The cellular and molecular basis of store-operated calcium entry

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The impact of calcium signalling on so many areas of cell biology reflects the crucial role of calcium signals in the control of diverse cellular functions. Despite the precision with which spatial and temporal details of calcium signals have been resolved, a fundamental aspect of the generation of calcium signals — the activation of 'store-operated channels' (SOCs) — remains a molecular and mechanistic mystery. Here we review new insights into the exchange of signals between the endoplasmic reticulum (ER) and plasma membrane that result in activation of calcium entry channels mediating crucial long-term calcium signals.

Calcium signals control a vast array of cellular functions ranging from short-term responses, such as contraction and secretion, to longer-term control of transcription, cell division and cell death[1,2]. In most non-excitable cells, the generation of receptor-induced cytosolic calcium signals is complex and involves two interdependent and closely coupled components: the rapid, transient release of calcium from stores in the ER and then slow and sustained entry of extracellular calcium[2–12]. Through the activation of phospholipase C (PLC) subtypes, G protein-coupled receptors and tyrosine kinase-coupled receptors generate the second messengers inositol 1,4,5-trisphosphate ($InsP_3$) and diacylglycerol (DAG). The former functions as a chemical message that diffuses rapidly within the cytosol and interacts with $InsP_3$ receptors ($InsP_3R$) located on the ER, which function as calcium channels to release calcium stored in the ER lumen and generate the initial calcium signal phase[1,2,4]. The resulting depletion of calcium stored within the ER lumen functions as the primary trigger for a message that is returned to the plasma membrane (PM), resulting in the relatively slow activation of SOCs, which allow entry of external calcium [3,5,8,10–14]. This sustained calcium entry phase mediates longer term cytosolic calcium signals and provides a means to replenish intracellular stores[3,8,9,11,15]. The other product of PLC activation, DAG, also has important effects on calcium entry channels[16–18]. Although the molecular identity of SOCs has not been determined, certain members of the TRP family of cation channels[19] display properties intriguingly similar to SOCs[17,18,20–23]. Two prevailing coupling scenarios to activate SOCs, involving either a diffusible chemical messenger[24–31] or physical interactions between ER and PM[5,32], are depicted in Fig. 1 (top). However, the nature of the ER-derived signal to activate SOCs remains unresolved.

SOCs mediate capacitative calcium entry

The original concept of capacitative calcium entry (CCE) was introduced to describe the process whereby depletion of intracellular calcium stores caused movement of extracellular calcium into cells[13]. Originally, the model predicted that external calcium was conveyed into stores[13]. However, it subsequently became clear that calcium moving through SOCs enters the cytoplasm[14]. The term 'capacitative' is appropriate as it is possible that close interactions between the ER and the PM underlie SOC activation[33–35]. In certain cell types, including haematopoietic cells, SOCs carry a highly calcium-selective, non-voltage-dependent and inwardly rectifying current[8,36-38], termed the calcium release-activated calcium current, or I_{CRAC}[38]. This current is very small (24 fS[8,36,38]) and strongly inhibited by calcium, probably reflecting an important calcium feedback to limit the CRAC channel[8,37,39].

A larger current observed in the absence of external calcium[40] has been attributed partially to operation of the TRPM7 channel (formerly known as TRP-PLIK or LTRPC7), a distinct magnesium-sensitive ubiquitously expressed member of the TRP channel family[41–43]. Whereas the CRAC channel functions in some cells, it is by no means universal to all cells that exhibit SOC activity, and there are a number of different currents that seem to be related to store-depletion[8]. Channels with larger conductances and somewhat less cation-selectivity have been characterized in A431 cells[44], endothelial cells[45] and pancreatic acinar cells[46]. The store-depletion-activated current identified in endothelial cells can be stimulated by $InsP_3$ (ref. 45), and evidence (discussed later) suggests $InsP_3Rs$ may mediate TRP channel and SOC activation[47–53].

The function of SOCs

In most non-excitable cells, as well as in many excitable cells, calcium signals are activated in response to PLC-coupled receptors through production of $InsP_3$ and rapid induction of calcium release through $InsP_3$ receptors in the ER membrane[1,2,7]. Calcium release from the ER provides a fast but limited calcium signal; thereafter, PM and ER calcium pumps work in concert to reduce the levels of cytosolic calcium. In virtually all cells in which $InsP_3$ mediates calcium release, the emptying of stores triggers activation of SOCs that mediate controlled calcium entry from outside the cell. This is an important component of the calcium signal: first, to extend the temporally limited calcium signal that can be effected through release from ER; second, to provide the means to allow replenishment of calcium stored within the ER lumen.

In some cells, SOC-mediated calcium entry is a major component of calcium signalling. For example, T lymphocytes contain only limited calcium stores and CCE mediated by the CRAC channel represents a substantial portion of calcium signals induced by the PLC-γ-coupled T cell receptor. Calcium entry controls rapid exocytotic release of lytic granules in cytotoxic T cells[11], and in helper T cells calcium entry is crucial for the long-term activation of cytokine genes[11]. CCE is the sole mechanism for mediating these calcium-dependent responses in T lymphocytes[54]. The B cell receptor also couples to CCE and the sustained calcium entry controls developmental transition, maturation and function of B cells[55]. Also, in dendritic cells, the CRAC channel has been shown to be the major calcium entry pathway mediating calcium-dependent maturation and migration[56]. In many other non-excitable cells, CCE may provide a smaller contribution towards receptor-induced cytosolic calcium signalling but can mediate important longer-term calcium elevation and contribute to generation of calcium oscillations and waves[5,7,8]. Sustained changes in store-operated calcium entry are

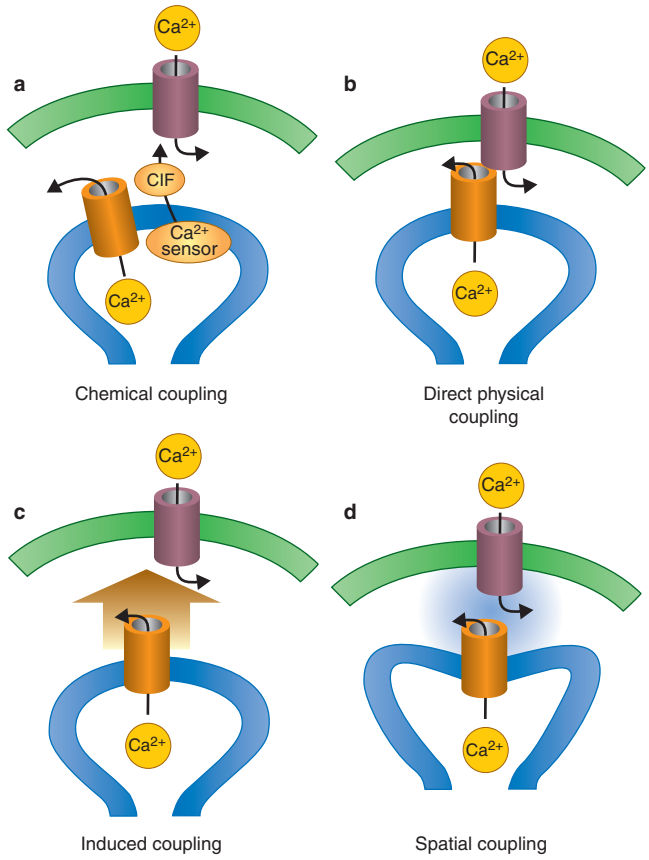

Figure 1 **Coupling models for activation of store-operated channels (SOCs).** **a**, 'Chemical coupling', involving the release of a diffusible calcium influx factor (CIF) from the ER in response to release detected by a presumed luminal calcium sensor. **b**, 'Direct physical coupling', involving a direct interaction between components of the ER and PM, shown hypothetically as a complex between calcium release and entry channels. **c**, **d**, Two variations on the physical coupling theme. 'Induced coupling' (**c**), in which a dynamic process concomitant with store-depletion induces the formation of new conformationally coupled ER–PM junctions. **d**, 'Spatial coupling', in which a spatially close organization exists between ER and PM, permitting short-range signalling to effect SOC activation within a restricted space (shaded).

important for regulating diverse cellular activities, ranging from exocytosis in mast cells[57] to the maintenance of tone in smooth muscle cells[58]. Long-term modification of CCE probably contributes to the complex patterns of calcium signals that control gene expression in lymphocytes, as well as in other cells[59,60]. Thus, whereas the transcription factors NFκB and JNK are activated during the calcium release phase of calcium signals, activation of NF-AT (nuclear factor of activated T cells) occurs specifically during the prolonged phase of calcium entry corresponding to CCE activation[11,61,62]. Calcium signals are also crucial for cell cycle regulation, control of cell proliferation and the onset of apoptosis[1,15], and CCE seems to be important in mediating these events[8,63–65].

In addition to providing temporally sustained calcium signals, store-operated calcium entry may mediate spatially confined calcium signals. For example, calcium-regulated adenylyl cyclase subtypes in the PM are selectively modified by calcium that enters through SOCs[66,67]. A similar proximal relationship with SOCs seems to hold for the calcium-stimulated PLC-δ1 enzyme[68] and nitric oxide synthase[69]. Such results suggest that calcium from SOCs enters initially into a spatially occluded cytosolic environment from which diffusion of calcium may be restricted.

Just as important as mediating calcium signals directly, SOCs are crucial in mediating the replenishment of calcium stored within the ER[3,8,9,12]. The calcium released through $InsP_3Rs$ is rapidly extruded from cells by PM calcium pumps, resulting in emptied stores. SOCs therefore provide a means to refill the calcium stores. Although passing initially into the cytosol, calcium entering cells through SOCs is efficiently channelled into the ER[3,6,8]. In polarized pancreatic acinar cells, calcium release mediating secretion occurs at the apical pole, whereas CCE occurs predominantly at the basal membrane[70]. Calcium enters the cytosol but is directly accumulated within ER close to the basal membrane and 'tunnelled' within the ER lumen to the remote apical pole for release[70]. Again, there seems to be close functional coupling between SOCs and the ER. In cells undergoing continuous oscillations of calcium, the entry of calcium to replenish repeatedly releasing calcium stores becomes even more crucial. However, during oscillations, the relationship between released and re-entering calcium is complex[1,7,71]. SOC-mediated replenishment of ER calcium is more significant than simply preserving a calcium signalling pool. Thus, luminal calcium itself controls ER functions such as protein translocation, folding and trafficking[72,73]. Also, changes in ER calcium content profoundly modify cell growth and entry into apoptosis[1,15,63–65,74,75]. The intimate relationship between calcium entry through SOCs and ER luminal content means that regulation of SOCs may effect important control over ER function and cell growth.

The coupling mechanism for SOC activation

The original conformational coupling model[15,32] predicted that SOC activation might involve direct interactions between the ER and the PM (Fig. 1b). An alternative to this has been the chemical coupling model based on studies indicating that store depletion induces release of a diffusible SOC-activating messenger from the ER that could be detected in the cytosol[24] (Fig. 1b). Although recently there has been some more support for such a messenger[25–27], candidate messenger molecules from the cytosol have not been identified. Evidence has indicated that metabolites of the cytochrome P-450 epoxygenase pathway are potential mediators of SOC activation[30,76]. Furthermore, modifiers of the lipoxygenase pathway were shown to modify I_{CRAC} in RBL cells[31], although the direct actions of lipoxygenase metabolites could not be observed. Whereas the weight of evidence generally supports the theory of physical coupling between the ER and PM to activate SOCs, to date there is no proof that direct physical coupling is required. Indeed, we should not exclude the possibility that a chemical mediator, perhaps working at short-range, is involved in local coupling between the ER and PM. Consequently, we define two variations on the direct physical coupling theme (Fig. 1b). First, a mechanism termed 'induced coupling' (Fig. 1c), in which we consider a dynamic process concomitant with store-depletion induces the formation of new conformationally coupled junctions between ER and PM. Second, a model termed 'spatial coupling' (Fig. 1d), in which we consider that a spatially close organization exists between the ER and PM (as opposed to a direct molecular interaction), permitting short-range signalling within a restricted space to effect SOC activation. These definitions are described and further refined as we explore the evidence that characterizes the process of coupling to activate SOCs.

Evidence for a local organization between $InsP_3$-sensitive calcium stores and SOCs has been obtained in different systems. In polarized epithelial cells, $InsP_3$ production and calcium release were confined locally to the same regions as SOC-mediated calcium entry[77]. Similarly, local application of receptor agonists onto the surface of oocytes induced localized calcium release from stores and activated PM SOCs in the same area[78]. Similar conclusions were also drawn from studies with RBL cells[79]. The efficient sequestration of calcium within the ER after entry through SOCs in pancreatic acinar cells[70] also suggests a close association between SOCs

and the ER. These studies suggest peripheral ER stores localized near PM couple to activate SOCs, consistent with a physical coupling model, but do not distinguish between direct versus permanent coupling.

It is tempting to draw analogies between the coupling of SOCs with the coupling between release channels and entry channels in skeletal muscle triad junctions. Support for this analogy derives from information suggesting that the $InsP_3R$ itself may be the ER membrane component required for SOC activation. However, caution is appropriate in relating the two coupling processes. The triad junction has evolved to provide exceedingly rapid coupling to activate calcium release from muscle SR. In contrast, CCE is a slowly developing process[8,37]. The time-dependence of SOC activation is of the order of tens or hundreds of seconds. This has been observed in numerous patch-clamp measurements of I_{CRAC} activation[8,36,37,40,80,81], as well as from measurements of intracellular calcium concentration[33,82,83]. The slow 'development' of CCE in response to store-emptying does not mean 'activation' of SOCs is a slow process *per se*. Indeed, from studies on the activation of I_{CRAC} in response to rapid photolytic release of caged $InsP_3$, significant channel activation was observed as early as 100 msec after photolysis[80]. However, this early channel activity was only a small fraction of the total activity that continued to develop over the next 10–30 s. Thus, one obvious question concerns what accounts for the slow development of SOCs. One possibility is that the emptying of calcium stores is itself protracted, reflecting calcium release from areas remote from the PM[70]. Militating against this hypothesis are results from the use of ionophores[33] or the intraluminal calcium buffer, TPEN[81]. These agents are expected to lower calcium levels rapidly and indiscriminately within the ER network. However, they induce SOC activation with similar slow time-dependence. Another explanation for slow SOC activation is that the process involves translocation and/or docking between ER and PM[33,34]. Thus, whereas fast muscle triads are structurally organized 'precoupled' junctions, the activation of SOCs may result from induction of new coupling entities through 'induced' coupling (Fig. 1c). Indirect support for such a hypothesis comes from numerous studies that reveal the involvement of a GTP-regulatory step in the activation of SOCs[8,84–86] consistent with operation of a GTP-binding protein-mediated trafficking event.

Although there is evidence for the existence of junctional densities between peripheral ER cisternae and the PM[5,87], it is unclear whether such entities contain localized calcium channels or pumps. However, studies suggest that close proximity between the ER and the PM may be necessary for activation of SOCs. Induction of cortical actin polymerization, or translocation of existing actin filaments to form a dense cortical actin band subjacent to the PM, displaced ER from the vicinity of the PM and prevented coupling to activate SOCs[33]. This indicates that physical separation of the ER and the PM prevents the SOC coupling process and suggests that it involves close contact between the two membranes. These data are consistent with observations in *Xenopus laevis* oocytes, in which physically applied cell membrane distention prevented coupling to activate SOCs by creating distance between the ER and the PM[34]. This study provided evidence that Rho GTPase-mediated cytoskeletal assembly and trafficking, as well as the docking machinery involving SNAP-25, are involved in SOC activation. In platelets[88] and hepatocytes[89], the actin cytoskeleton seems to be a significant mediator of coupling in the activation of SOCs. In endothelial cells, an interaction between the spectrin cytoskeleton and the anchoring protein 4.1 seem to be required for SOC activation[90]. However, a role for the cytoskeleton in mediating SOCs was not observed in RBL-1 cells[91].

There is growing evidence (see below) that G protein-coupled receptors, PLC and calcium entry channels exist within organized domains of the PM[22,92–94] and that such organization occurs within cholesterol-rich caveolin-containing lipid raft domains[95]. In addition, localized high concentrations of calcium entering through SOCs are detected by calcium-sensitive adenylyl cyclase (described above) in caveolae[96]. Caveolar microdomains are reported to be juxtaposed with the $InsP_3R$[94,95], providing evidence that coupling between PM and ER membranes may itself occur at these functionally specific junctional units. Generally, these findings suggest that an organized and spatially structured interaction between the ER and the PM is required for activation of SOCs. However, there is no evidence that components within these two membranes must be directly or permanently coupled. Indeed, results indicate that after physical separation of the two membranes, the successful coupling event to activate SOCs can be re-established[33–35,88]. This evidence supports a model in which coupling is considered to be an 'induced' event (Fig. 1c). However, the nature of such an induced coupling event is open to interpretation. Some considered a 'secretion' model, in which vesicular fusion causes insertion of channels into the PM[34]. In contrast, others considered a 'secretion-like' model, in which SOC-activation results from reversible interactions between the ER and the PM[33,88]. This model has a precedent[33,35] in the 'kiss and run' mechanism, by which secretory vesicle release from cells can occur through a reversible docking event not involving actual membrane fusion[97].

In both these models, dynamic trafficking events are hypothesized to underlie SOC induction. Although there is no direct structural evidence for trafficking events after store emptying, it is known that closely interacting regions between the ER and the PM exist in cells[5,87]. As the cytoskeleton and docking machinery may be important in the maintenance of such regions, it is probable that these components may be disrupted by the modifications described above. Such closely interacting domains between ER and PM have been hypothesized to form diffusionally restricted intervening spaces, which may be the sites of coupling for activation of SOCs[5,9,12,13,87,98]. We have termed this a 'spatial-coupling' model (Fig. 1d), and it provides for the possibility that a chemical mediator that activates SOCs may operate at a short range within the restricted space. Such a mediator could be calcium itself[9,12,98]. Indeed, as described earlier, the CRAC channel is highly sensitive to calcium and fully blocked at calcium levels exceeding the normal resting cytosolic calcium concentration[8,37,39]. Leakage of calcium from the ER into a diffusionally restricted space could maintain SOCs in their closed state; after store-emptying and concomitant decreased leak into the space, channel inhibition would be alleviated to allow calcium entry. As blockade of ER calcium pumping induces SOC activation, this model would predict that ER pumps are located remotely from the restricted spaces. It is interesting that calcium entering cells through SOCs may indeed reach locally high concentrations[99], consistent with its entry into a diffusionally restricted region. However, a significant question concerns how SOCs remain open for an extended time, as the build-up of entering calcium would be expected to have inhibitory effects on the channels. It is possible that under conditions of store emptying, the channel becomes modified to reduce its calcium sensitivity. Alternatively, coupling within a restricted space may not be mediated by calcium, but instead by a close-acting calcium influx factor (CIF). The spatially restricted occurrence of such an agent could explain the difficulties encountered in identifying potential CIF candidates.

The molecular identities of SOCs

Much recent interest has been directed towards the TRP-family of ion channels and their possible relationship with SOCs[20–22]. The TRP channel was first identified as an essential component of phototransduction in *Drosophila melanogaster*[19]. In *Drosophila*, rhodopsin couples through the G protein G_q to activate PLC-β (encoded by the *norpA* gene), and through a mechanism that remains unresolved, mediates opening of channels in the membrane that mediates the light-induced depolarizing current[92]. *Drosophila* mutants with the transient receptor potential phenotype are deficient in a gene that

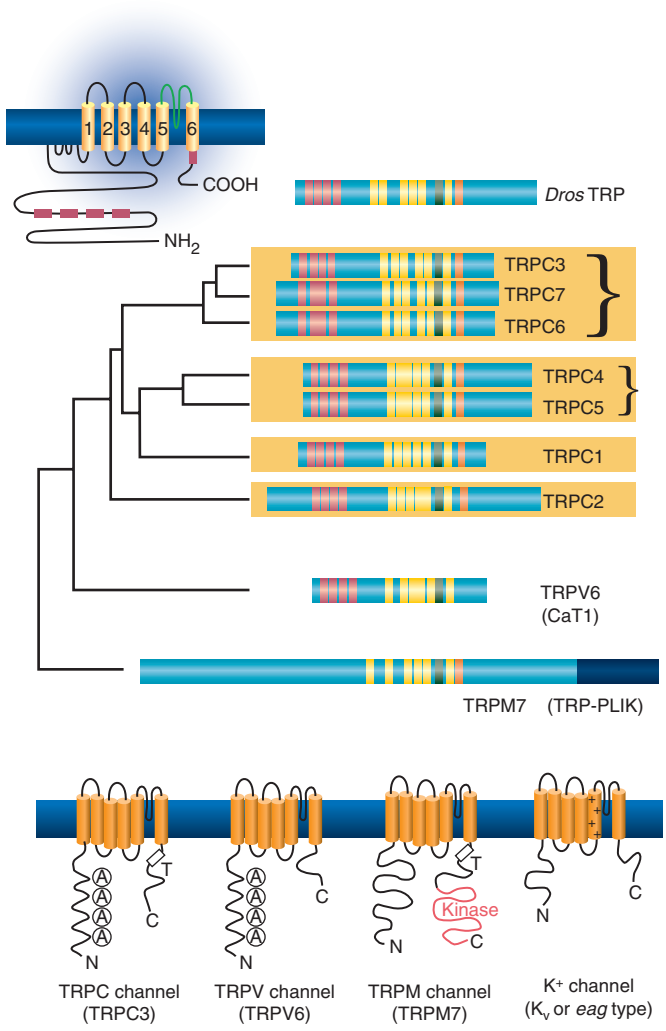

Figure 2 **Phylogenetic relationship between TRP channels implicated in CCE.** Proteins are aligned around the 'pore-forming' loop (green). Also shown are other transmembrane segments (yellow), ankyrin-like repeats (purple), the kinase domain of TRPM7 (blue) and the 'TRP-box' (red). The TRP-box is a small highly conserved region (amino acid sequence EWKFAR) found in *Drosophila* TRP, as well as in mammalian TRPC and TRPV channels. A topological comparison of the structures of three TRP subtypes and a potassium channel are shown below. 'A' denotes ankyrin-like repeats.

encodes the TRP protein, a receptor-activated cation channel that is somewhat calcium-selective and almost exclusively expressed in photoreceptor cells of the eye[22,92]. Generation of the light-induced current requires both the TRP channel and another similar TRP-like (TRPL) channel[92]. Both channels seem to function together within a large signalling complex that also includes rhodopsin, Gq protein, PLC and calmodulin[22,92,100]. The signalling proteins seem to be arranged through interactions with the multiple PDZ domains of the scaffolding protein, INAD which itself self-associates into large complexes[22,100]. As TRP mediates a longer-term entry of calcium activated downstream of PLC, it was considered that its activation would proceed after $InsP_3$-induced release of calcium stores and that TRP could be the prototype for SOCs .

The search for mammalian counterparts of TRP was successful and a total of seven closely related genes encoding TRP channel counterparts have been identified[17,18,22,101–103] (termed TRPC1–TRPC7; see Fig. 2 and refs 22,104). The 'C' denotes 'canonical', as these genes are all closely related to the original *Drosophila* TRP channel[104]. The seven mammalian TRPC channels can be divided phylogenetically into four subgroups (Fig. 2), the channels within each subgroup being closely related in both structure and function[20,22]. A group of six transmembrane-spanning segments and an additional pore-forming hydrophobic domain between segments 5 and 6 are conserved throughout the *Drosophila* TRP and mammalian TRPC channels. The topology of this pore-forming domain is similar to that of voltage-gated channels[22], although the conserved voltage-sensing positively charged residues in the fourth transmembrane segment of the voltage-gated channels are notably absent in TRP channels (Fig. 2). Both *Drosophila* TRP and mammalian TRPC channels retain a number of conserved ankyrin homology sequences towards the amino terminus that may assist in association with cytoskeletal elements. Other members of the broader TRP-related channel family[104], in particular, members of the TRPV (named after the prototype vanilloid-gated ion channel) and TRPM subfamilies (named after the original member, melastatin) have also been implicated in CCE (Fig. 2).

The role of TRP channels in mediating CCE is intriguing and controversial. TRP channels are ubiquitously expressed and multiple TRP channel subtypes exist in most cells[17,18,20–23]. One difficult problem in assessing function of TRP channels is that analysis of overexpressed channels is usually performed against a background of endogenous SOCs and TRP channels. Similarly, reduction of TRP channel expression by antisense or knockout approaches can be confused by the functional overlap and redundancy between endogenous TRP channel subtypes. The TRP superfamily is coupled to respond to an extraordinary array of signals, including temperature, pain, oxidative stress, hormones and light[23]. Moreover, individual TRP channel subtypes seem to receive input from multiple sources[17,18]. One unifying characteristic among the *Drosophila* TRP and most mammalian TRPC channels is their response to receptor-activated PLC[20–22], classifying these channels as 'receptor-operated' (ROCs). However, PLC activation and concomitant $InsP_3$ production assures calcium store release. Therefore, it is difficult to say whether TRPC channels function as SOCs, as opposed to ROCs. The 'hallmark' that is used to define store-operated calcium entry is a response to store-depletion alone using calcium pump blockers (for example, thapsigargin) or ionophores (for example, ionomycin)[6,8]. However, we should consider carefully that emptying stores with these agents is not a physiological process *per se*. Physiologically, stores are released after PLC-coupled receptor activation. Furthermore, the consequences of PLC activation are not limited to store-release alone (see below).

There have been numerous reports describing experiments in which overexpressed mammalian TRPC channels appear activated in response to store-emptying with calcium pump blockers or ionophores. These include studies on TRPC1 (refs 105–107), TRPC2 (ref. 108), TRPC3 (refs 47, 48, 103, 109, 110), TRPC4 (refs 111–112), TRPC5 (ref. 113), TRPC6 (ref. 114) and TRPC7 (ref. 115). In addition, there are many studies indicating that endogenously expressed TRPC channels contribute to CCE. Such conclusions are drawn from a variety of approaches to modify endogenous TRPC expression, including antisense methodology[116–121], gene knock-out systems[122,123] or use of dominant-negative mutants[124]. However, there are equally numerous reports indicating that TRPC channels overexpressed in mammalian cells are activated by PLC-coupled receptors, but are apparently not activated by store-depletion in response to thapsigargin or ionomycin. Such results have been reported for TRPC1 (ref. 125), TRPC3 (refs 16, 50, 126–129), TRPC4 (refs 130–132), TRPC5 (refs 130, 133, 134), TRPC6 (refs 16, 135, 136) and TRPC7 (ref. 137). Similar conclusions on the apparent store-independence of TRPC channels have also been drawn from studies in which endogenous TRPC channel expression was modified by antisense procedures[136,138] or gene knock-out approaches[139]. In the interest of space we have not provided detailed experimental descriptions of this extensive literature.

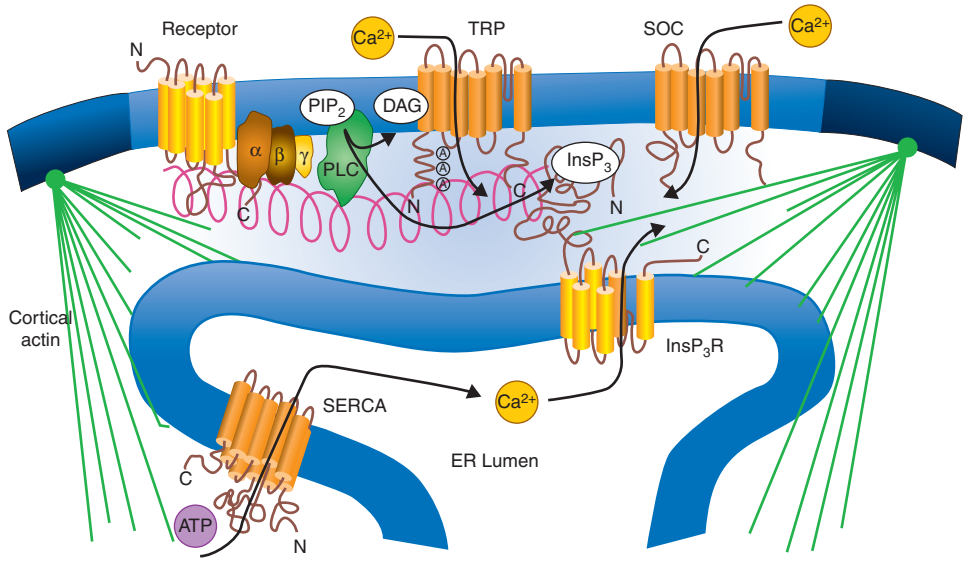

Figure 3 **The hypothetical calcium signal-coupling domain.** The complex is based on the model of Montell[22], describing the signalling molecules in *Drosophila* retina. G protein-coupled receptors activate PLC-β, resulting in the cleavage of $PtdInsP_2$ and the generation of membrane-retained DAG and cytosolic $InsP_3$. DAG can directly activate TRP channels. The target for $InsP_3$ — the ER-localized $InsP_3R$ — may be closely coupled to TRP channels, forming part of a theoretical ER–PM complex. Such junctions may be similar to skeletal muscle triads and/or they may form a diffusionally restricted intervening space. The molecular identity of SOCs is unknown and its depiction is hypothetical. In this model, the signalling complex is stabilized by cortical actin or an equivalent of the PDZ domain-containing scaffold protein, INAD (shown as a purple coiled structure), which is known to organize the *Drosophila* retinal complex. SERCA denotes the SR/ER calcium ATPase pump.

However, comprehensive listings of the relationship between TRPC channels and calcium stores are provided in recent excellent reviews[12,17,18,20,21].

At first sight, the abundance of apparently conflicting information on the coupling of TRPC channels to store-emptying is overwhelming. However, determining whether TRP channels are directly activated by store-depletion, as opposed to modifying or being modified by endogenous SOCs, is difficult. In certain cells, high endogenous TRPC channel expression has allowed more definitive analysis. For example, TRPC3 channels are highly expressed in pontine neurons and allow passage of sodium and calcium ions in response to PLC-coupled receptors, but are not activated by store-emptying[140]. The TRPC2 channel is highly localized to rat vomeronasal sensory microvilli cells in which calcium stores are absent, suggesting that gating of TRPC2 is independent of calcium store depletion[141]. In contrast, the endogenous TRPC2 channel in mouse sperm is reported to be activated by thapsigargin-induced store-emptying[142]. Thus, from native TRPC channel analysis, we can conclude that the coupling phenotype is cell-dependent, and that store-dependence may be a function of available cellular machinery.

A further explanation for the variation in observed activation of TRPC channels is that the channels themselves undergo assembly into multimeric structures[22]. Indeed, it was originally observed that coexpression of the *Drosophila* TRP and TRPL channels in mammalian cells resulted in a store-operated phenotype, whereas, expressed individually, the channels were store-independent[143]. However, as discussed later, whereas changes in the pore-properties and regulation of the channels can be observed after coexpression, in general, marked changes in the mechanism of activation (for example, store-dependence) of coexpressed TRPC channels have not been demonstrated[144–146]. Another complicating factor in assessing expressed TRPC channels is the calcium released from stores or entering through native SOCs, which could itself be responsible for activation under conditions of store-emptying. For example, increased cytosolic calcium directly enhanced TRPC3 channels[126,147] independently of store-emptying and calcium entry through SOCs was shown to stimulate overexpressed TRPL channels[148].

Recent evidence suggests that the canonical TRPs are not the only SOC contenders. The TRPV family[23,104,149] contains at least one member, the TRPV6 channel (formerly named CaT1 or ECaC2), with pore properties and coupling functions that resemble CRAC channels[150]. TRPV6, and its close relative TRPV5[149], both closely resemble the overall topology of TRPC channels (Fig. 2), retaining multiple N-terminal ankyrin repeats. Functionally, TRPV6 channels resemble CRAC channels with respect to cation selectivity and single channel conductance. Importantly, TRPV6 channels expressed in CHO cells could be activated by store-depletion using either $InsP_3$ or thapsigargin, although the store-dependence was highly dependent on expression level[150]. A subsequent study reported that although the TRPV6 and CRAC channels have similar pore properties, no activation of the TRPV6 channel was observed in HEK293 cells after ionomycin-induced store release[151]. Furthermore, 2-aminoethoxydiphenyl borate (2-APB), an effective biphasic modifier of CRAC channels[152–154], seemed to be largely ineffective on TRPV6 channels[151]. However, it was recently reported that both the function and pharmacological properties of TRPV6 are highly expression-dependent[155]. Thus, at low expression levels, the channel was store-dependent and sensitive to 2-APB, similar to CRAC channels, whereas at higher expression levels, the TRPV6 channel was constitutively active and insensitive to 2-APB.

Finally, the TRPM subfamily also has SOC connections[156]. Notably, TRPM7 is an interesting channel identified from yeast 2-hybrid screening as a PLC-binding partner that contains a large carboxy-terminal kinase-like domain[156] (Fig. 2). TRPM7 is ubiquitously expressed[41,156] and seems to be essential for cell viability[41]. Although apparently not store-operated[41,42,156], TRPM7 is notable in having channel properties that closely overlap with those of CRAC channels. It is now recognized as mediating a significant component of the current originally defining I_{CRAC}, permitting a more refined characterization of CRAC channel properties[42,43]. TRPM7 is a magnesium-inhibited cation channel with pharmacological properties that are distinct from the CRAC channel[43].

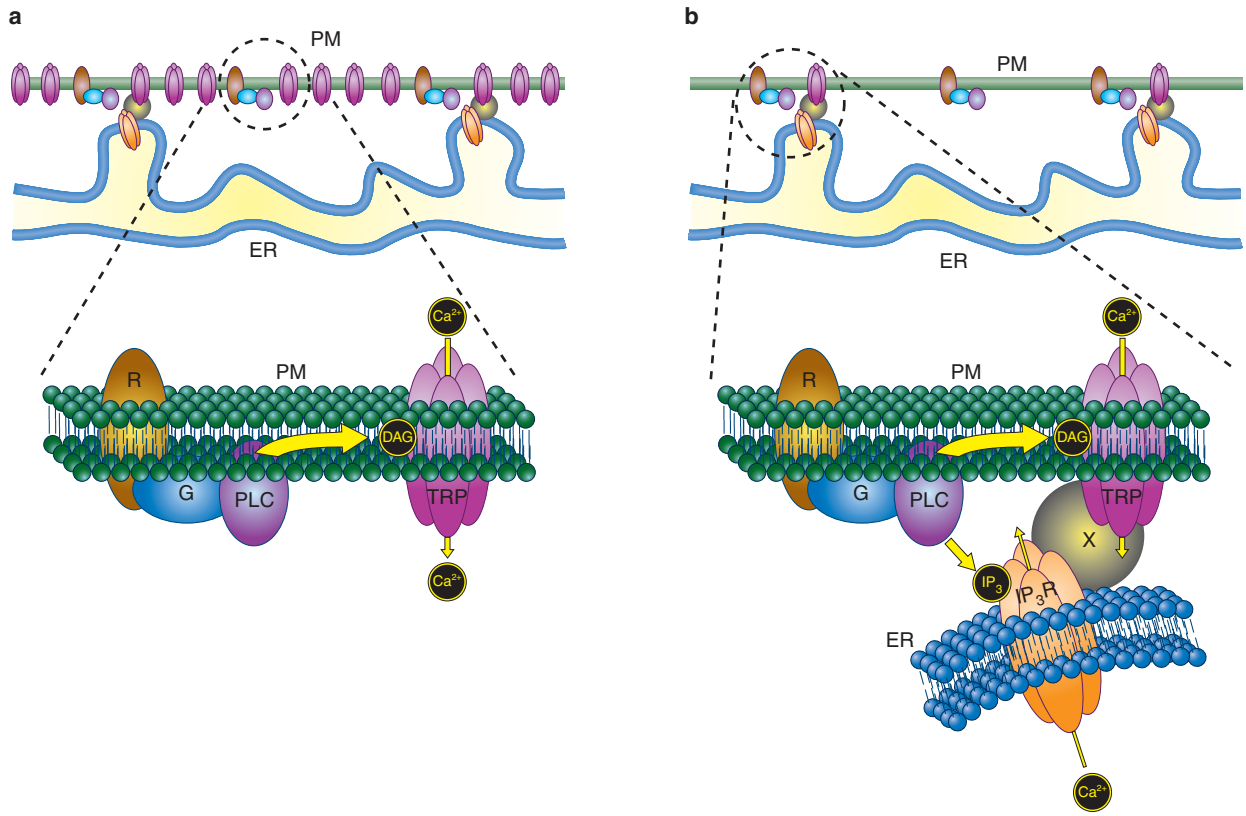

Figure 4 **Coupling of TRP channels expressed in vertebrate cells.** Two scenarios are depicted based on the level of TRP channel expression. **a**, High-level expression: TRP channels are depicted as homomeric structures that exceed the levels of endogenous coupling machinery. This results in a phenotype predominantly uncoupled from stores, but activated in response to PLC-derived DAG. **b**, Low-level expression: TRP channels are expressed at levels that permit stoichiometric coupling with the cell machinery, allowing mediation of store-operated calcium entry. A higher proportion of expressed TRP channels now take on a store-coupled phenotype, although they are still responsive to DAG. The low-level expression is depicted as a heterotetrameric TRP channel assembly resulting from possible assembly with endogenous TRP channels. The TRP channels are shown coupling to $InsP_3Rs$ through a theoretical coupling entity denoted as X.

Although the TRPM7 channel may not convey the store-operated calcium current, it defines a paradigm that is shared among many TRP channels, that is, response to multiple inputs[20–23]. Thus, the TRPM7 channel seems to respond directly to ions, nucleotides, lipids and proteins, and itself functions as both a channel and a kinase[23]. The channel is highly sensitive to magnesium and is regulated by adenine and guanine nucleotides[41–43]. Opening of the TRPM7 channel is positively controlled by phosphatidylinositol bisphosphate ($PtdInsP_2$); hence PLC-coupled receptor activation causes channel inactivation[157]. This suggests that the TRPM7 channel and SOCs are controlled reciprocally. Additionally, the channel can interact directly with PLC-β and PLC-γ enzymes[156,157] and may receive receptor-derived input through such interactions. This paradigm of response to multiple inputs can clearly be extended to the TRPC channels. Thus, both $InsP_3$ and DAG are involved in activation of TRPCs. There is considerable evidence that the action of $InsP_3$ through $InsP_3Rs$ has direct input into TRPC channel activation (see below). The closely related TRPC3, TRPC6 and TRPC7 subgroup of channels (Fig. 2) are directly activated by DAG and its cell-permeant analogues[16,21,50,129,137]. This action of DAG is independent of PKC[16,137] and mediated by an N-terminal domain in the TRPC6 channel[158]. The response to DAG seems subgroup-specific as overexpressed TRPC1, TRPC4 and TRPC5 channels are unresponsive to DAG[16,130], although endogenous TRPC4 channels were reported as DAG-modified[138]. DAG also seems to activate overexpressed *Drosophila* TRPL channels in insect Sf9 cells[159]. Interestingly, native fly retinal TRP and TRPL channels can be directly activated by polyunsaturated fatty acids (linolenic and arachadonic acids), perhaps derived from the breakdown of PLC-generated DAG[160]. As described earlier, the *Drosophila* TRP channels exist within structured membrane domains that are organized by the PDZ-containing scaffold protein, INAD, and are likely to be in close contact with the rhodopsin-triggered phototransduction machinery[92,100]. Less is known about the organization of TRPC channels in mammalian cells, but there are indications that the PDZ-containing NHERF protein interacts with TRPC channels[161] and may organize a spatial relationship with G protein-coupled receptors, PLC and the cytoskeleton in an analogous fashion[162]. Overall, TRP channels probably exist in an organized signalling domain, in which input from effector proteins is specifically targeted towards them (Fig. 3). The evidence that this signalling domain may also receive direct input from components in the ER membrane is considered in the final section.

Organization of functionally-coupled SOCs and TRPs

By its nature, CCE involves the integration of signals between two membranes. Significant new insights into this process have arisen from studies indicating that the $InsP_3R$ may be pivotal in the activation of calcium entry, as originally predicted[5,32]. Certainly, the $InsP_3R$ is strategically placed for this purpose, being the sensor of the original receptor-generated $InsP_3$ signal, the mediator of the initial calcium release signal, and by virtue of cytosolic and luminal calcium sensing[5], able to respond to calcium changes on both sides

of the ER membrane. Its topological similarity to ryanodine receptors, which couple to Ca_v entry channels, provides a paradigm for considering an equivalent coupling between $InsP_3$ receptors and calcium entry channels. However, whereas structural evidence for TRP channel–$InsP_3R$ interactions is compelling, a role for $InsP_3$ receptors in SOC activation is unproven.

The involvement of $InsP_3Rs$ in mediating PM calcium entry was first reported in excised inside-out patches from Jurkat cells[163]. The demonstration that $InsP_3$ activated calcium channels in excised patches from endothelial cells suggested that these channels were the same as those activated in intact cells by depletion of calcium stores[45]. By single-channel analysis, low-conductance ('miniature') calcium-selective channels in A431 cells were shown to be activated by $InsP_3$ (ref. 164). Whole cell experiments using HEK293 cells stably expressing TRPC3 channels[127] revealed that TRPC3 channels were stimulated by PLC-coupled receptors, and to a lesser extent by store-depletion[47]. After excision of patches, reduced TRPC3 channel activity could be restored by application of both $InsP_3$ and $InsP_3Rs$, indicating that TRPC3 channels require ligand-bound $InsP_3Rs$ for function[47]. $InsP_3R$ constructs containing the N-terminal $InsP_3$-binding domain were sufficient to couple to TRPC3 channels and render them activatable by $InsP_3$ (ref. 48). The remainder of the $InsP_3R$ was required to confer store-sensitivity by sensing the degree of store-emptying. A relatively short segment within the C terminus of TRPC3 was required for the $InsP_3R$ interaction and two specific $InsP_3R$ sequences close to the $InsP_3$ binding domain interacted with TRPC3[49]. Peptides from the interacting regions of TRPC3 and $InsP_3Rs$ reduced activation of SOCs in normal cells, suggesting that native SOCs are activated through $InsP_3Rs$[49]. Studies revealed that $InsP_3Rs$ interact with an inhibitory calmodulin-binding site on TRPC3, displacing calmodulin and thereby activating the channel[165,166]. $InsP_3Rs$ were also shown to interact with TRPC4 channels, an interaction that is specific to alternative TRPC4 splice variants[167]. TRPC1 is reported to specifically interact with type-II $InsP_3Rs$, but not type-I or type-III $InsP_3Rs$, and this interaction is activated by store-depletion[53]. Moreover, this interaction was prevented *in vivo* by the same modifications of the cortical actin cytoskeleton[52] that were shown to prevent coupling to activate endogenous SOCs[33,50]

Despite this rather compelling account of TRPC–$InsP_3R$ interactions, the role of $InsP_3Rs$ in physiological CCE is less clear. The actions of the two distinct $InsP_3R$ antagonists, 2-APB and xestospongin C, in blocking both endogenous CCE and receptor-induced TRPC3 activation was considered evidence that $InsP_3Rs$ mediate activation of both channels[50]. However, more compelling were studies demonstrating that in the DT40 B-lymphocyte triple $InsP_3R$ knockout variant line, CCE was identical to that observed in wild-type cells[129,152,153,168,169]. Despite exhaustive searching (including examination of transcripts, full-length proteins or fragments thereof, $InsP_3$-binding activity or physiological $InsP_3R$-mediated calcium release in intact or permeabilized cells), no $InsP_3Rs$ were detected in these cells[129,152,168,169]. Moreover, the blocking action of 2-APB on CCE was unaltered by elimination of $InsP_3Rs$[152,153,169]. These and other studies indicated the action of 2-APB was either on the channel[91,154,169] or, more interestingly, on the coupling machinery for SOCs[153,155] (see below). The DT40 cells provided one further twist in the $InsP_3R$-coupling chronicle. TRPC3 channels expressed rapidly and with high efficiency in DT40 cells were activated by PLC-coupled receptors independently of $InsP_3R$ expression and store-emptying[129]. Intriguingly, TRPC3 channels expressed more slowly and at lower efficiency in the same cells were partially dependent on $InsP_3R$ expression and wholly dependent on store emptying[109]. The stark dichotomy of these findings may actually serve to illuminate the black box that mediates SOC coupling (see below)

In *Drosophila* phototransduction, a role for $InsP_3Rs$ in mediating TRP activation is unlikely. Genetic elimination of the single $InsP_3R$ in *Drosophila* does not alter TRP activation[170,171]. As *Drosophila* phototransduction is completely dependent on PLC, logic would predict that the other PLC product, DAG, is the mediator. As described earlier, polyunsaturated fatty acids can activate TRP channels[160] and could be derived from DAG. However, there is no evidence that DAG exerts a direct effect on the TRP channel. Thus, despite its prominence as a paradigm for understanding calcium entry, the exact nature of the coupling to activate the *Drosophila* TRP channel remains a mechanistic mystery. In mammalian cells, the ryanodine receptor (RR) has also been shown to undergo coupling to TRPC3 channels[172], and it was suggested that in DT40 $InsP_3R$-knockout cells, RRs might substitute in the coupling of SOCs[173]. However, the lack of observable RR function in DT40 cells and the identity of SOC activation in the DT40 wild-type and $InsP_3R$-knockout cells[12,129,152] argue against this.

Topologically, TRP channels resemble a spectrum of hexahelical ion channels, including the potassium K^v channels (Fig. 2), known to exist as functional tetramers. Importantly, tetramers may include heterogeneous channel subunits, and the makeup of heterotetramers can determine many functional parameters, including trafficking, membrane retention, ionic specificity and regulatory properties of the channels[22]. Evidence is accumulating that members of the TRP superfamily undergo similar heterotetrameric association[20,22,146,174] and it is reasonable that the multitude of TRP channel responses could reflect specific heterotetrameric assembly. A precedent for this view arises from the observation that *Drosophila* TRP and TRPL channels coexpressed in mammalian cells have a store-dependent phenotype[143]. It is probable that the *Drosophila* retinal light-induced current reflects specific associations between TRP and TRPL proteins, and also a recently identified third retinal protein, TRP-γ[175]. Nevertheless, the phenotype of store-dependence does not coincide with any known function of the TRP channels in native *Drosophila* retina. The mammalian TRPC channels are ubiquitously expressed among tissues, and most individual cell types endogenously coexpress multiple TRPC subtypes[20–22]. Therefore, the premise that heteroassembly occurs and defines functional TRPC phenotype is an enticing possibility. This premise derives support from a study where coexpression of TRPC1 and TRPC5 resulted in channels with ionic selectivity and voltage dependence properties distinct from the individual channels[144]. However, there was no indication of store-dependence[144]. Furthermore, coexpression of TRPC1 and TRPC3 revealed channel activity with altered cation selectivity and calcium-induced feedback but, again, no evidence of store-dependence[145]. Neither study provided direct evidence for the formation of heteromeric complexes. However, recent studies have elegantly characterized the heteromeric associations between different classes of mammalian TRPC channels by examining subcellular trafficking and fluorescence resonance energy transfer between subunits[146]. A fundamental principle arising from these studies is that the associations between TRPC channels are non-stochastic and follow conservative combinatorial coexpression patterns.

SOC activation: reflections and reconciliations

From the discussions above, the intrinsic functions of TRP channels depend on two organizational parameters: first, self-association in the formation of tetramers; second, interaction with organizing regulatory elements. The latter include a combination of scaffolding/adaptor proteins and cytoskeletal elements; indeed, by virtue of their ankyrin repeats, TRP channels may function as organizing proteins in their own right. Within this structured framework, TRP channels are organized in a complex of signalling proteins, including receptors, G proteins, PLC and $InsP_3Rs$, referred to as a 'signalplex'[22,100]. TRP channel opening probably reflects integration of the signals generated from each associated protein.

With this in mind, we return to the experimental dichotomy[109,129] of TRPC3 channel coupling observed in DT40 cells described above. The coupling phenotype seems to rest solely on

expression conditions. Acute TRPC3 channel expression at high levels results in a 'receptor-operated' phenotype, in which the channels are exclusively activated by PLC-coupled receptors (through DAG production), and entirely independent of $InsP_3Rs$ and calcium stores[129]. However, extended TRPC3 channel expression at lower levels results in a 'store-operated' phenotype, in which the channels operate in an exclusively store-dependent manner and in which $InsP_3Rs$ clearly have input[109]. Assuming that the cells have a limited quantity of the machinery required for store-coupling (be it junctions, $InsP_3Rs$ or linking proteins), then the level of expression of the channels themselves determines the degree of store-coupling efficiency. At high levels of channel expression, an excess of channels relative to coupling machinery results in the majority of channels being uncoupled from stores (see Fig. 4b). At lower expression levels, the stoichiometry of the channel and coupling machinery permits a high proportion of coupled channels (Fig. 4b).

This interpretation of the coupling of TRPC3 channels is directly congruous with explanations provided from analysis of the coupling of TRPV6 channels to store emptying[150]. In this case, low-level expression results in significant store coupling, whereas a constitutively activated phenotype is observed at higher expression levels. These results are consistent with studies of expression of TRPV6 channels in RBL cells[155]. In this case, although it has pore properties distinct from native CRAC channels, the TRPV6 channel has two clearly distinct phenotypes: at low-level expression, it is store-coupled and blocked by 2-APB, at high expression levels, it is constitutively active (store-independent) and 2-APB-insensitive. The latter phenotype corresponds exactly to earlier work[151] that presumably reflected channel expression beyond the capacity of the coupling machinery. An interesting corollary from the TRPV6 study[155] is that 2-APB, known as an effective CCE modifier in all cells (whether mediated by the CRAC channel or unidentified SOCs)[152–154,169], has an action which is itself dependent on TRPV6 expression level. The interpretation is that 2-APB, rather than a stoichiometric channel blocker, may be targeting the coupling machinery itself[155]. This conclusion is in good agreement with earlier studies[153], and may provide an important means to finally unlock the molecular mystery of the store-coupling process.

Overall, whereas the CRAC channel itself has not been identified at the molecular level, there is little doubt that a number of different TRP channels can function as SOCs. As integrators of multiple signals, the input from stores is but one of the signals to which TRP channels respond. The molecular complex within which TRP channels operate probably contains the store-coupling machinery as one of several components. However, the degree to which the input from this machinery can be integrated by TRP channels seems highly cell-dependent, providing a rationalization for the elusive nature of this crucial signal-coupling pathway. □

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