

Immobile IP₃ Receptor Clusters: Building Blocks For IP₃-Evoked Ca²⁺ Signals

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Co-regulation of IP₃ receptors (IP₃Rs) by IP₃ and cytosolic Ca²⁺ allows them to mediate regenerative signals, amongst which are Ca²⁺ puffs. These reflect the near-simultaneous opening of a few IP₃Rs within a small cluster. A long-standing conundrum is the observation that while most IP₃Rs appear to be mobile, Ca²⁺ puffs repeatedly initiate from a limited number of fixed sites. Using gene-editing to attach GFP to endogenous IP₃Rs in HeLa cells has allowed the distribution of IP₃Rs and the Ca²⁺ signals they evoke to be imaged simultaneously. This approach shows that most endogenous IP₃Rs are loosely assembled into small clusters, most of which are mobile. However, the Ca²⁺ puffs evoked by histamine or photolysis of caged IP₃ invariably initiated at immobile IP₃R clusters adjacent to the plasma membrane (PM). Hence, only a small fraction of cellular IP₃Rs are ‘licensed’ to respond. The licensed IP₃R clusters sit alongside the sites where store-operated Ca²⁺ entry (SOCE) occurs, suggesting that the IP₃Rs may allow local regulation of SOCE.

Keywords: Ca²⁺ signal, Ca²⁺-induced Ca²⁺ release, endoplasmic reticulum, IP₃ receptor, spatial organization, store-operated Ca²⁺ entry.

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INTRODUCTION

Inositol 1,4,5-trisphosphate receptors (IP₃Rs) and their cousins, the ryanodine receptors (RyRs), are the largest of all ion channels. Each forms a large-conductance Ca²⁺ channel with relatively weak selectivity for Ca²⁺ over K⁺ (Van Petegem 2014, Foskett et al. 2007). Both of these major families of intracellular Ca²⁺ channels are expressed predominantly within the endoplasmic or sarcoplasmic reticulum (ER or SR), where the functional channel is assembled from four closely related subunits. For IP₃Rs, the channel can comprise four identical subunits or a mixture drawn from the products of the three IP₃R genes and their splice variants (Joseph et al. 2000). RyRs are invariably homomeric. The IP₃R subtypes differ in their expression between cell types, in their subcellular distributions, their association with accessory proteins and in their affinities for IP₃ and modulation by additional intracellular signals (Prole & Taylor 2016). The subtypes may also fulfil different biological roles. However, the similarities between IP₃R subtypes are more striking than the differences. The most important of the shared features is the co-regulation of all IP₃Rs by IP₃ and cytosolic Ca²⁺: both ligands are required for the IP₃R channel to open. The simplest scheme envisages that binding of IP₃ primes IP₃Rs to bind Ca²⁺, with Ca²⁺ binding then triggering channel gating (**Fig. 1A**). This interplay is important because it endows IP₃Rs with a capacity to amplify, through Ca²⁺-induced Ca²⁺ release (CICR), the Ca²⁺ signals evoked by IP₃Rs or other Ca²⁺ channels. Higher concentrations of cytosolic Ca²⁺ inhibit IP₃Rs. There is, therefore, a biphasic dependence of IP₃R gating on cytosolic Ca²⁺ concentration: low Ca²⁺ concentrations are stimulatory, while higher concentrations inhibit. RyRs are also biphasically regulated by cytosolic Ca²⁺ (Van Petegem 2016). It is worth noting, since the observations are still cited, that it had been suggested that IP₃R2 and IP₃R3 were not biphasically regulated by cytosolic Ca²⁺ (Hagar et al. 1998, Ramos-Franco et al. 1998). However, subsequent work established that all IP₃Rs are biphasically regulated by cytosolic Ca²⁺ (Foskett et al. 2007, Taylor & Tovey 2012), although there are subtle differences between IP₃R subtypes in the interplay between IP₃ and Ca²⁺.

The structural basis of IP₃R activation has been most explored for IP₃R1, but the considerable amino acid sequence similarity between IP₃R subtypes suggests that the mechanisms of activation are likely to be similar for all subtypes. Indeed, the basic architecture of the activation mechanisms is probably similar for IP₃Rs and RyRs (Seo et al. 2012, Van Petegem 2016). For IP₃Rs, activation begins when IP₃ binds to the clam-like IP₃-binding core (IBC), which lies towards the N-terminal of the primary sequence of each subunit (**Fig. 1B**). The IBCs of all four subunits must bind IP₃ before the channel can open

(Alzayady et al. 2016). The 4- and 5-phosphate groups of IP₃, which are essential features of all IP₃R ligands, interact with basic residues lining opposite sides of the inner surface of the clam (Bosanac et al. 2002). This allows IP₃ binding to partially close the clam. Since one side of the clam, the α -domain, adheres tightly to the N-terminal domain of the IP₃R (the so-called suppressor domain, SD), clam closure causes movement of the SD. We suggest that this movement weakens interactions between IP₃R subunits, leading ultimately to channel gating (Seo et al. 2012). However, details of the structural links between the initial conformational changes around the IBC and SD, Ca²⁺ binding, and dilation of the pore that allows Ca²⁺ to pass from the ER into the cytosol, are not yet resolved. A major step towards revealing these details is the cryo-EM structure of IP₃R1 in a closed state (Fan et al. 2015). A notable feature of this structure, which may point the way towards a gating mechanism, is the rod-like α -helical C-terminal domain, which extends directly from TMD6, through a perpendicular linking domain, to make contact with the SD of an adjacent subunit (Fan et al. 2015).

The most pertinent points for subsequent discussion are the obligate co-regulation of IP₃Rs by IP₃ and cytosolic Ca²⁺; the large Ca²⁺ conductance of IP₃Rs (Foskett et al. 2007); the large size of an IP₃R (11,000 residues, ~1.2 MDa) with its mushroom-like cytosolic region extending some 20 nm from the ER membrane (Fan et al. 2015); and the observation that neither RyRs nor IP₃Rs are randomly distributed within intracellular membranes (Franzini-Armstrong 2018, Thillaiappan et al. 2017, Jayasinghe et al. 2018).

RECRUITMENT OF CA²⁺ SIGNALS BY IP₃-REGULATED CICR

High-resolution optical imaging, first with confocal microscopy and then with total internal reflection fluorescence microscopy (TIRFM), has revealed the subcellular organization of IP₃-evoked Ca²⁺ signals in cells loaded with a fluorescent Ca²⁺ indicator and EGTA to restrain the regenerative propagation of Ca²⁺ signals by CICR (Bootman et al. 1997, Wiltgen et al. 2010, Parker & Smith 2010). These ‘optical patch-clamp’ methods resolve the brief openings of individual IP₃Rs (as ‘Ca²⁺ blips’); the co-ordinated opening of several (typically fewer than 10) IP₃Rs within small clusters (‘Ca²⁺ puffs’); and the regenerative propagation of intracellular Ca²⁺ waves, which initiate more frequently as the IP₃ concentration increases (Smith & Parker 2009, Thurley et al. 2014). This hierarchy of Ca²⁺ release events is assumed to arise from CICR as higher concentrations of IP₃ progressively prime more IP₃Rs to respond to Ca²⁺ diffusing to them from nearby active IP₃Rs (**Fig. 1C**). The functional significance of the hierarchy comes from the changing nature of the Ca²⁺ signals as the stimulus intensity increases, and the opportunities that provides for encoding Ca²⁺ signals in

both spatial and temporal domains. Hence, the Ca^{2+} signal evolves from large focal increases in cytosolic $[\text{Ca}^{2+}]$ delivered to targets adjacent to IP_3Rs , to a global signal that can activate more remote targets, and these global signals can encode stimulus intensity in the frequency of the resulting Ca^{2+} spikes (Thurley et al. 2014). The extent to which IP_3 -evoked Ca^{2+} signals are amplified by this CICR mechanism, and so progress through the hierarchical pathway, depends on both the concentration of IP_3 and the separation of IP_3Rs .

Where Ca^{2+} blips have been resolved, they arise from sites that appear either immobile or to move with diffusion coefficients ($D = 0.003 \mu\text{m}^2\text{s}^{-1}$) (Wiltgen et al. 2010) at least ten-times slower than those of IP_3Rs determined from fluorescence recovery after photobleaching (FRAP) or single-particle tracking of tagged IP_3Rs (Smith et al. 2014, Thillaiappan et al. 2017). It is not yet clear whether Ca^{2+} blips arise from lone IP_3Rs or from IP_3Rs within clusters that fail to ignite the activity of their neighbours. Ca^{2+} puffs also initiate at sites that remain immobile for many minutes (Wiltgen et al. 2010, Bootman et al. 1997, Keebler & Taylor 2017, Thillaiappan et al. 2017). The pioneering studies of IP_3R puffs suggested there were no more than a handful of initiation sites per cell (Bootman et al. 1997, Smith et al. 2014, see Smith & Parker 2009), but recent work suggests they may be more abundant with perhaps a hundred sites per cell (Keebler & Taylor 2017). 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 complement of IP_3Rs . There is, therefore, a conundrum in that most IP_3Rs (typically $\sim 70\%$) 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 (Parker & Smith 2010, Smith et al. 2014). To gain further insight into this problem, we used gene-editing to attach enhanced green fluorescent protein (GFP) to the endogenous $\text{IP}_3\text{R1}$ of HeLa cells (Thillaiappan et al. 2017). We demonstrated that all $\text{IP}_3\text{R1}$ s, the major subtype in HeLa cells, were tagged with GFP, the tagged $\text{IP}_3\text{R1}$ s were functional and assembled with other IP_3R subtypes, and they were expressed in clusters within ER membranes. Subsequent sections of this short review focus on our analyses of these tagged endogenous IP_3Rs insofar as they illuminate our understanding of the relationship between the geography of IP_3Rs and the Ca^{2+} signals they elicit.

WHAT IS AN IP_3 RECEPTOR CLUSTER?

Functional analyses of Ca^{2+} puffs (Dickinson et al. 2012), single-particle tracking of over-expressed mEos2- $\text{IP}_3\text{R1}$ (Smith et al. 2014) and patch-clamp recordings from the outer

nuclear envelope (Rahman et al. 2009, Vais et al. 2011, Rahman et al. 2011) suggest that typical puff sites are likely to include 2-9 functional IP₃Rs. There is, however, considerable heterogeneity in the number of active IP₃Rs, both between sites and between successive Ca²⁺ puffs at the same site (Smith & Parker 2009). This suggests that not all IP₃Rs within a cluster are recruited during each Ca²⁺ puff.

Our single-step photobleaching analyses of endogenous GFP-IP₃R1 in HeLa cells suggest that most IP₃Rs form clusters, with up to ~40 IP₃Rs in each, and a mean of ~8 IP₃Rs per cluster (Thillaiappan et al., 2017) (**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) (Smith et al. 2014). These small IP₃R clusters, which we suggest may be the elementary structural units of IP₃R signalling, are expressed throughout the cell. A surprising observation is the apparent independence of each cluster. There is no evident mixing of IP₃Rs between mobile and immobile clusters, and we observe clusters apparently moving past each other without losing their identities. Hence, once IP₃Rs are assembled into a cluster, it seems to be a long-lasting relationship. Super-resolution analyses of the distribution of IP₃Rs within clusters suggests that many of the component IP₃Rs are too far apart to interact with each other directly (**Fig. 2A**). We suggest, therefore, that IP₃R 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. 2A**). The notion that IP₃Rs might be relatively loosely distributed within their stable clusters sits comfortably with several features of Ca²⁺ puffs. Firstly, although the rising phase of Ca²⁺ puffs is usually very brisk, consistent with rapid recruitment of closely spaced IP₃Rs, it is sometimes possible to discern steps in the rising phase, suggestive of a looser coupling (Smith & Parker 2009). Secondly, and notwithstanding the blurring of signals as Ca²⁺ diffuses away from IP₃Rs, the dimensions of Ca²⁺ puffs (~500 nm) are much larger than needed to accommodate ten or fewer IP₃Rs (each ~20 nm across). Thirdly, although puff sites are immobile, the peak of the Ca²⁺ signal wanders by up to 300 nm within the site during a puff (Wiltgen et al. 2010), suggesting that active IP₃Rs may be as much as 300 nm apart. Finally, even the most mobile of Ca²⁺ blips ($D = 0.003 \mu\text{m}^2\text{s}^{-1}$) (Wiltgen et al. 2010), which may represent IP₃Rs within clusters that fail to ignite a Ca²⁺ puff, would be expected to move only about 35 nm during the typical duration of a Ca²⁺ puff (i.e. well within the confines of the loose cluster). Hence, we suggest that most IP₃Rs, whether mobile or immobile, are corralled into loose confederations by scaffolding complexes that typically hold ~8 IP₃Rs in a long-lasting relationship.

We concluded from our previous patch-clamp analyses of IP₃Rs in the outer nuclear envelope, which is continuous with the ER, that low concentrations of IP₃ cause IP₃Rs to assemble into small clusters (Rahman et al. 2009, Rahman et al. 2011). Others have challenged this conclusion by suggesting that IP₃R clusters assemble without the need to increase the intracellular IP₃ concentration (Vais et al. 2011, Smith et al. 2009b), and our results from HeLa cells also show that stable IP₃R clusters are present in unstimulated cells (Thillaiappan et al. 2017). How might these observations be reconciled? It may be that the nuclear envelope is not an appropriate model for the ER, or basal levels of IP₃ may be sufficient to ensure assembly of IP₃R clusters in unstimulated cells (Smith et al. 2009b). However, a more attractive possibility, which we have yet to address experimentally, is that most IP₃Rs are already assembled into loose corrals in unstimulated cells, and IP₃ then promotes tighter clustering within the corral. Although preliminary analyses failed to provide support for this scheme (Smith et al. 2014), it deserves closer attention since such local ‘huddling’ would not increase the size of each pre-formed cluster, but it would be expected to improve the CICR-mediated recruitment of IP₃Rs within a cluster. Hence, IP₃ might initiate Ca²⁺ puffs by stimulating gating of IP₃Rs and by enhancing CICR by causing loosely pre-clustered IP₃Rs to huddle.

CA²⁺ PUFFS OCCUR AT IMMOBILE IP₃ RECEPTOR CLUSTERS NEAR THE PLASMA MEMBRANE

Both direct measurements, using FRAP (Fukatsu et al. 2004, Pantazaka & Taylor 2011, Ferreri-Jacobia et al. 2005) or single-particle tracking (Smith et al. 2014, Thillaiappan et al. 2017), and evidence that stimuli can regulate IP₃R clustering (Wilson et al. 1998, Tateishi et al. 2005, Iwai et al. 2005, Chalmers et al. 2006, Tojyo et al. 2008, Rahman et al. 2009, Geyer et al. 2015) attest to the mobility of IP₃Rs within ER membranes. Typically, these studies suggest that most IP₃Rs are mobile (mobile fractions, M_f, typically ~70%) and that most movement is by diffusion. A caveat remains that most such studies have observed over-expressed IP₃Rs, with the attendant risk that they may not faithfully report behaviours of endogenous IP₃Rs. Using single-particle tracking of endogenous GFP-IP₃Rs in HeLa cells to record the movement of IP₃R clusters, we observed that most clusters (~70%) were mobile, while the remainder remained immobile for periods of many minutes. Within the population of mobile clusters, most clusters moved by diffusion ($D \sim 0.03 \mu\text{m}^2 \cdot \text{s}^{-1}$), but a small fraction of the mobile clusters (~10%) moved directionally along microtubules, driven by kinesin and dynein motors (Thillaiappan et al. 2017). Our results are similar to those obtained using

single-particle tracking of over-expressed mEos2-IP₃R1, in suggesting that ~70% of IP₃R clusters are mobile, but they differ in that only we observed directional movement along microtubules. The difference may be due to the different cell types used or to over-expression of mEos2-IP₃R1 masking the small number of actively moving IP₃R clusters. The key points are that most native IP₃R clusters are clustered, and most of these clusters are mobile.

In HeLa cells, almost all Ca²⁺ puffs initiate close to the plasma membrane (PM) (Thillaiappan et al. 2017), consistent with similar observations in SH-SY5Y cells (Smith et al. 2009a). Furthermore, the pattern was similar whether the Ca²⁺ signals were evoked by histamine to stimulate endogenous signalling pathways, or by photolysis of caged-IP₃ to allow uniform release of IP₃ throughout the cytosol. Indeed, both we (Keebler & Taylor 2017) and others (Lock et al. 2017) have shown that endogenous signalling pathways and photo-released IP₃ activate the same Ca²⁺ puff sites. By simultaneously recording the Ca²⁺ puffs evoked by IP₃ and the underlying distribution of endogenous GFP-IP₃R clusters in HeLa cells, we showed that Ca²⁺ puffs initiate only at immobile clusters of IP₃R clusters (Thillaiappan et al. 2017). The observation is important, because IP₃R clusters are expressed throughout the cell, not just near the PM, and most IP₃R clusters are mobile. Hence, only a small subset of the few thousand IP₃R clusters in a cell, namely those within immobile clusters adjacent to the PM, is ‘licensed’ to respond to IP₃ (**Fig. 2B**). There must, therefore, be an additional level of regulation of IP₃R clusters that endows them with the competence to respond to IP₃. The nature of that regulation has yet to be resolved.

In addition to causing an increase in cytosolic [Ca²⁺], activation of IP₃R clusters also causes a decrease in [Ca²⁺] within the ER, and that leads to activation of store-operated Ca²⁺ entry (SOCE) (Hogan 2015). The core features of SOCE are now clear: they involve direct interactions between the ER Ca²⁺ sensor, stromal interaction molecule 1 (STIM1), and the PM Ca²⁺ channel, Orai1. Loss of Ca²⁺ from the luminal EF-hands of STIM1 causes STIM1 to cluster and expose cytosolic domains that bind to phosphatidylinositol 4,5-bisphosphate (PIP₂) and Orai1. Binding of STIM1 to Orai1 opens its channel, allowing Ca²⁺ to flow into the cell. Hence, loss of ER Ca²⁺ causes STIM1 to be captured, through its interaction with PIP₂ and Orai1, within narrow junctions where the ER and PM are no more than ~20 nm apart (Hogan 2015, Prakriya & Lewis 2015). Our results from HeLa cells show that the sites to which STIM1 translocates after loss of Ca²⁺ from the ER are immediately adjacent to (but not perfectly coincident with) the immobile near-PM IP₃R clusters that we know to be the sites where Ca²⁺ puffs occur (Thillaiappan et al. 2017). Because IP₃R clusters project about 20 nm from the ER membrane (Fan et al. 2015), they may be too large to enter the narrow ER-PM

junctions wherein STIM and Orai interact. This physical exclusion may account for the lack of perfect colocalization of STIM1 with immobile IP₃R clusters, but it leaves unexplained our observation that immobile near-PM IP₃R clusters are preferentially juxtaposed to the ER-PM SOCE junctions (Thillaiappan et al. 2017). Future work will need to identify the tether that positions the licensed IP₃R clusters adjacent to SOCE junctions. We can, however, speculate on the possible physiological significance of the juxtaposition.

We suggest that immobile near-PM IP₃R clusters sit alongside SOCE junctions and face the PM (**Fig. 2B**). Since PIP₂ recruits STIM to ER-PM junctions, we suggest that the licensed IP₃R clusters are located immediately alongside the substrate (PIP₂) from which endogenous signalling pathways will generate IP₃. Activation of SOCE requires substantial loss of Ca²⁺ from the ER (Brandman et al. 2007, Prakriya & Lewis 2015), yet while regulating SOCE the ER must also fulfil numerous additional functions, many of which require luminal Ca²⁺ (Berridge 2002). We speculate that the positioning of licensed IP₃R clusters alongside SOCE junctions might allow IP₃R activation to locally deplete the ER and activate SOCE without trespassing into the other Ca²⁺-requiring functions of the ER. Finally, SOCE is acutely regulated by Ca²⁺ passing through the low-conductance Orai1 channel (Prakriya & Lewis 2015). If IP₃Rs, with their very large Ca²⁺ conductance, were too close to SOCE junctions, they might disrupt this local feedback regulation. Hence, having the licensed IP₃Rs that will respond to IP₃ alongside, rather than within, SOCE junctions may provide the best compromise between local regulation of SOCE by local depletion of ER Ca²⁺ stores, while retaining acute auto-regulation of SOCE by Ca²⁺ passing through Orai channels (**Fig. 2B**).

CONCLUSIONS

IP₃Rs are co-regulated by IP₃ and cytosolic Ca²⁺. This allows them to mediate regenerative intracellular Ca²⁺ signals as IP₃ primes IP₃Rs to respond to Ca²⁺ (**Fig. 1C**). Ca²⁺ puffs, which report the near-simultaneous opening of a small number of IP₃Rs within a cluster, are the smallest units of these regenerative Ca²⁺ signals. Each cluster is a loose, but stable, confederation of a small number of IP₃Rs that must be held together by an as yet unidentified scaffold. Most IP₃R clusters are mobile, but only immobile clusters immediately adjacent to the PM initiate Ca²⁺ puffs. The additional signal that provides these licensed IP₃Rs with competence to respond to IP₃ is unknown. Nor is it resolved whether the large population of mobile IP₃R clusters contributes to Ca²⁺ signals evoked by more intense stimulation. Since the licensed IP₃R clusters sit alongside the ER-PM junctions where SOCE occurs, we speculate that they may contribute to local regulation of SOCE.

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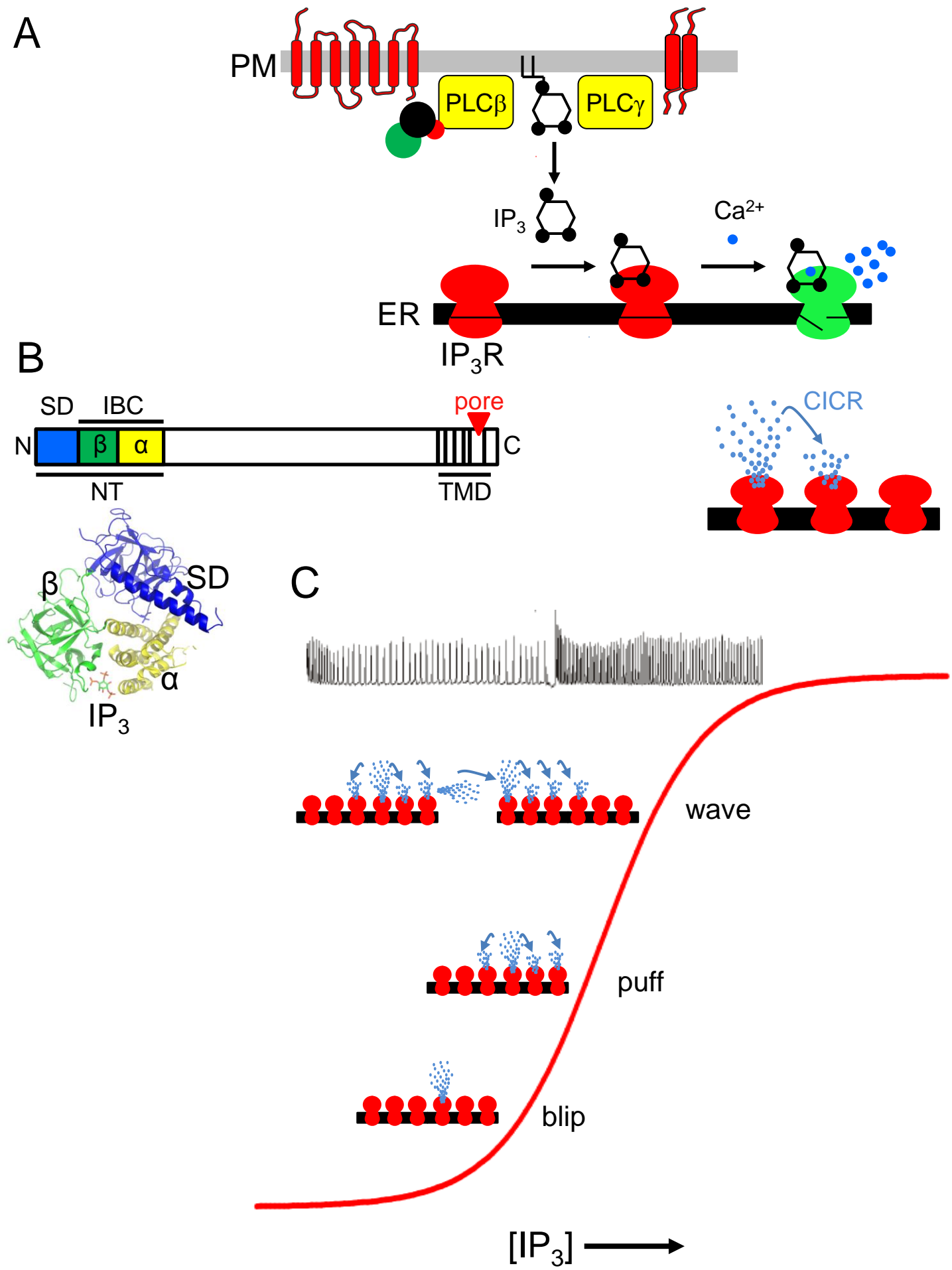
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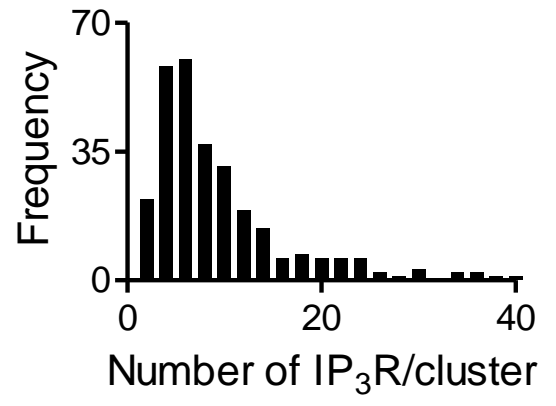
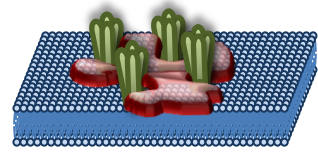
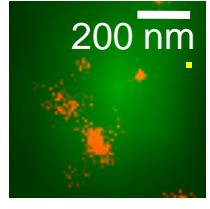
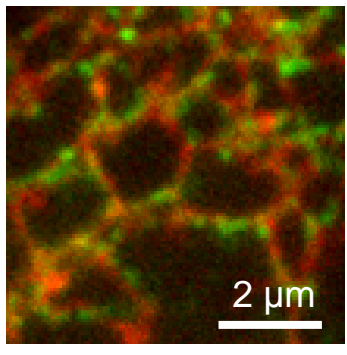
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Figure 1. Hierarchical recruitment of IP₃-evoked Ca²⁺ release. (A) Many receptors in the plasma membrane (PM) stimulate phospholipase C (PLC), with concomitant formation of IP₃. Binding of IP₃ to each of the four subunits of a tetrameric IP₃R primes the IP₃R to bind Ca²⁺, and that Ca²⁺ binding then triggers channel gating. Co-regulation of IP₃R by IP₃ and Ca²⁺ endows them with the capacity to mediate IP₃-regulated Ca²⁺-induced Ca²⁺ release (CICR). (B) IP₃ binds within the clam-like cleft formed by the α - and β -domains of the IP₃-binding core (IBC), causing partial closure of the clam. This clam closure causes re-orientation of the suppressor domain (SD) and thereby initiates channel gating. The pore of the channel is formed by transmembrane domains (TMD) towards the C-terminal of the primary sequence. Structure from Seo *et al.* (2012). (C) By tuning the gain on CICR by IP₃Rs, IP₃ allows hierarchical recruitment of intracellular Ca²⁺ release events.

Figure 2. Immobile IP₃R clusters at the PM are licensed to respond to IP₃. (A) TIRFM image shows a portion of a HeLa cell in which endogenous IP₃R1s are tagged with GFP; the ER is shown in red. STORM image of a single mobile IP₃R cluster shows the localization of GFP-IP₃R1 in red, and the underlying TIRFM image in green. The yellow box beneath the scale bar shows the approximate dimensions of a single IP₃R tetramer. The image shows that IP₃Rs are often relatively loosely associated within clusters, even though individual clusters retain their individuality for prolonged periods. Single-step photobleaching analyses suggest that IP₃R clusters contain variable numbers of tetrameric IP₃Rs, but with a mean of ~8 IP₃Rs/cluster. Results from Thillaiappan *et al.* (2017). (B) We suggest that most IP₃R clusters are mobile and inactive (red), but licensed IP₃R clusters (green) are immobilized alongside the ER-PM junctions where SOCE occurs. This, we suggest, may allow local regulation of SOCE through local depletion of the ER by licensed IP₃Rs, while retaining acute feedback regulation of SOCE activity by Ca²⁺ passing through Orai channels. See text for further details.



A**B**