IP_3 receptors and Ca^{2+} entry

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Highlights

- IP_3Rs mediate Ca^{2+} release from ER and thereby store-operated Ca^{2+} entry (SOCE)
- IP_3Rs parked near ER-PM junctions may locally deplete ER and facilitate SOCE
- In *Drosophila*, IP_3Rs also regulate SOCE downstream of depleting the ER of Ca^{2+}
- In some cells, small numbers of PM IP_3Rs directly mediate Ca^{2+} entry

ABSTRACT

Inositol 1,4,5-trisphosphate receptors (IP_3R) are the most widely expressed intracellular Ca^{2+} release channels. Their activation by IP_3 and Ca^{2+} allows Ca^{2+} to pass rapidly from the ER lumen to the cytosol. The resulting increase in cytosolic [Ca^{2+}] may directly regulate cytosolic effectors or fuel Ca^{2+} uptake by other organelles, while the decrease in ER luminal [Ca^{2+}] stimulates store-operated Ca^{2+} entry (SOCE). We are close to understanding the structural basis of both IP_3R activation, and the interactions between the ER Ca^{2+}-sensor, STIM, and the plasma membrane Ca^{2+} channel, Orai, that lead to SOCE. IP_3Rs are the usual means through which extracellular stimuli, through ER Ca^{2+} release, stimulate SOCE. Here, we review evidence that the IP_3Rs most likely to respond to IP_3 are optimally placed to allow regulation of SOCE. We also consider evidence that IP_3Rs may regulate SOCE downstream of their ability to deplete ER Ca^{2+} stores. Finally, we review evidence that IP_3Rs in the plasma membrane can also directly mediate Ca^{2+} entry in some cells.

Keywords:

Ca^{2+} entry

Ca^{2+} puff

Ca^{2+} signal

Endoplasmic reticulum

IP_3 receptor

Ryanodine receptor

Store-operated Ca^{2+} entry

STIM

1. Introduction

All animal cells maintain steep Ca^{2+} concentration gradients across the plasma membrane (PM) and across the membranes of at least some intracellular organelles, most notably the endoplasmic reticulum (ER). Other intracellular organelles, including the Golgi apparatus [1], nuclear envelope [2] and lysosomes [3] also provide intracellular Ca^{2+} stores. Regulated opening of Ca^{2+}-permeable channels within these membranes allow cells to call upon either finite intracellular Ca^{2+} stores to generate cytosolic Ca^{2+} signals or the effectively unlimited pool of extracellular Ca^{2+}. The resulting Ca^{2+} signals then regulate many different cellular activities, the nature of which may depend on the source of the Ca^{2+} [4]. A diverse array of Ca^{2+}-permeable channels is expressed in the PM of different cells, including channels regulated directly by extracellular stimuli, membrane potential, temperature changes, stretch and various intracellular signals. By contrast, only two major families of related intracellular Ca^{2+} channels are thought to be responsible for linking extracellular stimuli to release of Ca^{2+} from the ER. The first of these are ryanodine receptors (RyR), which are opened either by their direct interaction with a voltage-sensing Ca^{2+} channel in the PM (RyR1 in skeletal muscle) or else by Ca^{2+} (RyR2 and RyR3), most commonly delivered to them when depolarization evokes Ca^{2+} entry through voltage-gated Ca^{2+} channels in the PM, in heart for example [5]. RyR may also be stimulated by cyclic ADP-ribose, but this is contentious [6]. The second major family of intracellular Ca^{2+} release channels, and the focus of this review, comprises the inositol 1,4,5-trisphosphate receptors (IP_3R) [7]. These are more widely expressed than RyR, with almost all animal cells expressing at least one of the three IP_3R subtypes. Since IP_3Rs open only when they have bound IP_3 [8], they link receptors in the PM that stimulate phospholipase C (PLC), and thereby IP_3 formation, to release of Ca^{2+} from the ER (**Fig. 1A**).

One of several recurrent themes in Ca^{2+} signalling is the interaction between different sources of Ca^{2+} [9]. In heart, for example, Ca^{2+} entering the cell across the PM triggers Ca^{2+} release from the sarcoplasmic reticulum (SR) through RyR2. Similar amplification of Ca^{2+} entry signals by Ca^{2+}-induced Ca^{2+} release (CICR) through RyRs occurs in other cells too. An even more widespread interaction between ER and PM underlies store-operated Ca^{2+} entry (SOCE), wherein loss of Ca^{2+} from the ER stimulates Ca^{2+} entry through PM channels. Abundant evidence has vindicated the idea, first developed by Jim Putney [10], that store depletion is itself sufficient to activate SOCE. Foremost amongst this evidence is the oft-repeated demonstration that depletion of ER Ca^{2+} stores by inhibiting their Ca^{2+} pumps with thapsigargin or cyclopiazonic acid is sufficient to stimulate SOCE [11, 12]. An obvious

appeal of this scheme is that IP_3, by releasing Ca^{2+} from the ER, coordinates both phases of the Ca^{2+} signal: an initial Ca^{2+} release followed by a sustained SOCE. When the stimulus provoking IP_3 production is removed, the IP_3Rs close, the ER Ca^{2+} stores can refill, and SOCE is switched off.

In this short review, we consider three further features of the relationship between IP_3Rs and Ca^{2+} entry. We first describe results suggesting that physiological stimulus intensities may selectively deplete an ER sub-compartment that preferentially regulates SOCE. We then consider evidence that IP_3Rs, independent of their ability to release Ca^{2+} from the ER, may contribute to regulation of SOCE. Finally, we describe the presence of IP_3Rs within the PM, where they can contribute directly to Ca^{2+} entry. We begin with short overviews of IP_3Rs and SOCE.

2. **Regulation of IP_3Rs and SOCE**

2.1. Regulation of IP_3Rs

IP_3Rs are tetrameric structures, assembled from closely related subunits (IP_3R1-3). The subunits differ in their distributions, affinities for IP_3, and modulation by the many additional signals that regulate IP_3Rs [13]. Discussion of the latter lies beyond our present scope, but it is noteworthy, in the context of our focus on SOCE and IP_3Rs, that both cAMP [14] and cAMP-dependent protein kinase (PKA) [15, 16] regulate IP_3Rs, while SOCE regulates the activity of some adenylyl cyclases [17]. Despite the differences between IP_3R subtypes, they all share core properties. All IP_3Rs form large-conductance Ca^{2+} channels with relatively weak selectivity for Ca^{2+} over K^+ [18]. They are all expressed predominantly within the ER, although they are also present in the nuclear envelope [2], Golgi apparatus [1], and perhaps more rarely, in the PM [19] (Section 5). Most importantly, all IP_3Rs are co-regulated by IP_3 and cytosolic Ca^{2+} [9, 18]. Both ligands are required for an IP_3R channel to open. The simplest scheme suggests that binding of IP_3 primes IP_3Rs to bind Ca^{2+}, and Ca^{2+} binding then triggers channel opening (**Fig. 1B**) [20, 21]. This interplay is important because it allows IP_3Rs to amplify, through CICR, the Ca^{2+} signals evoked by neighbouring IP_3Rs or by other Ca^{2+} channels (**Fig. 1C**) (Section 3.1). Higher concentrations of cytosolic Ca^{2+} inhibit IP_3Rs [22, 23]. There is, therefore, a biphasic dependence of IP_3R gating on cytosolic Ca^{2+} concentration: low Ca^{2+} concentrations are stimulatory, while higher concentrations inhibit [22, 23].

The IP_3R has a structure reminiscent of a square mushroom [24, 25] (**Fig. 1A,D**). Most of the stalk is embedded in the ER membrane and the cap, with a diameter of ~25 nm, extends

~13 nm into the cytosol. The large size is relevant because it may exclude IP_3Rs from the narrow junctions between the ER and the PM [26], but at other junctions, between ER and mitochondria or lysosomes for example, it puts the head of the IP_3R, where Ca^{2+} exits, close to the neighbouring organelle. Within the ER membrane, there are thought to be 24 transmembrane domains (TMDs), six from each IP_3R subunit, although a recent report suggests the possible presence of two additional TMDs between TMD1 and TMD2 [25]. The structure of this transmembrane region is similar in RyRs and, to a lesser extent, in voltage-gated cation channels. The ion-conducting path is lined by the four tilted TMD6 helices, with a short (~1 nm) 'selectivity filter' at the luminal end, through which hydrated cations must pass in single-file. The selectivity filter, its supporting pore-loop helix and a flexible luminal loop are all formed by residues linking TMD5 to TMD6. Near the cytosolic end of TMD6, a narrow hydrophobic constriction blocks the movement of ions in the closed channel. The hydrophobic side-chains of these residues must move for the pore to open. Opening of RyR is associated with splaying and bowing of TMD6, such that the occluding hydrophobic side-chain is displaced [27]. A similar mechanism may open the IP_3R pore. TMD6 extends well beyond the ER membrane (~1.5 nm) and then terminates in a short α-helical bundle (the linker, LNK) that includes a Zn^{2+}-finger motif and aligns parallel with the ER membrane [24, 25]. Hence, structures formed by the TMD5-6 loop guard the luminal entrance to the pore, whereas the cytosolic exit is formed by the extended TMD6. Each of these regions is enriched in acidic residues that probably contribute to the cation selectivity.

Activation of an IP_3R begins when IP_3 binds to the clam-like IP_3-binding core (IBC) (**Fig. 1E**). This is located towards the N-terminal of the primary sequence of each subunit, and comprises two domains (α and β), with the pocket between them providing the positively charged residues that interact with IP_3 [28]. All four subunits must bind IP_3 before the channel can open [8]. The N-terminal region forms a triangular structure at the cap of the mushroom. The N-terminal domain (suppressor domain, SD; also known as β-trefoil domain 1, BTF1) and IBC-β (BTF2) line the cytosolic exit of the channel. Adjacent subunits interact through a loop within the SD that contacts the neighbouring IBC-β [24, 25, 29, 30]. IBC-α lies behind this structure at the tip of a series of largely α-helical domains (ARM 1-3) that extend in a boomerang-like shape to meet the LNK domain (**Fig. 1F**). IP_3 binding causes the IBC clam to partially close [25, 29, 31], and this conformational change is linked to IP_3R gating through the SD, but the exact sequence is unresolved. It may be that movement of IBC-α relative to a firmly anchored IBC-β/SD re-orients the ARM domains [25], or it may be that tethering of IBC-α to the SD causes re-orientation of the SD and disruption of inter-

subunit interactions [27, 29, 32]. In any event, it is clear that IP_3 binds some 7 nm away from the hydrophobic constriction of the closed pore, and the IP_3-evoked conformational changes must pass through the contacts between ARM3 and LNK domains (**Fig. 1F**).

ARM3 terminates in the intervening lateral domain (ILD), which is sandwiched between the cytosolic structures and the TMDs, runs largely parallel with the ER membrane, and comprises two β-strands (which sit immediately beneath ARM3) followed by a helix-turn-helix motif that links to TMD1 (**Fig. 1F**). The LNK domain (an extension of TMD6) sits between the β- and α-helical components of the ILD. Hence, interleaved structures formed by extensions of ARM3 (ILD) and TMD6 (LNK) form the critical nexus between the cytosolic region and the pore of the IP_3R. Mutations within ILD disrupt IP_3R function [30], and the LNK domain contributes a conserved residue to a Ca^{2+}-binding site at the base of the ARM3 domain (**Fig. 1F**) [25]. This Ca^{2+}-binding site, formed by residues at the interface of the cytosolic (base of ARM3) and pore (LNK domain) regions, suggests an appealing, but untested, link between Ca^{2+} and gating of the IP_3R. A high-resolution structure of IP_3R3 recently identified another Ca^{2+}-binding site, which is also formed by residues provided by different domains across a domain interface (ARM1 and ARM2) [25]. It is not yet clear how (or whether) either of these Ca^{2+}-binding sites relates to stimulation and inhibition of IP_3Rs by cytosolic Ca^{2+}. It is, however, intriguing that both sites are formed by residues contributed by different domains, consistent with IP_3-evoked rigid-body movements of domains influencing whether Ca^{2+} binds to these sites.

We are getting closer to understanding how IP_3 binding to the IBC causes pore residues some 7 nm distant to move and allow Ca^{2+} to pass from the ER lumen to the cytosol. IP_3R activation is initiated by closure of the clam-like IBC, the conformational consequences of which must pass through the critical nexus formed between the cytoplasmic and pore domains at the ILD-LNK complex. Recalling that IP_3 primes IP_3Rs to bind Ca^{2+}, which then triggers channel opening [21] (**Fig. 1B**), we can speculate that clam closure is communicated to either of the Ca^{2+}-binding sites, at the ARM1-ARM2 interface or at the LNK-ARM3 nexus. The conformational changes evoked by Ca^{2+} binding must then pass through the ILD-LNK complex to cause movement of the hydrophobic residues in TMD6 that occlude the closed pore (**Fig. 1F**).

2.2. Regulation of SOCE

SOCE is stimulated by loss of Ca^{2+} from the ER. It is a feature of most, and perhaps all, animal cells. After a long history, which included suggestions that IP_3R might

conformationally couple empty Ca^{2+} stores to PM Ca^{2+} channels [33], it is now clear that there are two essential players in SOCE [34-36]. Stromal interaction molecule (STIM) is the sensor of ER Ca^{2+} content, and Orai is the low-conductance, highly Ca^{2+}-selective PM channel regulated by STIM [12]. The two homologues of STIM (STIM1 and 2) differ in their Ca^{2+} affinities and efficacy in activating Orai [37, 38]. STIM1 is probably the major link between receptor-evoked store-depletion and SOCE activation [39], although it may cooperate with STIM2 [37]. Three genes encode Orai proteins (Orai1-3), all three of which can form homo- or hetero-hexameric STIM-gated Ca^{2+} channels, although there are subtle differences in their behaviours [12]. Each Orai subunit has 4 TMDs and cytosolic N- and C-termini. The Ca^{2+}-selective pore of Orai is lined by TMD1 from each of the six subunits. Until recently, the oligomeric state of the functional Orai1 channel was contentious [12], but elegant use of concatameric constructs has confirmed that the functional channel [40], like the high-resolution structure [41], is a hexamer. Furthermore all six C-termini of the Orai hexamer must bind STIM1 for the channel to open [42]. STIM is expressed (though not exclusively) in ER membranes, it has a single TMD, a luminal N-terminal that includes the Ca^{2+}-sensing canonical EF-hand, and a C-terminal cytosolic region. The latter includes a polybasic C-terminal tail through which STIM interacts with PM lipids, probably phosphatidylinositol 4,5-bisphosphate (PIP_2); and the CRAC-activation domain (CAD), through which STIM activates Orai. It is noteworthy, for future discussion (Section 4), that *Drosophila* STIM lacks the polybasic C-terminal tail.

STIM is a dimer in unstimulated cells. Dissociation of Ca^{2+} from the EF hand of STIM promotes either strengthening of the existing association within a dimer or further oligomerization. This then triggers unmasking of the cytosolic CAD domain and polybasic tail [43]. The latter allows STIM1 to be trapped, as puncta, by PM lipids at largely preformed junctions, where the ER membrane and PM are about 10-20 nm apart. The separation, maintained by scaffold proteins [44-47], is important because it allows STIM, with its CAD domain, to reach out from the ER to the C-termini of Orai subunits in the PM. But it is significant also because the gap is probably too narrow to accommodate IP_3Rs (Section 3.1). Within the ER-PM junctions, STIM1 traps Orai, so that each is retained within the junction, where they form coincident puncta. At the cytosolic end of TMD4 of Orai, there is a long α-helix that aligns parallel to the PM and pairs with its partner from the neighbouring TMD4 [41] to provide three pairs of anti-parallel helices with which the CAD domain of STIM interact to open the channel. Since Orai can bind several STIM, and STIM is itself

multimeric, the STIM-Orai interaction can assemble a tightly packed cluster of Orai channels within the ER-PM junction [38, 48].

While STIM and Orai are the essential components of SOCE, many additional proteins associate with them to regulate their activities or modulate the ensuing Ca^{2+} signals [49]. These include septins, which contribute to organizing the ER-PM junction [50]; STIMate, which enhances STIM activity at ER-PM junctions [51]; Sigma 1 receptors, which associate with STIM and attenuate its coupling to Orai [52]; a complex of junctate and junctophilin-4 [53, 54] that assists STIM recruitment; CRACR2A [55] and SARAF [56], which provide cytosolic Ca^{2+} regulation of the association between STIM and Orai; and POST, which is recruited to junctions by STIM and then regulates Ca^{2+} extrusion by the PM Ca^{2+} pump [57]. Regulation of the ER-PM junctions themselves provides another level of control for SOCE [58].

In the context of the present review, two features of SOCE are particularly pertinent. First, under physiological conditions SOCE is usually activated by loss of Ca^{2+} from the ER through IP_3Rs. Second, activation of SOCE depends steeply on the ER free Ca^{2+} concentration, such that substantial loss of Ca^{2+} from the ER is required before SOCE is detectably activated by STIM1 [39, 59, 60]. We return to these issues in the next section.

3. Licensed IP_3Rs may selectively activate SOCE

3.1. Immobile IP_3Rs adjacent to the PM are licensed to respond

High-resolution optical imaging, pioneered by Ian Parker, has revealed the subcellular organization of IP_3-evoked Ca^{2+} signals in cells loaded with a fluorescent Ca^{2+} indicator [61-63]. These 'optical patch-clamp' methods reveal the brief openings of individual IP_3Rs (as 'Ca^{2+} blips'); the co-ordinated opening of several (typically fewer than 10) IP_3Rs within small clusters ('Ca^{2+} puffs'); and the regenerative propagation of intracellular Ca^{2+} waves, which initiate more frequently as the IP_3 concentration increases [64, 65]. This hierarchy of Ca^{2+} release events is assumed to arise from CICR as higher concentrations of IP_3 prime more IP_3Rs to respond to Ca^{2+} diffusing to them from nearby active IP_3Rs (**Fig. 1C**). The large local Ca^{2+} signals delivered by Ca^{2+} puffs, which are a feature of all IP_3R subtypes [66], may allow local control of cytosolic effectors with relatively low affinity for Ca^{2+}. Hence, as the stimulus intensity increases, the nature of the cytosolic Ca^{2+} signal changes from local to global, and that provides opportunities for encoding Ca^{2+} signals in both spatial and temporal domains [65]. The extent to which IP_3-evoked Ca^{2+} signals are amplified by this CICR mechanism, and so progress through the hierarchical sequence, depends on the concentration

of IP_3 and the separation of IP_3Rs. It is important to recognise, therefore, that subcellular geography may be as important as stimulation of biochemical pathways in shaping intracellular Ca^{2+} signals.

Ca^{2+} puffs initiate at sites that remain immobile for many minutes [26, 61, 62, 66-68]. The pioneering studies of Ca^{2+} puffs suggested there were no more than a handful of such initiation sites per cell [61, 64, 69], but recent work suggests they are more abundant with perhaps a hundred sites per cell [66, 67]. Nevertheless, it is clear that Ca^{2+} puffs repeatedly initiate at sites that remain immobile for many minutes, and which include only a small fraction of the total cellular complement of IP_3Rs. There is, therefore, a conundrum in that most IP_3Rs (typically ~70%) appear to be mobile, yet IP_3-evoked Ca^{2+} signals initiate at fixed sites, leading Parker and his colleagues to speculate that anchoring of immobile IP_3Rs into clusters may prime them to respond to IP_3 [63, 69]. To gain further insight into this problem, we used gene-editing to attach enhanced green fluorescent protein (GFP) to the endogenous IP_3R1 of HeLa cells [26]. We demonstrated that all IP_3R1s, the major subtype in HeLa cells, are tagged with GFP, the tagged IP_3R1s are functional, assemble with other IP_3R subtypes, and they are expressed in clusters within ER membranes [26].

Single-step photobleaching analyses of endogenous GFP-IP_3R1 in HeLa cells suggest that most IP_3Rs form clusters, with up to ~40 IP_3Rs in each, and a mean of ~8 IP_3Rs per cluster [26] (**Fig. 2A**). The dimensions of a cluster, typically several 100 nm across, are similar to the dimensions estimated from a single-particle tracking analysis (~400 nm) [69]. These small IP_3R clusters, which we suggest may be the elementary structural units of IP_3R signalling [66], are expressed throughout the cell. Super-resolution analyses of the distribution of IP_3Rs within the clusters shows that many of the component IP_3Rs are too far apart to interact directly with each other (**Fig. 2B**). We suggest, therefore, that IP_3R clusters are loose confederations held together by scaffolding complexes that might involve cytosolic or ER proteins, lipid microdomains, or contacts between ER and other organelles (**Fig. 2C**). A surprising observation is the apparent independence of each cluster. There is no evident mixing of IP_3Rs between mobile and immobile clusters, and we observe clusters apparently moving past each other without losing their identities. Hence, once IP_3Rs are assembled into a cluster, it seems to be a long-lasting relationship. The observation that IP_3Rs are rather loosely distributed within their clusters aligns with several features of Ca^{2+} puffs. Firstly, although the rising phase of Ca^{2+} puffs is usually brisk, consistent with rapid recruitment of closely spaced IP_3Rs, it is sometimes possible to discern steps in the rising phase, suggestive of a looser coupling [64]. Secondly, and notwithstanding the blurring of signals as Ca^{2+}

diffuses away from IP_3Rs, the dimensions of Ca^{2+} puffs (~500 nm) are much larger than needed to accommodate ten or fewer IP_3Rs (each ~20 nm across). Thirdly, although puff sites are immobile, the peak of the Ca^{2+} signal wanders by up to 300 nm within the site during a puff [62], suggesting that active IP_3Rs may be as much as 300 nm apart. We conclude that most IP_3Rs, whether mobile or immobile, are corralled into loose confederations by scaffolding complexes that typically hold ~8 IP_3Rs in a long-lasting relationship.

Measurements, using FRAP [70-72] or single-particle tracking [26, 69], and evidence that stimuli can regulate IP_3R clustering [2, 73-78] attest to the mobility of IP_3Rs within ER membranes. Typically, these studies suggest that most IP_3Rs are mobile (mobile fractions, M_f, typically ~70%) and that most movement is by diffusion. Using single-particle tracking of endogenous GFP-IP_3Rs in HeLa cells to record the movement of IP_3R clusters, we observed that most clusters (~70%) were mobile, while the others remained immobile for periods of many minutes. Within the population of mobile clusters, most moved by diffusion, but a small fraction of the mobile clusters (~7%) moved directionally along microtubules, driven by kinesin and dynein motors [26]. Hence, most native IP_3Rs are clustered, and most of these clusters are mobile.

However, in HeLa cells, almost all Ca^{2+} puffs initiate close to the PM [26], consistent with similar observations in SH-SY5Y cells [79, 80]. Furthermore, the pattern is similar whether the Ca^{2+} signals are evoked by histamine to stimulate endogenous signalling pathways, or by photolysis of caged-IP_3 to allow uniform release of IP_3 throughout the cytosol. Indeed, both we [67] and others [81] have shown that endogenous signalling pathways and photo-released IP_3 activate the same Ca^{2+} puff sites. Simultaneous recording of the Ca^{2+} puffs evoked by IP_3 and the underlying distribution of endogenous GFP-IP_3Rs showed that Ca^{2+} puffs initiate only at immobile clusters of IP_3Rs [26]. This observation is important, because IP_3R clusters are expressed throughout the cell, not just near the PM, and most IP_3R clusters are mobile. Hence, only a small subset of the few thousand IP_3Rs in a cell, namely those within immobile clusters adjacent to the PM, is 'licensed' to respond to IP_3. There is, therefore, an additional level of regulation of IP_3Rs that endows them with the competence to respond to IP_3. The licensing factor has yet to be identified.

3.2. Licensed IP_3Rs abut ER-PM junctions where SOCE occurs

In addition to increasing cytosolic Ca^{2+} concentration, activation of IP_3Rs also causes a decrease in the luminal Ca^{2+} concentration of the ER, and that leads to activation of SOCE [47] (Section 2.2). There is, however, a problem in that the ER is widely thought to be

luminally continuous [82, 83], and yet substantial depletion of ER Ca^{2+} stores is required to cause detectable SOCE [39, 59, 60]. Furthermore, low-intensity stimulation, which evokes minimal overall loss of ER Ca^{2+}, evokes cytosolic Ca^{2+} oscillations that are sustained only when SOCE is active [39]. How might cells activate SOCE without globally depleting their ER of Ca^{2+} and so compromising additional ER functions, like protein folding?

Our results from HeLa cells show that the sites to which STIM1 translocates after loss of Ca^{2+} from the ER are immediately adjacent to (but not perfectly coincident with) the immobile near-PM IP_3R clusters that we know to be the sites where Ca^{2+} puffs occur (**Fig. 2D**) [26]. Because IP_3Rs project about 13 nm from the ER membrane [24] (Section 2.1), they may be too large to enter the narrow ER-PM junctions where STIM and Orai interact. This physical exclusion may account for the lack of perfect colocalization of STIM1 with immobile IP_3R clusters, but it leaves unexplained our observation that immobile near-PM IP_3R clusters are preferentially juxtaposed to the ER-PM SOCE junctions [26]. Future work will need to identify the tether that positions licensed IP_3R clusters adjacent to SOCE junctions. We can, however, speculate on the possible physiological significance of the juxtaposition.

We suggest that immobile near-PM IP_3R clusters sit alongside SOCE junctions and face the PM (**Fig. 2E**). Since PIP_2 recruits STIM to ER-PM junctions, the licensed IP_3R clusters are located immediately alongside the substrate (PIP_2) from which endogenous signalling pathways will generate IP_3. It is not however clear, given the compartmentalization of PIP_2 within the PM [84], STIM1 and PLC interact with the same pool of PIP_2. Activation of SOCE requires substantial loss of Ca^{2+} from the ER [39, 59, 60], yet while regulating SOCE the ER must also fulfill numerous additional functions, many of which require luminal Ca^{2+} [85]. We speculate that the positioning of licensed IP_3R clusters alongside SOCE junctions might allow IP_3R activation to locally deplete the ER and activate SOCE without trespassing into other Ca^{2+}-requiring ER functions. Finally, SOCE is acutely regulated by Ca^{2+} passing through the low-conductance Orai channel [12]. If IP_3Rs, with their very large Ca^{2+} conductance, were too close to SOCE junctions, they might disrupt this local feedback regulation. Hence, having the licensed IP_3Rs that will respond to IP_3 alongside, rather than within, SOCE junctions may provide the best compromise between local regulation of SOCE by local depletion of ER Ca^{2+} stores, while retaining acute auto-regulation of SOCE by Ca^{2+} passing through Orai channels (**Fig. 2E**) [26].

4. IP_3Rs also regulate SOCE independent of their ability to release ER Ca^{2+}

Hitherto, the contribution of IP_3Rs to SOCE has been thought to depend only on their ability to release Ca^{2+} from the ER and thereby activate STIM1 (**Fig. 3A**). But several findings suggest an additional role for IP_3Rs. In *Drosophila*, just as in vertebrates, SOCE is mediated by STIM and Orai, although *Drosophila* has only a single gene for each. SOCE evoked by thapsigargin is attenuated in *Drosophila* neurons with mutant IP_3Rs. The obvious explanation for the defect would be that functional IP_3Rs normally contribute to the Ca^{2+} leak unmasked by inhibiting the ER Ca^{2+} pump with thapsigargin, and in their absence the stores empty less completely causing reduced activation of STIM [86, 87]. However, pan-neuronal knock-down of the single IP_3R subtype expressed in *Drosophila* and several mutant alleles of the IP_3R attenuated thapsigargin-evoked SOCE without affecting thapsigargin-evoked Ca^{2+} release from the ER [88]. These results suggest a role for IP_3Rs downstream of their ability to deplete the ER of Ca^{2+} (**Fig. 3B**). STIM and Orai expression are normal in neurons with mutant IP_3Rs, but their association after store depletion, whether assessed by immunoprecipitation or translocation to puncta, is attenuated. This suggests that IP_3Rs stabilize the interaction of STIM with Orai [88], consistent with evidence that both the attenuated SOCE [88] and systemic phenotypes of *Drosophila* IP_3R mutants are rescued by neuronal over-expression of STIM and Orai [87].

How might evidence that IP_3R contributes directly to downstream regulation of SOCE in *Drosophila* be reconciled with evidence from avian DT40 B cells, where graded depletion of Ca^{2+} stores evoked indistinguishable SOCE in cells with and without IP_3Rs (DT40-KO cells) [88]? One possibility is that the IP_3R is one of several proteins that influence STIM-Orai interactions (Section 2.2), and adaptive changes to the other modulators allow fully functional SOCE when IP_3Rs are lost from vertebrate cells. An alternative possibility is that modulation of SOCE by IP_3Rs is restricted to invertebrates. *Drosophila* STIM, for example, lacks the polybasic tail through which mammalian STIM is tethered to PIP_2. Perhaps the IP_3R, which can also bind to PIP_2 [89], provides an alternative tether for *Drosophila* STIM [88]. A third possibility is that the SOCE measured in DT40 cells after loss of IP_3Rs is not mediated by Orai, but by different Ca^{2+}-permeable channels [90, 91]. There is, therefore, a need to both define the properties of the IP_3R that intervene downstream of store depletion and to establish the extent to which such regulation extends beyond *Drosophila* (**Fig. 3B**).

5. Plasma membrane IP_3Rs can also mediate Ca^{2+} entry

SOCE is the almost universal partner of IP_3-evoked Ca^{2+} release, and we have argued that the distribution of licensed IP_3Rs may allow physiological stimuli to cause sufficient depletion of

small ER compartments near the PM to activate SOCE (**Fig. 3A**, Section 3.2). We have further suggested that IP_3Rs may play modulatory roles downstream of store depletion (**Fig. 3B**, Section 4). Receptors that stimulate PLC, and so formation of IP_3, can also activate additional PM Ca^{2+} channels. Some transient receptor potential (TRP) channels, for example, are regulated by G proteins, PIP_2, diacylgycerol, Ca^{2+} or, more contentiously [92], by store depletion. Hence, there are many opportunities for cells to activate Ca^{2+} entry pathways in parallel with Ca^{2+} release from the ER by IP_3Rs and their inextricable link to SOCE (Section 2.2). In this final section, we briefly consider evidence that IP_3Rs within the PM can provide an additional parallel route for Ca^{2+} entry.

In DT40 cells, stimulation of PLC evokes both Ca^{2+} release from intracellular stores and Ca^{2+} entry. Both phases of the response require IP_3Rs [19, 93-95]. In DT40 cells, as in most cells, pharmacological depletion of ER Ca^{2+} stores activates SOCE, which can be detected using Ca^{2+} indicators [19, 91, 96, 97] or by whole-cell patch-clamp recording of CRAC (Ca^{2+}-release activated Ca^{2+}) currents [90, 98-101]. The SOCE is blocked by low concentrations of Gd^{3+} [102]. However, concentrations of Gd^{3+} that completely block thapsigargin-evoked SOCE, only partially block the Ca^{2+} entry evoked by stimulating PLC through the B-cell receptor (BCR) [19, 95]. Furthermore, whereas thapsigargin fails to evoke Ba^{2+} entry, activation of the BCR does [94, 95]. Hence, in DT40 cells there is an IP_3R-dependent Ca^{2+} entry that is clearly not SOCE, and which contributes about 50% of the BCR-evoked Ca^{2+} entry [19]. The non-SOCE pathway is restored to DT40-IP_3RKO cells by expression of functional IP_3R [19]. In whole-cell patch-clamp recording from DT40 cells, IP_3 stimulates opening of large-conductance cation-selective channels. These are absent from cells without IP_3Rs, restored by expression of functional IP_3Rs, and most decisively, the conductance of these PM channels is predictably affected by point mutations within the pore [19]. Further confirmation that IP_3Rs within the PM carry these currents (rather than IP_3R regulating another channel from the ER) was provided by expressing IP_3R with an α-bungarotoxin-binding site inserted into a luminal loop. When expressed in DT40 cells, this binding site would be within the ER for intracellular IP_3R, but in the extracellular space for IP_3R in the PM. Intracellular IP_3 activated the mutant channel in the PM, and its conductance was increased by addition of extracellular, but not intracellular, α-bungarotoxin [19]. The only tenable conclusion, namely that IP_3R straddles the PM and provides the pore through which IP_3-activated currents pass, has been confirmed by others [15, 103, 104].

A striking feature of the PM IP_3R is the reliability with which DT40 cells 'count' small numbers of functional IP_3R into the PM. In both native DT40 cells and those transfected with

IP_3R, IP_3Rs are invariably detected in the PM, but there are always very few in each cell, typically 1-3 IP_3R/cell and never more than 5 IP_3R/cell. The presence of so few IP_3Rs in the PM might arise through imperfect ER retention, with a few IP_3Rs 'escaping' to the PM. We would then expect such rare events to follow a Poisson distribution, in which case many cells would be predicted to have no PM IP_3Rs. However, we invariably detected at least one PM IP_3R in each cell, suggesting that IP_3R expression within the PM is actively regulated rather than a failure of ER retention [19, 105]. The tiny numbers of IP_3Rs, given their very large conductance relative to Orai, are sufficient to provide substantial Ca^{2+} entry signals. Furthermore, even when IP_3R are massively over-expressed in the ER, there is no change in the number of functional IP_3R detected in the PM [19]. These observations suggest that cells count IP_3R into the PM, with just 2-3 from the 10,000 IP_3R expressed in each DT40 cell being targeted to the PM. We had expected such counting to rely on feedback from active IP_3R within the PM regulating trafficking of IP_3R. But neither the cytosolic Ca^{2+} signal evoked by IP_3Rs nor any IP_3-evoked conformational change within the IP_3R is required for IP_3Rs to be reliably counted into the PM [105]. How, without any apparent feedback monitoring, DT40 cells reliably count small numbers of functional IP_3R into the PM remains a mystery.

We conclude that in addition to their long-recognised role in mediating release of Ca^{2+} from the ER [106], IP_3R can also be expressed in the PM [19, 95, 105, 107, 108] and mediate Ca^{2+} entry (**Fig. 3C**). In DT40 cells, half the Ca^{2+} entry evoked by activation of the BCR occurs via SOCE mediated by some 10,000 or so Orai channels, and the other half is carried by the 1-3 IP_3R expressed in the PM [19]. The two very different modes of Ca^{2+} entry – dribbling into cells though low-conductance Orai channels or gushing in through IP_3Rs – will generate very different local Ca^{2+} signals that might differentially regulate intracellular events.

6. Conclusions

IP_3-evoked Ca^{2+} release and SOCE exist in an almost inseparable functional relationship in most cells. IP_3Rs licensed to respond to IP_3 are anchored alongside the ER-PM junctions where SOCE occurs and that, we suggest, may allow the licensed IP_3Rs to locally and substantially deplete the ER of Ca^{2+} and to thereby activate STIM1 and SOCE (**Fig. 3A**). It seems reasonable to think of the ER-PM junction and the associated licensed IP_3Rs as the autonomous and fundamental signalling unit for SOCE. In addition to their role in linking cell-surface receptors to loss of Ca^{2+} from the ER, IP_3Rs may also intervene at a later stage by

regulating interactions between active STIM and Orai (**Fig. 3B**). The underlying mechanism and the generality of this contribution of IP_3Rs to SOCE need to be established. Finally, at least in some cells, IP_3Rs in the PM can directly mediate Ca^{2+} entry (**Fig. 3C**), which, given the weak cation selectivity of IP_3Rs, would also be associated with membrane depolarization.

Acknowledgements

Supported by the Wellcome Trust (101844) and Biotechnology and Biological Sciences Research Council UK (BB/P005330/1) to CWT; Department of Science and Technology (DST), Ministry of Science and Technology, India to GH; and by the DST INSPIRE Fellowship programme and an Infosys travel grant to PC.

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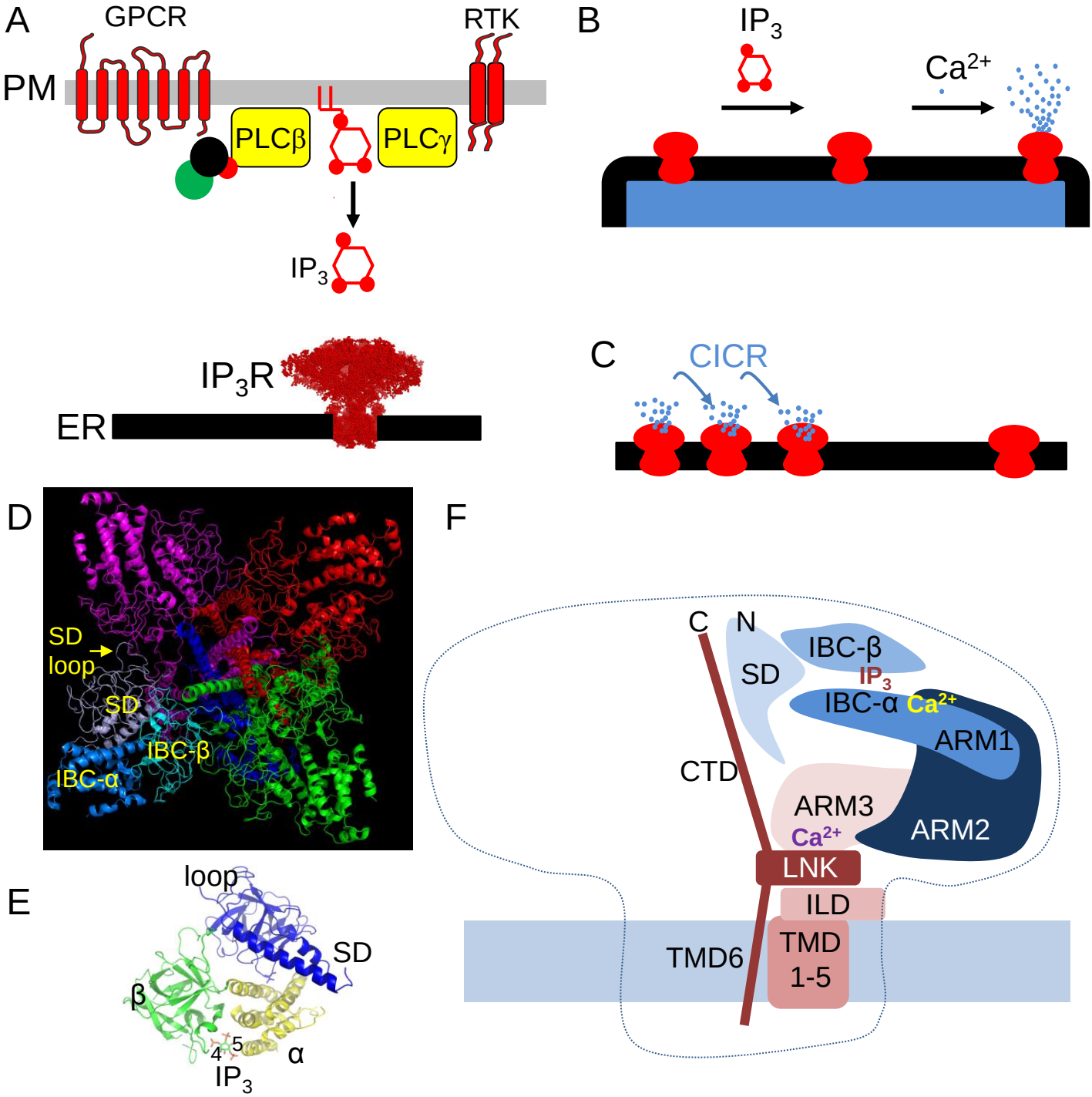
Fig. 1. Regulation of IP_3Rs by IP_3 and Ca^{2+}. (A) Receptors in the PM, including G-protein-coupled receptors (GPCRs) and receptor tyrosine kinases (RTKs), stimulate PLC and so production of IP_3, which then binds to the IP_3-binding site on each subunit of the tetrameric IP_3R. This leads to channel opening and release of Ca^{2+} from the ER. (B) IP_3 binding primes IP_3Rs to bind Ca^{2+}, and that leads to channel opening. (C) This regulation of IP_3Rs by IP_3 and Ca^{2+} allows them to propagate Ca^{2+} signals by CICR. (D) Cytosolic view of the closed IP_3R1 determined by cryo-EM [24], with the N-terminal domains of a single subunit highlighted and the loop through which the SD of one subunit and IBC-β of its neighbour interact. (E) IP_3 binds in the cleft between IBC-β and IBC-α. (F) Simplified view of a single IP_3R subunit showing some of the interactions between domains. The SD and IBC are at the top of the structure. Three large α-helical domains (ARM1-3) form the edges and underside of the mushroom. The only contact between the cytosolic and pore region occurs at the nexus between ARM3 with its C-terminal ILD domain and the C-terminal extension of TMD6 (LNK). Ca^{2+}-binding sites are formed by residues contributed across the interfaces between ARM1 and ARM2, and between LNK and the underside of ARM3 [25]. In one structure the C-terminal extends as an α-helix from LNK to the mushroom cap [24], but in another it is unresolved beyond LNK [25].

Fig. 2. Licensed IP_3Rs may locally deplete ER Ca^{2+} and stimulate SOCE. (A) Endogenously tagged EGFP-IP_3R1 form puncta in the ER membranes of HeLa cells, with an average of ~8 tetrameric IP_3Rs in each [26]. (B) Super-resolution (STORM) imaging shows that within puncta, IP_3Rs are quite diffusively distributed. (C) We suggest that scaffolds hold IP_3Rs within puncta. (D) Total internal reflection fluorescence (TIRF) image of an EGFP-IP_3R1-HeLa cell treated with thapsigargin to deplete Ca^{2+} stores showing distribution of IP_3Rs and STIM1. The enlargement shows (arrow heads) the juxtaposition of immobile IP_3R puncta and STIM1. (E) The ER-PM junction with its associated licensed IP_3Rs may comprise the basic functional unit for SOCE, with the licensed IP_3Rs causing the large local decrease in ER Ca^{2+} concentration required to activate STIM.

Fig. 3. IP_3Rs and Ca^{2+} entry. (A) Licensed IP_3Rs alongside the junctions where SOCE occurs may allow physiological stimuli to cause the large local decrease in ER Ca^{2+} concentration required to activate STIM1. (B) Even when the Ca^{2+} stores are empty, IP_3Rs may intervene to stimulate SOCE by enhancing interactions between STIM and Orai. The mechanisms are not fully resolved, but they appear to arise from IP_3R facilitating the coupling of STIM to Orai [88]. (C) In some cells, IP_3Rs in the PM directly mediate Ca^{2+} entry.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B
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C
CICR
D
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β
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IP3
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C
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ARM2
LNK
ILD
TMD6
TMD
1-5

Figure 1

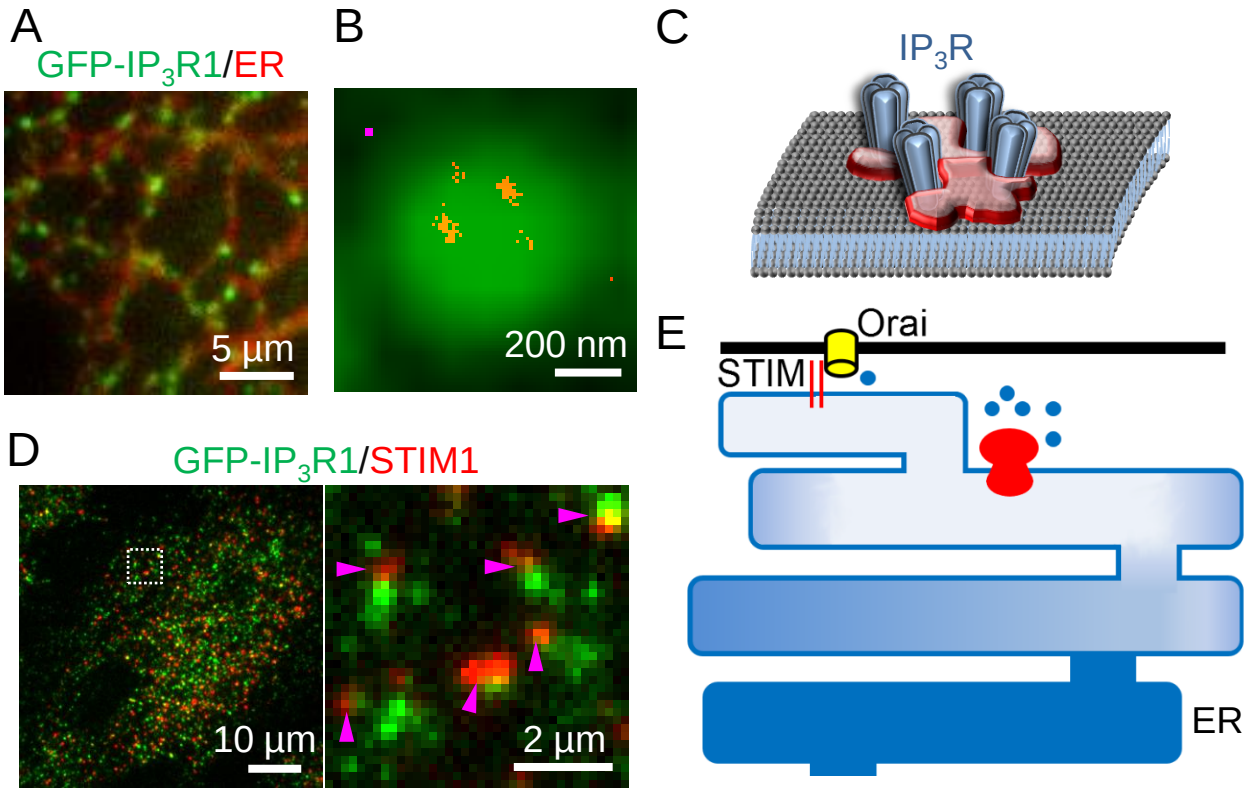
A
GFP-IP3R1/ER
5 µm
B
200 nm
C
IP3R
D
GFP-IP3R1/STIM1
10 µm
2 µm
E
Orai
STIM
ER

Figure 2

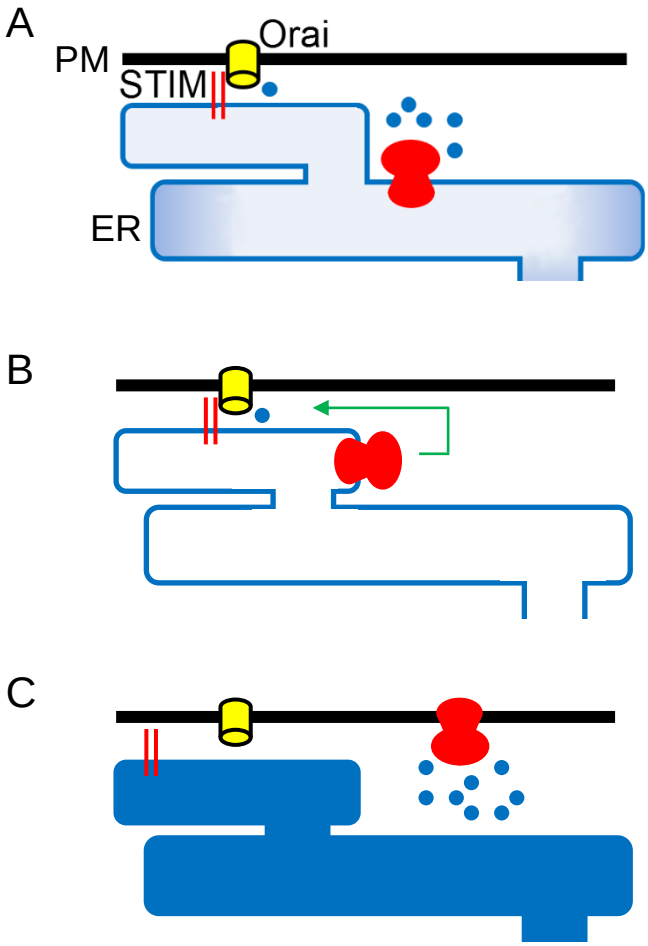

Figure 3