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Review

Calcium entry mediated by SOCs and TRP channels: variations and enigma

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Abstract

Ca^{2+} signals in response to receptors mediate and control countless cellular functions ranging from short-term responses such as secretion and contraction to longer-term regulation of growth, cell division and apoptosis. The spatial and temporal details of Ca^{2+} signals have been resolved with great precision in many cells. Ca^{2+} signals activated by phospholipase C-coupled receptors have two components: Ca^{2+} release from endoplasmic reticulum (ER) stores mediated by inositol 1,4,5-trisphosphate ($InsP_3$) receptors, and Ca^{2+} entry from outside the cell. The latter remains largely a molecular and mechanistic mystery. The activation of "store-operated" Ca^{2+} channels is believed to account for the entry of Ca^{2+}. However, debate now focuses on how much of a contribution emptying of stores plays to the activation of Ca^{2+} entry in response to physiological activation of receptors. Here we discuss recent information and ideas on the exchange of signals between the plasma membrane (PM) and ER that results in activation of Ca^{2+} entry channels following receptor stimulation and/or store emptying.

Keywords: Endoplasmic reticulum; Store-operated channel; Ca^{2+} signal

A vast array of cellular functions are controlled by Ca^{2+} signals. Such functions range from short-term responses such as contraction and secretion, to longer-term control of transcription, cell division, and cell death [1]. In most nonexcitable cells, the generation of receptor-induced cytosolic Ca^{2+} signals is complex involving two interdependent and closely coupled components: rapid, transient release of Ca^{2+} stored in the endoplasmic reticulum (ER), followed by slowly developing extracellular Ca^{2+} entry [1–3]. Receptors coupled to phospholipase C generate the second messengers, inositol 1,4,5-trisphosphate ($InsP_3$) and diacylglycerol (DAG). $InsP_3$ serves as a chemical message that diffuses rapidly within the cytosol to interact with $InsP_3$ receptors ($InsP_3R$) on the ER which function as Ca^{2+} channels to release luminal stored Ca^{2+} and generate the initial Ca^{2+} signal phase [1]. It has been considered that the resulting depletion of Ca^{2+} stored within the ER lumen serves as the primary trigger for a message which is returned to the plasma membrane (PM) resulting in the activation of "store-operated channels" (SOCs) which allow entry of external Ca^{2+} [2,4,5]. This entry of Ca^{2+} appears to be crucial for maintaining Ca^{2+} signals, mediating longer-term Ca^{2+} signals, and allowing stores to be replenished and hence preserving ER Ca^{2+} homeostasis [2,4–6]. The other product of PLC activation, DAG, serves as the signal to translocate and activate the major regulatory kinase, protein kinase C (PKC). In addition, DAG appears to have important PKC-independent effects on Ca^{2+} entry channels [7,8]. Although molecular identification of the channels

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mediating store-operated Ca^{2+} entry has not been accomplished, certain members of the transient receptor potential (TRP) family of cation channels display properties intriguingly similar to the operation of SOCs [9]. Electrophysiological analysis of channel activity activated by store-emptying has defined one current, the Ca^{2+} release-activated Ca^{2+} current (ICRAC), which operates mostly in small hematopoietic cells [4]. The activation of this small, highly Ca^{2+}-selective current, appears to require substantial Ca^{2+} store-depletion and there is some debate as to whether, physiologically, such an extensive depletion occurs other than in small cells with limited ER [10]. In several cell types, receptors can activate a highly Ca^{2+}-selective but distinct current which is mediated by arachidonic acid [11]. This current known as arachidonic acid regulated Ca^{2+}-selective current (ARC) can be activated by low agonist levels but is not activated by store-emptying alone [12]. In other cells, store-depletion appears to trigger activation of larger, less selective cation currents [13], which may have significance in modifying membrane potential [10].

1. How are Ca^{2+} entry channels coupled to receptor-activation and store-depletion?

A central question is how receptors couple to activate SOCs. A further question receiving much attention is whether the depletion of stores is the predominant signal and/or whether receptors can have direct input on the entry channels. As shown in Fig. 1, the paradigm for Ca^{2+} entry has been that the reduction in luminal Ca^{2+} (resulting from $InsP_3$-mediated Ca^{2+} release) is the trigger for Ca^{2+} release [2]. Thus, depletion of stores with the Ca^{2+} pump blocker, thapsigargin, is sufficient to trigger Ca^{2+} entry. However, we are learning that physiological receptor-induced $InsP_3$-mediated Ca^{2+} release in cells may not induce such a large extent of store-depletion. Thus, studies on the simultaneous measurement of luminal Ca^{2+} during the physiological activation of receptor-induced Ca^{2+} signals reveal only very modest depletion of Ca^{2+} stores [14]. This suggests either a small fraction of ER (perhaps close to the PM source of $InsP_3$) becomes highly depleted providing the signal for opening entry channels, or that the entry signal is not necessarily related to store-emptying. In Fig. 1, we depict Ca^{2+} entry channels as being influenced both by the stores and by more direct information deriving from the activated PM receptor. Arachidonic acid or DAG are potentially this sort of signal working through ARC channels or TRP channels. Considering the information that is received from the ER, this is either in the form of a chemical messenger released from the ER (referred to as a calcium influx factor or CIF), or in the form of a direct conformational coupling process between the ER and PM entry channel [2]. As discussed below, for TRPC channels, there is considerable evidence that they can interact with and be modified by $InsP_3$Rs in the ER [2].

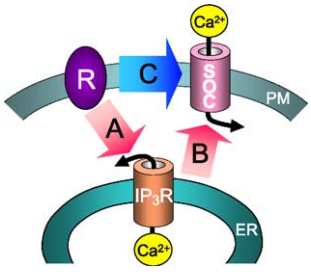

Fig. 1. Coupling in the activation of receptor-induced Ca^{2+} entry. The model shows the relationship between a store-operated Ca^{2+} channel (SOC) and receptor activation. Clearly, PLC-coupled receptors activate production of InsP3 which results in some level of ER Ca^{2+} store depletion (arrow A). Store-depletion induces activation of SOCs through an unknown coupling process (arrow B). However, the extent to which Ca^{2+} entry channels are influenced by stores when stores are depleted physiologically through InsP3 is unknown. It is clear that Ca^{2+} entry channels are under the influence of receptor-induced coupling at the plasma membrane (arrow C) mediated, for example, by arachidonic acid or DAG. In addition, there may be other adaptor or scaffold proteins in the plasma membrane involved in the coupling between receptors and Ca^{2+} entry channels.

Important to consider is that the time-dependence of SOC activation is of the order of tens or hundreds of seconds and this has been observed in numerous patch-clamp measurements of ICRAC activation and Ca^{2+} imaging experiments [2]. What accounts for the slow development of SOCs? One possibility is that the emptying of Ca^{2+} stores is itself protracted, reflecting Ca^{2+} release from areas remote from the PM [15]. Militating against this hypothesis are results from the use of ionophores [16] or the intraluminal Ca^{2+} buffer, TPEN [17]. Such agents are expected to lower Ca^{2+} rapidly and indiscriminately within the ER network, yet 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 [16,18]. Thus, in contrast to muscle triads which are structurally organized "precoupled" junctions, the activation of SOCs might result from induction of new coupling entities through the process we described earlier as "induced" coupling [2].

2. Are ER–PM interactions required for SOC activation?

Whereas there is evidence for the existence of junctional densities between peripheral cisternae of ER and the PM [19,20], it is not known whether such entities contain localized Ca^{2+} channels or pumps. However, studies have provided evidence that a close proximal relationship between ER and PM may be necessary in the pathway leading to SOC activation. Our experiments [16] revealed that induction of cortical actin polymerization or translocation of existing actin filaments to form a dense cortical actin band subjacent to the PM, in each case displaced ER from the vicinity of the PM and prevented coupling to activate SOCs. Coupling could be reestablished by cyto-

chalasin D-induced depolymerization of the cortical actin barrier. The results indicate that physical separation of ER and PM prevents the message originating from the ER which signals activation of SOCs, suggesting the coupling process involves close contact between the two membranes. These data agree with those of Yao et al. [18] using *Xenopus* oocytes in which physically applied distention of the cell membrane prevented coupling to activate SOCs by creating distance between the ER and PM. Moreover, Yao et al. provided evidence that Rho GTPase-mediated cytoskeletal assembly and trafficking, as well as the docking machinery involving SNAP-25, are each involved in SOC activation. Other evidence extends these observations and indicates that in platelets [21] and hepatocytes [22] the actin cytoskeleton is a significant mediator of coupling to activate SOCs. However, in our studies, we observed that the actin cytoskeleton was not required for store-operated Ca^{2+} entry [16]. Indeed, we could entirely disrupt the actin cytoskeleton with cytochalasin D and still see thapsigargin-induced Ca^{2+} entry. This signified to us that whatever structural domain between ER and PM was required for coupling, it was robust enough to withstand the highly disruptive process of cytoskeletal dismantlement. Moreover, if this close proximity between membranes is important for coupling, it does not appear be held together by the actin cytoskeleton. The findings from a number of labs paint a picture of an organized and spatially structured interaction between ER and PM being required for the activation of SOCs [2]. However, there is little direct 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 reestablished [16,18,21,23].

3. The role of the coupling domains and coupling proteins

It has been widely held by many in the field that the coupling process, be it conformational or chemical, takes place within spatially restricted domains at the PM. There is considerable evidence that Ca^{2+} entry channels (in particular, TRP channels) exist in a multimolecular complex with other functional proteins (including G proteins, PLC, and Ca^{2+} pumps) and adaptor/scaffold proteins [2,24]. A typical arrangement of the proteins involved in such a complex are shown in Fig. 2. In addition, there is evidence to support the location of entry channels and their attending proteins within specialized lipid-defined membrane domains (lipid rafts) [25]. Frequently, the existence of a "signalplex" of entry channels is based on analogies with the better characterized organization of TRP channels in *Drosophila* retina [26]. However, the highly specialized and organized phototransduction system in flies appears to function independently of Ca^{2+} stores or Ca^{2+} release channels [27]

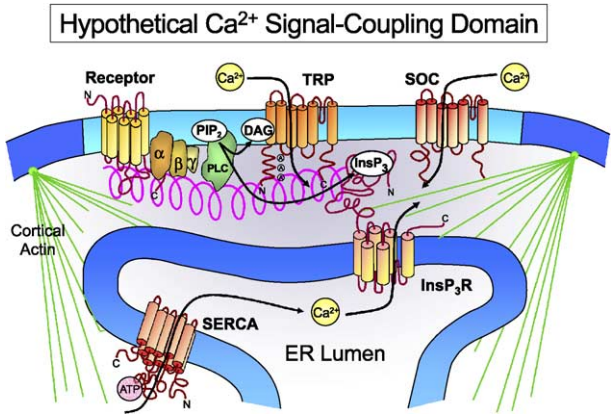

Fig. 2. A hypothetical Ca^{2+} signal-coupling domain. This domain is based on the model of Montell [26], describing the signaling molecules in *Drosophila* retina, although there is less evidence to support such a domain in mammalian Ca^{2+} signaling systems. GPCRs activate PLC-β resulting in cleavage of phosphatidylinositol bisphosphate (PIP2), and generation of membrane-retained DAG and cytosolic InsP3. DAG can directly activate TRP channels. The target for $InsP_3$, the ER $InsP_3R$, 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. The signaling complex is shown to be stabilized by cortical actin or an equivalent of the PDZ domain-containing scaffold protein INAD (shown as a purple coiled structure) known to organize the *Drosophila* retinal complex. SERCA: sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase pump.

and we should be wary of drawing such close analogies. Recently, it appears that regulation of Ca^{2+} entry channels may occur at the level of insertion and turnover in the membrane [28,29] and the macromolecular complex at the PM may have much to do with controlling this trafficking. The concept of conformational coupling rests heavily on the existence of coupling domains wherein ER (in particular, the $InsP_3R$ inserted in the ER membrane) may be juxtaposed with the PM (see Fig. 2). There have been few recent EM studies characterizing the occurrence of ER–PM densities and there is little direct information to place the $InsP_3R$ in such locations. While such entities would seem to be sparse in most cells, coupling domains wherein sensing of ER Ca^{2+} content is transmitted to the PM and/or entering Ca^{2+} can be directed efficiently into the ER, may necessarily be far and few between. Much study in the Ca^{2+} signaling field utilizes imaging at the light level which is largely inappropriate for resolving the putative coupling domains. Hence, it is probable that useful characterization of these entities will only come from reapplying EM analyses and trying to characterize the dimensions and constituents of such domains with high resolution.

In the fly retina, TRP channels appear to be organized by the PSD-95/Discs Large/ZO-1 (PDZ)-containing scaffold protein, INAD, and likely in intimate contact with the rhodopsin-triggered phototransduction machinery [27]. Less is known about organization of canonical TRP (TRPC) channels in mammalian cells, but there are indications that the PDZ-containing NHERF protein interacts with TRPC

channels [30] and may organize a spatial relationship with G protein-coupled receptors (GPCRs), PLC, and the cytoskeleton in an analogous fashion [31] as depicted in Fig. 2. It is likely that many different structural and adaptor proteins contribute to such a coupling complex. In recent studies, we determined that PLC-γ appears to play an adaptor-role independent of its enzymic activity in the coupling between receptors and the activation of Ca^{2+} entry [32]. Thus, experiments revealed that knocking down of PLC-γ 1 or PLC-γ2 isoforms prevented GPCRs (which activate PLC-β) from activating Ca^{2+} entry, even though there was still $InsP_3$-mediated Ca^{2+} release. In contrast, TG-induced Ca^{2+} entry was not affected. Using DT40 B-cells in which PLC-γ2 (the only isoform in B cells) was knocked out, again GPCR-induced Ca^{2+} entry was prevented without an effect on purely store-operated Ca^{2+} entry induced by TG. As shown in Fig. 3, using the same cells, we knocked-back in (i.e. transfected) PLC-γ2 and also a point mutated lipase-inactive mutant of PLC-γ2 (LIM). The B cell receptor

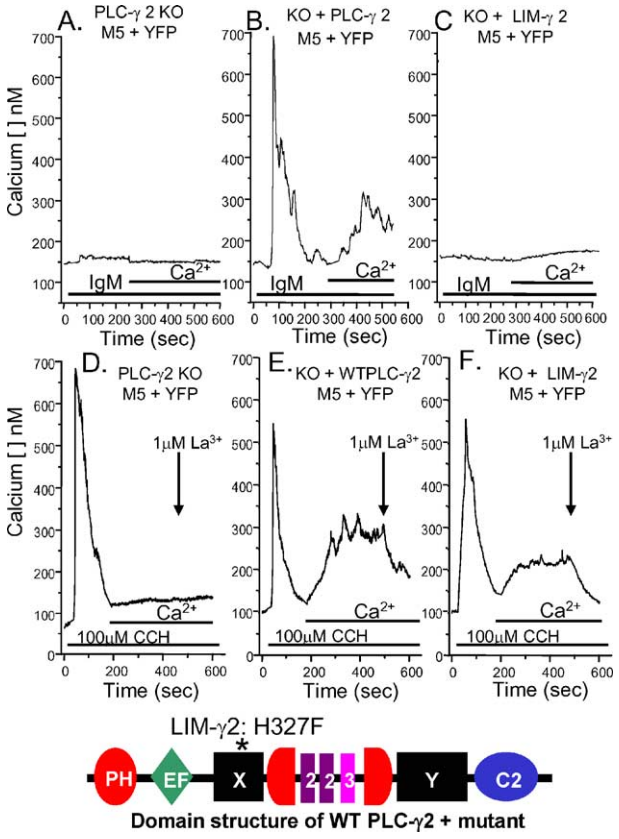

Fig. 3. A role for phospholipase C-γ2 in mediating receptor-induced Ca^{2+} entry. Wild-Type PLC-γ2 and a lipase-inactive mutant-γ2 (LIM-γ2) rescues receptor-induced Ca^{2+} entry in DT40 PLC-γ2 KO cells. Free Ca^{2+} measurements were made in KO cells transfected with either muscarinic M5 receptor (M5)+yellow fluorescent protein (YFP) alone, M5+YFP+rat PLC-γ2, or M5+YFP+rat-lipase-inactive mutant (LIM-γ2). Ca^{2+} stores were depleted by: (A–C) 3 μg/ml anti-IgM (IgM), in nominally Ca^{2+}-free medium followed by replacement with normal 1 mM Ca^{2+} medium; (D–F) 100 μM CCH (carbachol) in nominally Ca^{2+}-free medium followed by replacement with normal 1 mM Ca^{2+} medium, and addition of 1 μM La^{3+} (arrow). See Patterson et al. [32] for experimental details.

(BCR) in these cells activates the enzymic activity of PLC-γ2, and as expected the $InsP_3$-mediated Ca^{2+} release is restored in the PLC-γ2-knock-out cells by expression of wild-type PLC-γ2 but not by the lipase-inactive mutant (Fig. 3A–C). Significantly, whereas the PLC-γ2 knock-out cells were devoid of Ca^{2+} entry in response to the muscarinic receptor (a GPCR) (Fig. 3D), both wild-type and LIM PLC-γ2 restored Ca^{2+} entry in response to activation of the muscarinic receptor. The results indicate that PLC-γ2 plays a permissive role that is independent of its enzymic activity in mediating receptor—but not TG-induced Ca^{2+} release. PLC-γ isoforms have an array of protein-binding domains and appear to have a number of other adaptor roles independent of their enzymic activity [32]. Moreover, they are shown to interact directly with TRPC channels [32]. Thus, PLC-γ is an example of a PM protein that may fulfill the role of conveying information from the receptor directly to the Ca^{2+} entry channel (arrow C in Fig. 1). It also indicates an important distinction can exist in the activation of Ca^{2+} entry by receptors as opposed to that activated purely by store-emptying. As summarized diagrammatically in Fig. 4, in most cells there are two distinct receptor-mediated pathways that can activate Ca^{2+} signals. Either GPCRs functioning through PLC-β, or tyrosine kinase-coupled receptors that function through PLC-γ. Fig. 4 depicts a B cell in which the BCR complex, a series of kinases and adaptor proteins, are activated by BCR cross-linking, resulting in tyrosine phosphorylation and activation of PLC-γ. PLC-γ is playing a dual role of (a) mediating an enzymic production of DAG and $InsP_3$, and (b) mediating a permissive adaptor role that is necessary for GPCRs to activate Ca^{2+} entry channels. The implication from this is that PLC-γ may allow coupling to channels without necessarily requiring release of Ca^{2+} from stores. This point is further elaborated upon below.

4. The role of TRP channels: store-operated, receptor-operated, or both?

Much recent interest has been directed towards the TRP-family of ion channels and their possible relationship with SOCs [2,26,33]. A total of seven closely related genes encoding TRP channel counterparts to the original *Drosophila* TRP channels have been identified in mammals and designated TRPC1 through TRPC7 [26,34] (Fig. 5). The mammalian TRPC channels can be phylogenetically divided into four subgroups as shown, the channels within each subgroup being closely related in both structure and function [26]. Conserved throughout the *Drosophila* TRP and mammalian TRPC channels are a group of 6 trans-membrane-spanning segments, with an additional pore-forming hydrophobic domain between segments 5 and 6. The topology of this pore-forming domain is similar to that of voltage-gated channels [26], although the conserved voltage-sensing positively charged residues in the fourth

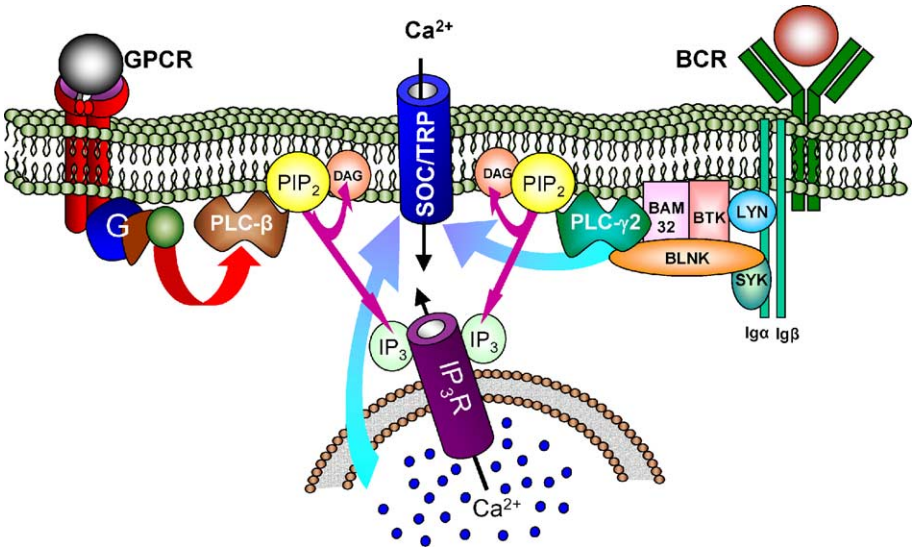

Fig. 4. Mediation of Ca^{2+} release and Ca^{2+} entry signals in response to G protein-coupled and tyrosine kinase-coupled receptors. The model depicts the dual signaling system in B cells involving either GPCRs coupling to the activation of PLC-β, or the B cell receptor which through the complex series of kinases and adaptor proteins shown, results in tyrosine phosphorylation and activation of PLC-γ2. PLC-γ plays a dual role of (a) mediating the enzymic production of DAG and $InsP_3$ in response to the BCR, and (b) mediating an adaptor role which is necessary for the ability of GPCRs to activate Ca^{2+} entry channels.

transmembrane segment of the voltage-gated channels, are notably absent in TRP channels (Fig. 5). Both *Drosophila* TRP and mammalian TRPC channels retain a number of conserved ankyrin homology sequences towards the N-terminus that may assist in association with cytoskeletal elements. The TRPC channels are part of a wider super-

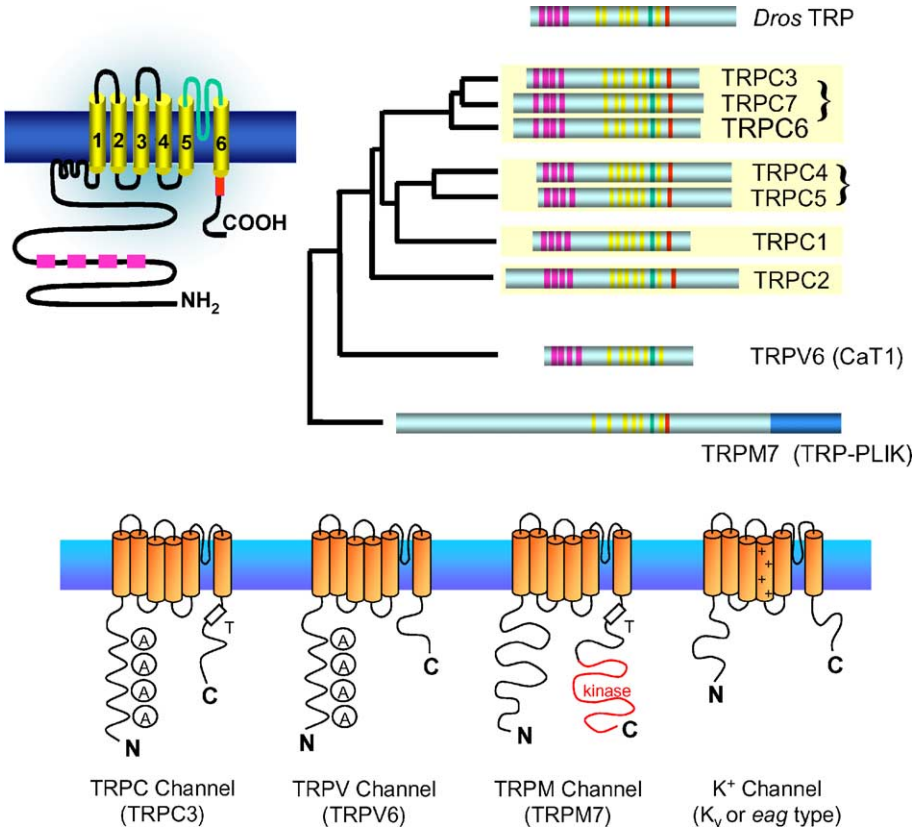

Fig. 5. Structural and phylogenetic relationship between TRP ion channels implicated in or related to store-operated Ca^{2+} entry. Proteins are aligned around the "pore-forming" loop (green). Also shown are other transmembrane segments (yellow), ankyrin-like repeats (purple), the kinase domain of TRPM7 (blue), and the "TRP-box" (red), a small highly conserved region (EWKFAR) in *Drosophila* TRP as well as mammalian TRPC and TRPV channels. A topological comparison of the structures of three TRP subtypes and a K^+ channel are shown below. "A" denotes ankyrin-like repeats.

family of TRP-related mammalian channel-like proteins which include several distinct subgroups [34]. Whereas much interest surrounds the possible role of TRPC channels in mediating store-operated Ca^{2+} entry, other members of the broader TRP-related family of channel proteins, in particular, members of the TRPV and TRPM subfamilies (Fig. 5), have also been implicated in this process.

The role of TRP channels in mediating store-operated Ca^{2+} entry is a controversial issue. TRP channels appear ubiquitously among cell types, and multiple TRP channel subtypes are expressed in most cells [2,33]. One problem with assessing the physiological role of TRP channels is that functional analysis of overexpressed channels is usually against a background of endogenous SOCs and TRP channels. Similarly, reduction of TRP channel expression by antisense or knock-out approaches can be confused by the functional overlap and redundancy with multiple endogenous TRP channel subtypes. One unifying characteristic among the *Drosophila* TRP and most members of the mammalian TRPC family, is their response to receptor-activated phospholipase C [26]. Thus, these channels can be classified as "receptor-operated" channels (ROCs). However, PLC activation and concomitant $InsP_3$ production assures at least some Ca^{2+} store release in most cell types. Therefore, the question of whether the TRPC channels function as SOCs as opposed to ROCs is difficult to answer and there is obvious overlap in these modes of entry. The "hallmark" that has been used to define store-operated Ca^{2+} entry is a response to store-depletion alone using Ca^{2+} pump blockers such as thapsigargin, or ionophores such as ionomycin [4]. However, we should consider carefully that store-emptying with these agents is not a physiological process per se. Physiologically, stores are released following PLC-coupled receptor activation, and, as considered later, the consequences of PLC activation are not limited to store-release alone.

There seems little disagreement that members of the quite diverse TRP family of channels do not yet fulfill the criteria of being either authentic CRAC or ARC channels. The closest characteristics to CRAC channels may be represented by the Ca^{2+}-selective TRPV5 and TRPV6 channels [35], but it is clear that there are a number of distinctions in channel properties from CRAC channels [36]. Whether exogenously expressed TRP channels operate in a "store-dependent" mode, is highly controversial, with studies claiming a number of TRPC, TRPM, and TRPV channels are activated when stores are emptied, and as many studies suggesting they are not (extensively reviewed in Ref. [2]). This abundance of apparently conflicting information on the coupling of TRPC channels to store-emptying is overwhelming at first sight. However, in heterologous expression systems in which the TRP channels are expressed alongside endogenous SOCs, the determination of whether TRP channels are directly activated by store-depletion as opposed to modifying or being modified by existing SOCs is difficult. In other words, it appears very hard to truly assess the TRPC channel phenotype in overexpression systems. In certain cell types, the high expression levels of specific TRPC channel subtypes has allowed more definitive analysis of endogenously expressed TRPC channels. For example, TRPC3 channels are highly expressed in pontine neurons and allow passage of Na^+ and Ca^{2+} ions in response to PLC-coupled receptors but are not activated by store-emptying [37]. A further explanation for variation in the observed activation of TRPC channels is that the channels themselves undergo assembly into multimeric structures [26]. Thus, it was originally observed that co-expression of the *Drosophila* TRP and TRPL channels in mammalian cells resulted in a store-operated phenotype, whereas singly expressed, the channels were store-independent [38]. However, whereas changes in the pore-properties and regulation of the channels can be observed after co-expression, in general, dramatic changes in the mechanism of activation (for example, store-dependence) of co-expressed TRPC channels have not been demonstrated [2,33].

Considering the above arguments on the physiological applicability of "emptying" stores artificially, we should be well served to remember that overexpressing channels and then subjecting them to such a severe stress condition as emptying stores with pump blockers or ionophores, is likely to result in spurious observations. Since many of the TRP channels are widely expressed, it is difficult to assess whether, using knock-out or knock-down approaches, there has been a definitive identification of the function of natively expressed TRP channels [10]. In some cases, Ca^{2+} signaling has been affected by such deletion approaches, but curiously, the "lost" currents do not correspond to those attributed to the overexpression of the corresponding channel. The properties of channels may be influenced not only by heteromeric makeup, but also by any one of the many proteins suggested to be in tight proximity in the molecular domains (or "signalplexes") referred to above. When overexpressed, particularly by transient overexpression which yields far higher channel levels than endogenous expression, it is very likely that channels are not assembled and organized as in native cells. We should also consider the importance of other classes of "receptor-activated" or "store-dependent" channels that, unlike CRAC, are not selective for Ca^{2+} ions. This is an increasingly voiced perspective [10] and it is likely that nonselective cation channels play crucial roles in mediating depolarization responses and hence contributing to the modification of a number of other voltage-dependent channels or electrogenic transporters (for example, the K^+ channels, Cl^- channels, or the Na–Ca exchanger).

For the TRPC family of proteins, it seems that their primary mechanism of activation is through PLC activation, making them functionally analogous to their closely resembling fly homologues. The role of $InsP_3$ and $InsP_3Rs$ in their activation is considered later. The other product of PLC, DAG, has a direct role in TRPC activation. Thus, the

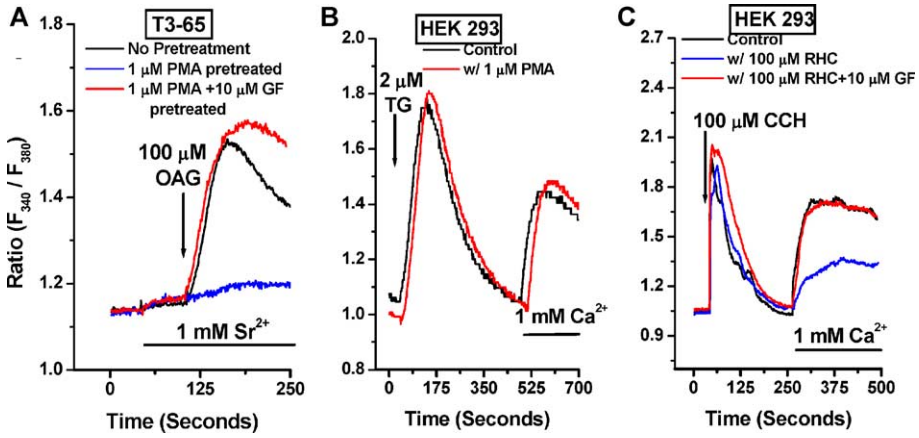

Fig. 6. Role of DAG and PKC in modulating the function of TRPC3 channels and endogenous SOCs. (A) TRPC3 channels in stably expressing T3-65 HEK293 cells are activated by the permeant diacylglycerol (DAG) analogue, 1-oleoyl-2-acetyl-in-glycerol (OAG). Treatment with the powerful PKC activator, PMA, completely blocks the stimulation by subsequent addition of DAG. However, the action of PMA is completely prevented by GF 109203X, a reliable aminoalkyl bisindolylmaleimide PKC blocker. In this case, the action of DAG is not only rapid, but longer lasting indicating that endogenously activated PKC likely results in the fast turnoff of TRPC3 channels. Thus, DAG has a bimodal action on TRPC3: short-term activation followed by later PKC-mediated deactivation. (B) Using control HEK293 cells, PMA has no effect on TG-induced Ca^{2+} entry. (C) In contrast, in the same cells, muscarinic receptor-induced Ca^{2+} entry is reduced 50% by the diacylglycerol lipase blocker, RHC-80267, suggesting that endogenous TRPC channels may contribute to physiological Ca^{2+} entry. See Venkatachalam et al. [45] for experimental details.

closely related TRPC3, TRPC6, and TRPC7 subgroup of channels (Fig. 4) are directly activated by DAG and its cell permeant analogues [7,39–42]. This action of DAG is independent of PKC [7,40] and is shown to be mediated by an N-terminal domain in the TRPC6 channel [43]. However, the response to DAG appears subgroup-specific; overexpressed TRPC1, TRPC4 and TRPC5 channels do not respond to DAG [7,44,45]. Interestingly, we recently determined that DAG has an important dual role in TRPC channels [45,46]. Thus we determined that, while not activating TRPC4 and TRPC5 channels, DAG in fact has a profound inhibitory effect on both channels. Moreover, this inhibitory effect was clearly mediated through PKC (in contrast to the stimulatory action on TRPC3 channels). The effect was not only seen with exogenously added DAG. Thus, we could modify endogenously produced DAG by manipulating both DAG kinase and DAG lipase the two major metabolizing enzymes controlling turnover of DAG [45]. As shown in Fig. 6, we were also able to show that this same PKC-mediated inhibition functions on TRPC3 chan-

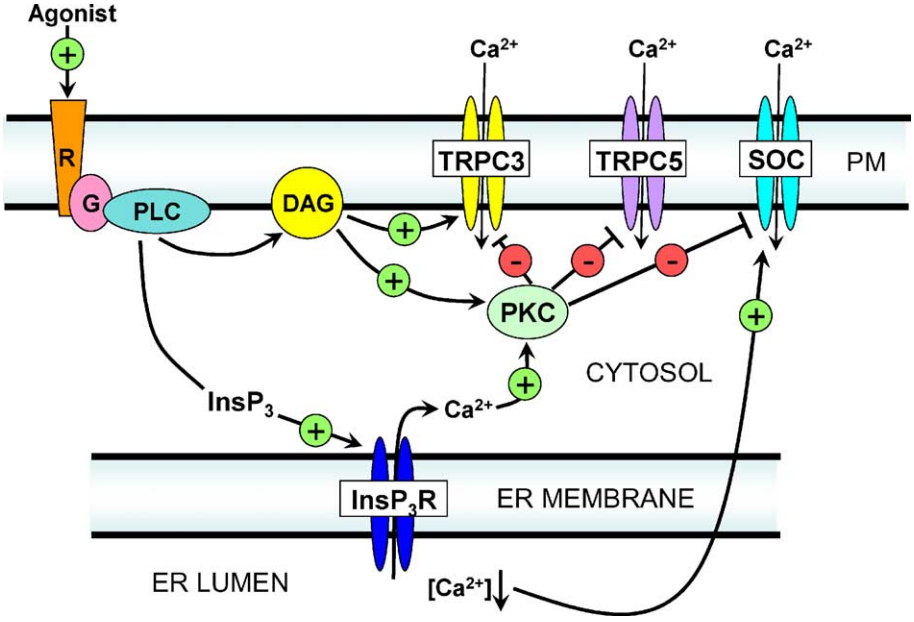

Fig. 7. Model depicting the actions of DAG and PKC on SOCs and TRPC channels. The TRPC channels all appear to be blocked by DAG-induced PKC, but only TRPC3 (and likely TRPC6 and 7) is activated. What accounts for receptor-mediated activation of TRPC5 channels is not known. See Venkatachalam et al. [45] for further details and discussion.

nels. As shown in Fig. 6A, TRPC3 channels in stably expressing T3-65 HEK293 cells are activated by the permeant DAG analogue, OAG. Treatment with the powerful PKC activator, PMA, blocks the stimulation by subsequent addition of DAG. However, the action of PMA is completely prevented by GF 109203X, a reliable aminoalkyl bisindolylmaleimide PKC blocker. In this case, the action of DAG is not only rapid, but longer lasting indicating that endogenously activated PKC likely results in the fast turnoff of TRPC3 channels. Thus, with TRPC3 channels, the single molecule has two opposing actions on the same channel. DAG clearly activates the channel through a PKC-independent mechanism. Thereafter, there appears to be a slower DAG-induced PKC-mediated inhibition of the channel, suggesting that this is an important feedback mechanism. This feedback mechanism takes on yet added significance considering that the Ca^{2+} entering the cytoplasm through the TRP channels will enhance the activation of PKC and hence the turning off of the channel. Interestingly, we were able to show that Ca^{2+} entry induced by receptors in nontransfected HEK293 cells was partially (50%) blocked by PKC activation (Fig. 6C), suggesting that endogenous TRPC-like channel activity may account for some of the entry. Significantly, TG-induced Ca^{2+} entry was unaffected by PKC (Fig. 6B). This provides another powerful example of differences between receptor-operated as opposed to purely store-operated Ca^{2+} entry. A summary of the actions of DAG and PKC on Ca^{2+} entry channels is shown in Fig. 7. Despite all this information, one question remains a mystery, that is, the question of what mediates the receptor-induced activation of TRPC4 and TRPC5 channels. Thus, enigmatically, they appear to be receptor-activated identically as the TRPC3/6/7 group of channels, yet they are not activated by DAG. Another aspect of the action of DAG is that it has been difficult to show that endogenously expressed TRPC3/6/7 channels are activated by DAG. Thus, we have to consider that DAG may not be the physiological activator of these channels. We and others have speculated that, in addition to DAG and $InsP_3$ production, another consequence of receptor-induced PLC activation is a decrease in PIP2. Hence, the possibility that the activation of TRPC channels may reflect decreased PIP2 levels is an area of investigation worthy of considerable attention.

5. Cross-talk between entry channels and release channels

By its nature, store-operated Ca^{2+} entry involves integration of signals between two membranes. Insights into the functioning of this process have arisen from studies indicating that the $InsP_3R$ may be a key player in the activation of Ca^{2+} entry as originally predicted by Irvine [47] and Berridge [19]. Certainly, the $InsP_3R$ is logistically strategic for this purpose being the sensor of the original receptor-generated $InsP_3$ signal, the mediator of the initial Ca^{2+} release signal, and, by virtue of cytosolic and luminal Ca^{2+} sensing [19], able to respond to Ca^{2+} 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 Ca^{2+} entry channels. However, whereas structural evidence for TRP channel-$InsP_3R$ interactions is compelling, the evidence for a role of $InsP_3$ receptors in SOC activation is controversial.

Kiselyov et al. [48] examined the function of mammalian TRPC3 channels stably expressed in HEK293 cells. In whole cell experiments, TRPC3 channel function was stimulated by PLC-coupled receptors, and to a lesser extent by store-depletion [48]. Upon 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 [48]. $InsP_3R$ constructs containing the N-terminal $InsP_3$ binding domain were sufficient to couple to TRPC3 channels and render them activatable by $InsP_3$ [49]. The remainder of the $InsP_3R$ was required to confer store-sensitivity by sensing the degree of store-emptying. Boulay et al. [50] extended this revealing a relatively short segment within the C-terminus of TRPC3 was required for interaction with the $InsP_3R$, and two specific $InsP_3R$ sequences close to the $InsP_3$ binding domain interacted with TRPC3. Peptides generated from the interacting regions of the TRPC3 channel and $InsP_3R$ induced a small but significant reduction in the activation of SOCs in normal cells, suggesting that native SOCs activate through $InsP_3Rs$ [50]. Studies have shown that $InsP_3Rs$ interact with an inhibitory calmodulin-binding site on TRPC3, displacing calmodulin and thereby activating the channel [51,52]. In addition to TRPC3, the $InsP_3R$ was shown to interact with TRPC4 channels and, interestingly, this interaction was not seen with a TRPC4 C-terminal splice variant, indicating that alternative splicing might regulate the $InsP_3R$/TRPC4 association [53]. TRPC1 is reported to specifically interact with type-II $InsP_3Rs$, but not type-I or type-III and this interaction is activated by store-depletion [54]. Moreover, this interaction was shown to be prevented in vivo by the same modifications of the cortical actin cytoskeleton [55] which were shown to prevent coupling to activate endogenous SOCs [16,39].

Even though this is quite compelling evidence for TRPC-$InsP_3R$ interactions, the question of the role of $InsP_3Rs$ in physiological store-operated Ca^{2+} entry is less clear. The actions of the two $InsP_3R$ antagonists, 2-aminoetyhoxydiphenyl borate (2-APB) and xestospongin C, to block both endogenous store-operated Ca^{2+} entry and receptor-induced TRPC3 activation was considered evidence for a mediating role of $InsP_3Rs$ in the activation of both channels [39]. However, more compelling were

studies revealing that in the DT40 B-lymphocyte triple $InsP_3R$ knock-out variant line, store-operated Ca^{2+} entry was entirely identical to that observed in wild-type cells [41,56–59]. Exhaustive searching for the presence of $InsP_3Rs$ in these knock-out cells by examination of transcripts, full-length proteins or fragments thereof, $InsP_3$-binding activity, or physiological $InsP_3R$-mediated Ca^{2+} release in intact or permeabilized cells, in all cases confirmed the absence of all $InsP_3Rs$ in these cells [41,56–58]. Moreover, the blocking action of 2-APB on CCE was unaltered by $InsP_3R$ elimination [57–59], this and further studies indicating that its action was either upon the channel [58,60,61] or, more interestingly, upon the coupling machinery for SOCs [59,62]. However, TRPC3 channels expressed rapidly and with high efficiency in DT40 cells were shown by Venkatachalam et al. [41] to be activated by PLC-coupled receptors independently of $InsP_3R$ expression and independently of store-emptying. Intriguingly, Vazquez et al. [63] observed that 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. While apparently enigmatic, coupling phenotype appears to rest on expression conditions [2,64,65]. On the one hand, 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 Ca^{2+} stores [41]. On the other hand, 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 the $InsP_3R$ clearly has input [63]. 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 channel expression levels, an excess of channels relative to coupling machinery results in the majority of channels being uncoupled from stores. At lower expression levels, the stoichiometry of channel and coupling machinery permits a high fraction of coupled channels. This explanation is elaborated upon at greater length in two recent reviews [2,65].

Returning to the coupling role of $InsP_3Rs$ in the activation of physiological receptor-induced Ca^{2+} entry, the original hypothesis of Irvine [47] and Berridge [19] predicted that that $InsP_3Rs$ in the ER involved in direct conformational coupling to PM entry channels might be non-conducting. Indeed, SOCs appear exquisitely sensitive to raised cytosolic Ca^{2+} [4] and their operation in the immediate vicinity of Ca^{2+} release would seem paradoxical. Using $InsP_3R$ knock-out cells, we obtained recent data indicating that pore-dead $InsP_3R$ mutants can indeed

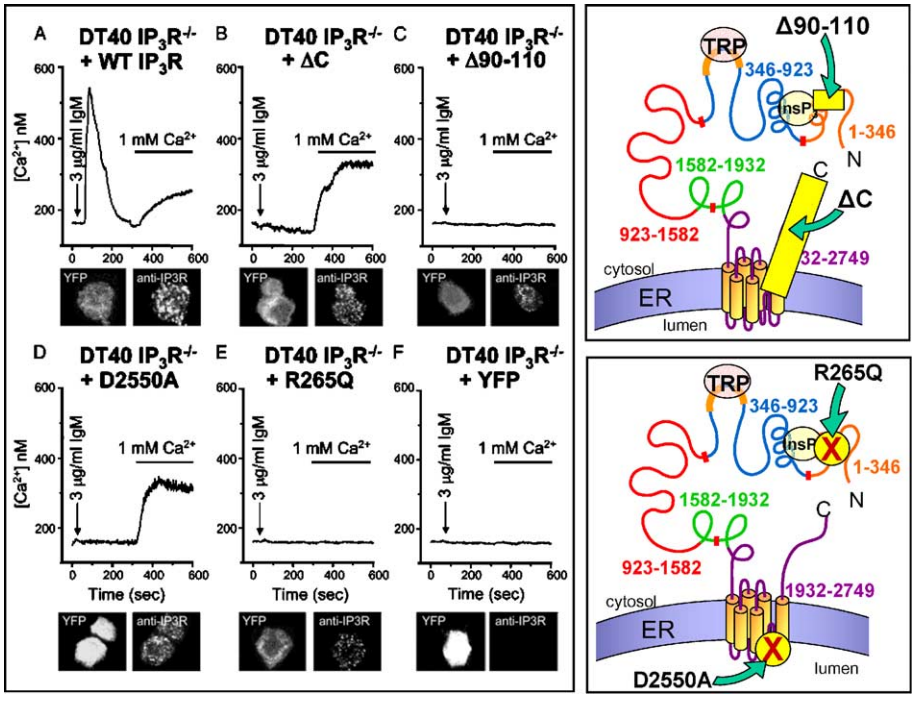

Fig. 8. Coupling to activate receptor-induced Ca^{2+} entry is mediated by a channel-deficient type 1 IP_3Rs in DT40 $IP_3R^{-/-}$ B cells but not by IP_3-binding-deficient type 1 IP_3Rs. Free Ca^{2+} measurements were made in DT40 $IP_3R^{-/-}$ knock-out cells transfected with either YFP plus WT IP_3R (WT; A), YFP plus C-terminal IP_3R mutant (C; B), YFP plus 90–110 IP_3R mutant (90–110; C), YFP plus D2550A IP_3R mutant (D2550A; D), YFP plus R265Q IP_3R mutant (R265Q; E), or YFP alone (F). Ca^{2+} stores were released in cells by 3 μg/ml anti-IgM (arrow) in nominally Ca^{2+}-free medium followed by replacement with anti-IgM and normal 1 mM Ca^{2+} medium (bar). DT40 $IP_3R^{-/-}$ knock-out cells, transfected as above, were prepared for immunocytochemistry and visualized by confocal microscopy, and they appear directly below their respective Ca^{2+} trace. (A–F) The left images corresponds to YFP expression, and the right images corresponds to IP_3R staining. See van Rossum et al. [66] for experimental details.

couple to mediate entry activated by receptors [66]. In these experiments, the DT40 triple knock-out cells devoid of all $InsP_3Rs$ were utilized and transfected with a series of mutated type 1 $InsP_3Rs$. As shown in Fig. 8, expression of wild-type $InsP_3R$ resulted in the reappearance of both release and entry of Ca^{2+} activated by BCR cross-linking (Fig. 8A). A C-terminal deletion mutant devoid of part of the pore-forming domain was unable to support Ca^{2+} entry as expected. Significantly, however, receptor-induced Ca^{2+} was observed with this mutant (Fig. 8B). In contrast, a mutant missing the 90–100 sequence within the $InsP_3$-binding domain, was unable to support either release or entry (Fig. 8C). We sought to use more precise mutants to verify these results. As shown in Fig. 8D, the D2550 point mutation which is devoid of any channel activity, was also able to support receptor-induced Ca^{2+} entry. However, the single R265Q point mutation in the $InsP_3$ binding domain which is unable to bind $InsP_3$, was unable to mediate either release or entry (Fig. 8E). Thus, the results indicate a consistent ability of the $InsP_3$ receptor to support receptor-induced Ca^{2+} entry only when the $InsP_3R$ retains its ability to recognize $InsP_3$. However, the $InsP_3R$ does not need to be able to conduct Ca^{2+} across the ER membrane in order to couple to activate Ca^{2+} entry. However, since thapsigargin-induced Ca^{2+} entry or CRAC channel activation both appear independent of $InsP_3Rs$ [57,60], this suggests a significant dichotomy between receptor—as opposed to purely "store-operated" Ca^{2+} entry. In addition, the original conformational coupling prediction appears to be supported by functional evidence.

6. Concluding comments

Finally, our concepts on the activation of receptor-induced Ca^{2+} entry are undergoing considerable rethinking and introspection It seems that stores are unlikely "emptied", but a signal likely derives from a modest diminution of luminal Ca^{2+} resulting in a change in the steady state entry of Ca^{2+}. It is also appears that physiological receptor activation can induce Ca^{2+} entry which does not necessarily require store-depletion, even though it may be usual that some albeit modest or restricted store-release accompanies receptor-activation. A number of different channel molecules are likely to be involved. Certain channels may be responsive to both the receptor- and store-induced signals, whereas the two channel activities so far defined, ARC and CRAC, appear to be more exclusive in their responses to these signals. Although we sometimes consider receptor- and store-derived signals as distinct, in reality they may be closely integrated. Moreover, the spectrum of responses from low agonist activation to global store depletion may provide cells with the ability to generate a broad and dynamic range of Ca^{2+} signal responses. The coupling to activate Ca^{2+} entry is a captivating signaling problem representing a most challenging combination of physical interactions and functional coupling between proteins organized within at least two distinct membranes. Solving this problem will provide valuable new information on the generation and possible manipulation of Ca^{2+} signals in all cells.

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