased the MscCa activity in recipient cells.

The finding that TRPC1 can participate in forming both SOCs and mechanosensitive channels suggests that either TRPC1 is a subunit for multiple channel types or that a single channel type formed by TRPC1 can be regulated by at least 2 separate mechanisms.

TRPC3

The initial study [60] to implicate endogenous TRPC3 as a mediator of native SOC activity was performed in endothelial cells from human umbilical vein (HUVEC). Depletion of intracellular Ca^{2+} stores, by the presence of $InsP_3$ in the patch pipette, was observed to induce a non-selective cation current that was slightly inward rectifying and carried a significant positive current at membrane potentials above +5 mV, characteristics that make it distinctively different from I_{CRAC} currents. This current could be dramatically inhibited by transfecting the HUVEC cells with a dominant negative mutant of TRPC3 (N-terminal domain, 1-302). Support for the involvement of endogenous TRPC3 in the formation of native SOCs was soon provided by a study in HEK-293 cells, where the expression of a TRPC3 antisense construct produced a significant reduction in thapsigargin-stimulated Ba^{2+} entry [45]. A subsequent report from the same laboratory showed that the expression of siRNA targeting TRPC3 could more efficiently suppress endogenous TRPC3 proteins levels, which in turn produced a more dramatic inhibition of thapsigargin-stimulated Ba^{2+} entry [54], as well as, a reduction of a store-operated current that has an I-V curve similar to that reported in HUVECs (with slightly more positive reversal potential, +15 mV) (Villereal, unpublished observations).

The next piece of evidence to support a role for endogenous TRPC3 in mediating native SOC activity came from studies in a rat osteoblastic-like cell line (ROS 17/2.8 cells), where it was demonstrated that intranuclear microinjection of TRPC3 antisense oligodeoxynucleotides could inhibit the rate and extent of thapsigargin-induced Mn^{2+} entry as a measure of SOC activity [61]. This group published a similar study a year later implicating endogenous TRPC3 in mediating native SOC activity in skeletal muscle cells [62].

Studies in H19-7 cells, a culture model for differentiating hippocampal neurons, presented a strong case for the involvement of endogenous TRPC3 in mediating SOC activity in cultured neurons [51]. It was observed that non-differentiated H19-7 cells express high levels of TRPC4 and TRPC7 and low levels of TRPC1 and TRPC3, but when placed under differentiating conditions, the levels of TRPC4 and TRPC7 drop dramatically, while those of TRPC1 and TRPC3 increase dramatically. Also, the thapsigargin-stimulated Ba^{2+} entry in H19-7 cells increases 3.5-fold under differentiating conditions [51], which correlates well with a dramatic increase in a store-operated current that has an I-V curve similar to the one reported in HUVEC cells (Villereal, unpublished observations). Expression of siRNA specifically targeting TRPC3 inhibits the native SOC activity in undifferentiated H19-7 cells and blocks the up-regulation of the native SOC activity in differentiating H19-7 cells [51].

A paper from another lab described an increased expression of TRPC3 in monocytes from spontaneously hypertensive rats, versus monocytes from normotensive rats, that correlated with an elevated thapsigargin-induced Ca^{2+} entry [63]. Expression of siRNA was found to significantly reduce both TRPC3 protein levels and thapsigargin-stimulated Ca^{2+} entry. Another study [53] investigated the role of TRPC3 in mediating SOC activity in parotid gland ductal cells (HSY), where previous studies had reported that Ca^{2+} store depletion initiates a non-selective cation current that is distinct from I_{CRAC} [64]. It was observed that expression of antisense to TRPC3 reduced both the endogenous TRPC3 protein levels and the native thapsigargin-stimulated current in HSY cells. An additional study in rat prostate smooth muscle

cells (PS1), demonstrated that expression of TRPC3 antisense could suppress the thapsigargin-activated Ca^{2+} entry [65].

The fact that 6 different laboratories have produced evidence for a role of endogenous TRPC3 in forming native SOCs in human umbilical vascular endothelial cells, HEK-293 cells, osteoblastic-like cells, skeletal muscle, hippocampal neurons, monocytes, parotid gland ductal cells, and prostate smooth muscle cells presents a very convincing argument that endogenous TRPC3 is a subunit of a native subtype of SOC. In those studies where store-operated currents were measured, the data argue that TRPC3 participates in forming a store-operated, non-selective cation channel that is distinctly different from the highly Ca^{2+} -selective CRAC channel.

Consistent with many of the TRPC3 overexpression studies, there also is evidence that endogenous TRPC3 proteins can participate in the formation of channels other than SOCs. In HEK-293 cells, the suppression of TRPC3 protein by expression of TRPC3 antisense or siRNA, inhibits an OAG-stimulated Ca^{2+} entry pathway, in addition to inhibiting the store-operated Ca^{2+} entry pathway [45,54,66]. The SOC and OAG-stimulated pathways appear to be separate channel types in HEK-293 cells, as suppression of TRPC1 protein levels by siRNA methods inhibits the thapsigargin-stimulated pathway, but has no effect on the OAG-stimulated pathway [54], and the thapsigargin-stimulated pathway is inhibited by 2-APB whereas the OAG-stimulated pathway is not [66]. In contrast, in HSY cells the TRPC3-dependent, thapsigargin-stimulated and OAG-stimulated pathways appear to be linked, as both thapsigargin-stimulated and OAG-stimulated currents are inhibited by suppressing TRPC1 proteins levels [53]. TRPC3 also was shown to be involved in both the thapsigargin-stimulated and the OAG-stimulated pathways in prostate smooth muscle cells, but that action appears to be via two separate pathways, as TRPC6 was required for the OAG-stimulated pathway, but not for the thapsigargin-stimulated pathway [65].

The finding that endogenous TRPC3 can participate in forming both native store-operated and native OAG-stimulated pathways indicate that it can participate in the formation of multiple channel types in the same cell population.

TRPC4

The initial studies of endogenous TRPC4 implicated this TRPC homolog in the formation of native SOCs in bovine adrenal cortex cells, since store-operated currents are suppressed by the expression of antisense targeting TRPC4 [67]. These findings differ from those discussed above for TRPC1 and TRPC3, as the store-operated current suppressed had an I-V curve similar to that for the highly Ca^{2+} selective CRAC channel. Subsequent studies indicated that in endothelial cells from $TRPC4^{-/-}$ mice the SOC activity was virtually absent [68]. The view that TRPC4 participates in forming SOCs was further substantiated by studies in mouse mesangial cells, where expression of TRPC4 antisense led to a large reduction in thapsigargin-stimulated Ca^{2+} entry [69], and in corneal epithelial cells, where expression of siRNA targeting TRPC4 inhibited cyclopiazonic acid-stimulated Ca^{2+} entry [70].

While there is ample evidence for the involvement of TRPC4 in forming store-operated channels, there is also good evidence that endogenous TRPC4 is used by some cells to form native channels other than SOCs. In HEK-293 cells, endogenous TRPC4 is not involved in mediating SOC activity [54,66], but appears to be involved in forming two types of ROCs. Knockdown of TRPC4 levels by antisense methods reduces the OAG-stimulated Ca^{2+} entry pathway, as well as the arachidonic acid-stimulated Ca^{2+} entry pathway [66]. These appear to be two separate pathways, as the OAG-stimulated pathway is inhibited by the knock down of TRPC3 levels, while the arachidonic acid stimulated pathway is not affected by knocking down TRPC3 levels [66]. TRPC4 is also not involved in forming SOCs in H19-7 cells, as suppression

of TRPC4 via siRNA methods has no effect on SOC activity in non-differentiated cells, or on the up-regulation of SOC activity in differentiating cells [51]. Also, in differentiating H19-7 cells, TRPC4 protein levels are dropping dramatically at a time when SOC activity is increasing by 3.5 fold [51]. In antral myocytes the addition of TRPC4 antibodies to the pipette solution led to a dramatic reduction of the CCh-stimulated current [71] which had an I-V curve more closely resembling the I-V relationships for the receptor-operated currents in cells overexpressing TRPC4, than the CRAC-like store-operated current discussed above. Additionally, in pulmonary aortic endothelial cells (PAEC), the expression of a dominant-negative TRPC4 mutant inhibits the native redox-sensitive cation conductance in PAECs, and the expression of an extracellularly HA-tagged TRPC4 makes the native redox-sensitive cation conductance become sensitive to inhibition by an anti-HA-antibody [72].

Thus, there is a considerable amount of evidence to support the involvement of endogenous TRPC4 in the formation of several different types of channels.

4. Possible reasons for conflicting results between TRPC overexpression and endogenous TRPC studies

Since all of the TRPC homologs have splice variants, it is possible that in some cases the form of the expressed TRPC homolog may differ from that utilized to make the native channel. To date, antisense and siRNA studies have not utilized a wide range of constructs that would allow one to test for the involvement of each different TRPC splice variant in the formation of native channels. However, the importance of collecting such information is indicated by a recent report demonstrating that the overexpression of TRPC3a (an elongated splice variant of TRPC3), but not TRPC3 itself, produces SOCs when overexpressed in HEK-293 cells [73].

The role of genetic variation between different recipient cells used in overexpression studies also needs to be considered in more depth. Virtually all mammalian cell types have some level of SOC and ROC activity, and therefore express some concentration of channel subunits and channel regulatory proteins. Do CHO and HEK-293 cells have comparable levels of these proteins, and for that matter, do the same cell lines grown in different laboratories have exactly the same genetic background? For example, the simple question of which TRPC homologs are expressed in HEK-293 cells does not elicit a uniform answer in papers from various labs [45, 54,74,75]. Similarly, in two early reports in HEK-293 only the I_{CRAC} subtype of SOCs was described [76,77], while in later studies multiple subtypes of SOCs in HEK-293 cells were recorded ([78] and Villereal, unpublished observations). Thus, it is important for investigators to know the TRPC protein expression profile of the recipient cells grown in their lab for use in overexpression studies. Given the recent evidence that heteromeric combinations of TRPC proteins may be involved in formation of certain channel types (see next section), it is important to know which endogenous TRPC proteins are present and potentially could assemble with the overexpressed TRPC homolog. For studies of the function of endogenous TRPC proteins, it is important to know both the TRPC expression profile and the store-operated and receptor-operated channel profile in the cells being utilized, as the knock down of TRPC homologs with siRNA may inhibit only one subtype of SOC (e.g., non-selective cation channel versus CRAC channel) or ROC (diacylglycerol-stimulated versus arachidonic acid-stimulated) present in the cells.

5. Evidence for the formation of heteromeric TRPC channels

The initial evidence that heteromeric combinations of TRP channel proteins could occur came from two studies in which two *Drosophila* TRP proteins were co-expressed. The first study showed that co-expression of TRP and TRPL in Xenopus oocytes produced a thapsigargin-stimulated current that was not observed in oocytes expressing either TRP or TRPL alone

[112], while the second study showed that TRP and TRPL could be co-immunoprecipitated from *Drosophila* photoreceptor cells, or from 293T cells, when they are co-expressed [113]. The co-expression of TRP and TRPL in 293T cells also produced a novel current not present in either TRP-expressing of TRPL-expressing cells.

The first reported heteromeric assembly of mammalian TRPC proteins was demonstrated by expressing TRPC1 and TRPC3 in 293T cells and showing that they could be co-immunoprecipitated [113]. In a later study, HEK-293 cells co-expressing TRPC1 and TRPC3 had channels that were unique, in terms of biophysical properties and mode of regulation, from the channels formed by the expression of either TRPC1 or TRPC3 alone [114]. In the absence of extracellular Ca^{2+}, these channels are constitutively active and can be further stimulated with diacylglycerol. However, since diacylglycerol does not stimulate a current in the presence of external Ca^{2+}, it is not clear how these channels are regulated under normal physiological conditions. A subsequent study identified a heteromeric complex of TRPC1 and TRPC5 in brain and showed that overexpression of TRPC1 and TRPC5 in HEK-293-M1 cells produced a unique non-selective cation conductance that was activated by CCh, but not by thapsigargin [88].

Subsequently, two studies were published that investigated in a systematic way the combinatory rules for TRPC channel subunit assembly. In the first study, several approaches, including FRET between two TRPC proteins labeled with either CFP or YFP, were utilized. These studies indicated that TRPC2 does not interact with other TRPC subunits, that TRPC1 can interact with TRPC4 or TRPC5, and that TRPC3/6/7 subfamily members only interact with each other [115]. Similar findings were published from a study investigating the assembly of TRPC homologs that were co-expressed in Sf9 insect cells, using co-immunoprecipitation to determine whether two TRPC homologs assemble into a channel complex. Results from those studies, and from co-immunoprecipitation studies in rat brain synaptosomal preparations [116], supported the general rules for TRPC subunit assembly developed in the earlier paper. However, as frequently occurs in biology, the rules for assembling heteromeric TRPC channels turned out to be more complex than initially reported. A systematic investigation of the assembly of TRPC channels in mammalian brain identified a novel combination of TRPC subunits which occurred in embryonic, but not adult, brain [117]. These channels were made of TRPC1 + (TRPC4 or TRPC5) + (TRPC3 or TRPC6). Thus, it appears that the rules developed for assembly of different TRPC proteins by co-expressing two TRPC proteins at a time may break down when more than two different TRPC channel subunits are expressed in the same cell.

6. Role of heteromeric TRPC assembly in the formation of native store-operated channels

Findings from several recent investigations of the subunit composition of native SOCs have converged to provide strong evidence for the participation of endogenous TRPC1 and TRPC3 in the formation of heteromeric native SOCs. As mentioned earlier, in H19-7 cells TRPC1, TRPC3 and store-operated Ca^{2+} entry are up-regulated in parallel during differentiation. The suppression of either TRPC1 or TRPC3 proteins levels by siRNA methods inhibits store-operated Ca^{2+} entry in undifferentiated cells, and blocks the up-regulation of SOC activity in differentiating H19-7 cells [51]. The co-suppression of both TRPC1 and TRPC3 protein levels gives no additional inhibition of SOC activity above that seen for suppression of either TRPC1 or TRPC3 alone, suggesting that TRPC1 and TRPC3 work in conjunction to mediate SOC activity. The demonstration that TRPC1 and TRPC3 can be co-immunoprecipitated from H19-7 cells further supports the notion of a heteromeric channel.

A subsequent paper in HSY cells supported the concept that endogenous TRPC1 and TRPC3 function as subunits of a heteromeric native SOC [53]. The addition of thapsigargin to HSY cells in whole cell voltage clamp mode stimulated a current with a linear I-V curve and a reversal potential of +5 mV. The suppression of either TRPC1 or TRPC3 by antisense methods decreased the thapsigargin-stimulated current and it was demonstrated that the immunoprecipitation of TRPC1 pulled down TRPC3, but not TRPC4. Conversely, the immunoprecipitation of TRPC3 brought down TRPC1.

Finally, a systematic investigation of the role of endogenous TRPC proteins in mediating the native SOC activity in HEK-293 cells indicated that the suppression of either TRPC1, TRPC3 or TRPC7 protein levels, via siRNA methods, inhibited SOC activity by comparable amounts. Furthermore, if multiple siRNA were expressed to co-suppress all three TRPC proteins, little additional inhibition was observed over that which occurred when TRPC1 alone was suppressed [54]. TRPC1, TRPC3 and TRPC7, but not TRPC4, could be co-immunoprecipitated from HEK-293 cells, using anti-TRPC1 antibodies. These data argue that TRPC1, TRPC3 and TRPC7 combine to form a heteromeric SOC in HEK-293 cells. While the potential role of TRPC7 was not tested in HSY cells, studies in H19-7 cells showed that suppression of TRPC7 protein had no effect on SOC activity, suggesting that, even though the SOCs in HEK-293 cells and H19-7 cells both are formed from TRPC1 and TRPC3, there is likely to be some differences in biophysical properties of their native SOCs, due to the participation of TRPC7 in forming native SOCs in HEK-293 cells.

Thus, in three different cell systems, there is strong evidence that TRPC1 and TRPC3 assemble into a heteromeric channel which mediates SOC activity. In HSY cells, the I-V curve is consistent with a store-operated current that represents a non-selective cation conductance, rather than the highly Ca^{2+}-selective CRAC current [53], a finding which is consistent with our results in HEK-293 cells and H19-7 cells (Villereal, unpublished observations).

The above findings raise an important question concerning the TRPC1 and TRPC3 overexpression studies in the literature. If both TRPC1 and TRPC3 are needed to form SOCs in three different cell systems, then why does overexpression of TRPC1 consistently result in enhanced SOC activity in recipient cells, while overexpression of TRPC3 usually gives an increased ROC activity? One simple answer would be that TRPC1 may be able to form a homomeric channel that can be regulated via store depletion, but has different properties than the native SOC subtype formed from TRPC1 and TRPC3. An important lesson from the voltage-gated Ca^{2+} channel field is that at least 5 subtypes of voltage-gated Ca^{2+} channels exist which have different biophysical and pharmacological properties, so one should not be surprised if there turns out to be a range of subtypes of SOCs. A second possibility is that in some cell types, endogenous TRPC3 proteins may be more abundantly expressed than endogenous TRPC1 proteins, which is certainly the case in our HEK-293 cells, and therefore overexpression of TRPC1 would be expected to increase the number of TRPC1/TRPC3 complexes, while overexpression of TRPC3 would not, but would instead lead to the production of homomeric TRPC3 channels that are receptor-operated.

7. Role of heteromeric TRPC assembly in the formation of other channel types

There are a growing number of papers which support the heteromeric combination of TRPC subunits in the formation of channel types other than SOCs. For example, in a rat prostate smooth muscle cell line, 1-adrenergic receptor agonists stimulate Ca^{2+} entry via a pathway that is inhibited (70-80%) by the suppression of either TRPC3 or TRPC6. Although the dual suppression of TRPC3 and TRPC6 was not tested, the 70-80% inhibition by the suppression of a single TRPC protein makes it highly unlikely that there are two parallel pathways with

additive Ca^{2+} entry, suggesting that TRPC3 and TRPC6 are combining to form a single Ca^{2+} entry pathway.

In HEK-293 cells, it appears that endogenous TRPC3 and TRPC7 participate in forming an OAG-stimulated channel, in addition to forming SOCs. The suppression of either TRPC3 or TRPC7 protein levels, but not TRPC1 levels, inhibits the OAG-stimulated Ba^{2+} entry by approximately 70% [54]. Additional siRNA evidence supports a channel subunit composition of TRPC3/ TRPC4/ TRPC6/ TRPC7 for this OAG-stimulated Ca^{2+} entry pathway in HEK-293 cells [54].

A recent paper demonstrated that TRPC3 and TRPC4 could be co-immunoprecipitated from pulmonary aortic endothelial cells, and that this co-immunoprecipitation could be reproduced in HEK-293 cells overexpressing TRPC3 and TRPC4 [72]. FRET experiments indicated a close proximity between the N-terminus of TRPC4 and the C-terminus of TRPC3. This observation differs from earlier FRET studies which indicated no interaction between TRPC3 and TRPC4, however those studies differed in that they were performed with both TRPC homologs labeled with CFP or YFP at the C-terminus [115]. In the more recent report, if TRPC3 and TRPC4 are both labeled at the C-terminus, or both labeled at the N-terminus, no FRET between the two channel subunits was observed [72]. Additional evidence for a heteromeric channel was provided by the observation that overexpression of TRPC3 with TRPC4 in HEK-293 cells produced a channel that is distinct in its properties from channels produced in either TRPC3-expressing, or TRPC4-expressing, cells. Finally, expression of an externally HA tagged TRPC4 mutant, in PAECs was found to transfer sensitivity to an anti-HA antibody to the native redox-sensitive cation conductance. When taken together, this evidence strongly supports the contention that TRPC3 and TRPC4 form a heteromeric redox-sensitive channel in PAECs.

In vascular smooth muscle cells, it was recently shown that sphingosine-1-phosphate (S1P) stimulates a cation conductance that appears to be mediated by a channel formed from the heteromultimerization of TRPC1 and TRPC5 [118]. TRPC1 and TRPC5 could be co-immunoprecipitated from saphenous vein samples, and the overexpression of TRPC1 and TRPC5 in HEK-293 cells produced a S1P-induced current which was not seen in cells overexpressing TRPC1. This current also could be biophysically distinguished from the current induced in cells overexpressing TRPC5 alone.

Another recent paper explored the molecular identity of ROCs in vascular smooth muscle (VSM) cells. It was found that when TRPC6 was immunoprecipitated using anti-TRPC6 antibodies, TRPC7 , but not TRPC4, was pulled down in the same complex, [104]. Expression of a dominant negative TRPC6 pore mutant was observed to inhibit the native ROC activity in VSM cells. A comparison of the Ca^{2+} sensitivity of native ROC activity in VSM cells to that of ROC activity in HEK-293 cells expressing either TRPC6, TRPC7 or TRPC6 + TRPC7 indicated that the Ca^{2+} response of the heteromeric TRPC6/TRPC7 channel more closely mimicked that of the native ROC in VSM cells. These combined biochemical and electrophysiological data suggest that a heteromeric TRPC6/TRPC7 channel is responsible for native ROC activity in VSM cells.

8. Heteromultimerization of other TRP proteins

TRPC family members are not the only TRP proteins which heteromultimerize, as some members of the TRPV family have also been reported to assemble into heterotetrameric channels. Similar to the study described earlier for TRPC proteins [115], an exhaustive analysis of the homo- and heteromultimerization of TRPV proteins was performed by labeling TRPV homologs, each with a different color variant of GFP (CFP and YFP), and looking for FRET between the same or different TRPV homologs [119]. It was reported that, except for TRPV5

and TRPV6, TRPV proteins prefer to assemble into homomeric channels. This preference for TRPV proteins to form homomeric channels may explain why the TRPV field has progressed so much faster than the TRPC field toward matching currents from overexpressed TRPV channels to those from native TRPV channels.

While a weak interaction was seen to occur between TRPV1 and TRPV2, the formation of homomeric TRPV1 and homomeric TRPV2 channels was preferred, when these two TRPV homologs were co-expressed. The observation of strong FRET efficiency between TRPV5 and TRPV6 is consistent with an earlier finding that co-expressed TRPV5 and TRPV6 can be co-immunoprecipitated from oocytes [120]. Additional evidence in that paper supports the formation of heterotetrameric channels of TRPV5 and TRPV6, with the biophysical properties of the formed channel being influenced by the ratio of TRPV6 to TRPV5 expressed. This suggests that a variety of native channels with a range of biophysical properties could exist by combining the endogenous TRPV5 and TRPV6 in various ratios.

9. Mechanism for the homomeric and heteromeric assembly of TRP channels

There have been only a few studies of the mechanism by which TRPC proteins interact within either homomeric or heteromeric TRPC channels. In the initial study, yeast two-hybrid assays were used to investigate the sites of homomultimerization of TRPC1 [17], and it was found that only the N-terminal domain of TRPC1 tested positive as a potential site to mediate TRPC1 homomultimerization. TRPC1 N-terminal domain mutants, in which the coil-coil domain or the ankyrin repeats were deleted, were tested for hybridization. It appears that the site of interaction for TRPC1 homomultimerization is the N-terminal coil-coil domains of adjacent TRPC1 subunits.

The mechanism for the co-assembly of TRPC1 and TRPC3 into heteromeric SOCs in HSY cells also was investigated utilizing a yeast two hybrid approach [53]. This assay identified interactions between the N-terminus of TRPC1 and the N-terminus of TRPC3 (NTRPC1 and NTRPC3), but not between NTRPC1 and CTRPC3, NTRPC3 and CTRPC1, or CTRPC1 and CTRPC3. Interactions observed in yeast two hybrid assays were confirmed in GST pull-down experiments where GST-NTRPC3 was observed to pull down full length HA-TRPC1 from extracts of HEK-293 cells expressing HA-TRPC1, and GST-NTRPC1 was observed to pull down full length HA-TRPC3 from extracts of HEK-293 cells expressing HA-TRPC3.

Another recent study investigated the domains that influence the homomeric assembly of TRPC4 channels [121]. The authors chose TRPC4 and TRPC6 as two subunits that are predicted not to interact, and then confirmed the predicted non-interaction by co-expressing TRPC4 and TRPC6 in HEK293T cells and finding that they do not co-immunoprecipitate. They then generated chimeras of TRPC6 by replacing certain domains with their corresponding domains from TRPC4. Each chimera was then co-expressed with TRPC4 and a determination made of whether it would co-immunoprecipitate with TRPC4. Chimeras of TRPC6 which contained the ankyrin repeats and coil-coil domains from TRPC4, but not chimeras containing only the ankyrin repeats or only the coil-coil domain of TRPC4, were found to co-immunoprecipitate with TRPC4. A second domain of interaction which involved the pore region and the C-terminal tail of TRPC4 also was demonstrated. Additional experiments were performed to investigate the sites for TRPC6 interaction within homomeric TRPC6 channels, and these studies showed that the N-terminus of TRPC6 interacts with the C-terminus of the neighboring TRPC6 upon homomultimerization.

The findings that certain TRPC proteins interact through their N-terminals to form homomeric and heteromeric channels is consistent with earlier findings in the TRPV field which indicated that both TRPV5 and TRPV6 interact via their N-terminal ankyrin repeat domains when they form homomeric TRPV5 or TRPV6 channels [122][123].

10. Concluding remarks

A recent shift of emphasis away from studying overexpressed TRPC proteins and toward studying endogenous TRPC proteins has resulted in a growing body of evidence indicating that a wide range of TRPC channels can be made via heteromeric combinations of TRPC proteins. The results from the investigation of endogenous TRPC proteins has solidified the evidence in favor of TRPC1 and TRPC3 being involved in the formation of store-operated channels, and has demonstrated that in some cell systems these two proteins assemble together to form native heteromeric SOCs. Studies of endogenously expressed TRPC proteins have also demonstrated that a given TRPC homolog (e.g. TRPC3) can be involved in the formation of multiple native channel types. Thus, it appears that through heteromultimerization of the 7 TRPC proteins, cells can assemble a wide range of channel types.

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Table 1

TRPC protein function in overexpressed channels versus native channels

Gene	Modes of activation	Overexpressed TRPC	Endogenous TRPC
TRPC1	Store-operated	[11,13-19]	[13-15,45-58]
	Stretch-activated	[59]	[59]
	Constitutively active	[12]	
TRPC2	Store-operated	[79]	[82]
	Receptor-operated	[80]	[83]
	Redox-sensitive	[81]	
TRPC3	Store-operated	[22-24,32]	[45,51,53,54,60-63,65]
	Receptor-operated	[21,25-32]	[45,54,65,66]
	Redox-sensitive	[72]	[72]
TRPC4	Store-operated	[40,41]	[56,67-70]
	Receptor-operated	[29,42-44]	[51,54,66,71]
	Redox-sensitive	[72]	[72]
TRPC5	Store-operated	[84-85]	[86]
	Receptor-operated	[42,85,87-91]	
	Lysophospholipid-stimulated	[92]	
TRPC6	Store-operated		[93]
	Receptor-operated	[27,94-100]	[52,65,101-105]
TRPC7	Store-operated	[106,107]	[54]
	Receptor-operated	[99,107-109]	[54,110,111]