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IP₃ receptors: take four IP₃ to open

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Abstract

Inositol 1,4,5-trisphosphate receptors (IP₃R) and ryanodine receptors are the channels responsible for Ca²⁺ release from the endoplasmic and sarcoplasmic reticulum. High-resolution structures of these enormous channels are beginning to reveal the inter-domain re-arrangements that link binding of their key regulators (IP₃ and/or Ca²⁺) to opening of the Ca²⁺-permeable pore. Activation of IP₃R is initiated when IP₃ causes its clam-like binding domain to close, thereby disrupting interactions with other domains that may then communicate with the pore. All four IP₃-binding sites within the tetrameric IP₃R must bind IP₃ before the channel can open. This has important consequences for the distribution of both IP₃ and IP₃R activity within cells, and it may contribute to the complex spatial organization of intracellular Ca²⁺ signals.

The endoplasmic reticulum (ER), which ramifies throughout the cell making contacts with many other intracellular membranes, is well placed to deliver Ca²⁺ signals rapidly to specific targets via its Ca²⁺-permeable channels. Opening of these channels allows Ca²⁺ to flow into the cytosol, causing the increase in cytosolic Ca²⁺ concentration that regulates many cellular processes. Pumps within the ER membrane eventually return Ca²⁺ to the ER lumen. Ca²⁺ fluxes across the plasma membrane can also generate Ca²⁺ signals, but cycling of Ca²⁺ across ER membranes is more economical, and it allows more targeted delivery to intracellular destinations. Within the ER, two related families of Ca²⁺ channels, inositol 1,4,5-trisphosphate receptors (IP₃R) and ryanodine receptors (RyR), mediate most Ca²⁺ signalling. Both IP₃R and RyR are stimulated by Ca²⁺, allowing them to propagate Ca²⁺ signals regeneratively as Ca²⁺ released by one channel ignites the activity of another (1). For IP₃Rs, IP₃ sets the gain on this potentially explosive Ca²⁺ release because IP₃Rs must bind IP₃ before they can respond to Ca²⁺. However, for IP₃R the IP₃-binding core (IBC) and pore lie at opposite ends of the primary sequence and far apart within the quaternary structure (Fig. 1), and the location of the stimulatory Ca²⁺-binding site is unknown (2). So how does IP₃, via its effects on Ca²⁺ binding, lead to opening of a distant pore? Structural analyses and a clever manipulation of IP₃R subunits have now revealed how IP₃ binding initiates IP₃R activation.

IP₃R and RyR are bigger than any other ion channel. Each channel is tetrameric, invariably a homo-tetramer for RyR, but IP₃R form homo- and heterotrimeric structures from three related subunits (IP₃R1-3) and their splice variants (3). The enormous size of these channels

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and the diversity of IP₃R tetramers have hampered structural analyses, although crystal structures of receptor fragments have provided valuable insight. High-resolution structures of the N-terminus of IP₃R, for example, showed how IP₃ is recognized and initiates IP₃R activation by closing the clam-like IBC, thereby displacing the N-terminal suppressor domain (SD) (4, 5). The SD is essential for channel gating (Fig. 1A). Similar analyses of RyR showed that the inactive N-terminal structures of IP₃R and RyR are similar (6). Indeed, the structures of the N-terminal and pore regions of the two receptors are sufficiently similar to allow chimeric IP₃R/RyR channels to function (5). Improvements to single-particle electron cryomicroscopy, notably use of direct electron capture cameras and improved image reconstruction algorithms, is transforming structural biology (7). Among the many beneficiaries of this 'resolution revolution' (7) are intracellular Ca²⁺ channels, with near-atomic resolution structures of RyR1 (8–10) and IP₃R1 (11) published in 2015. Each channel forms a mushroom-like structure anchored in the ER by its stalk. The N-terminal domains of each subunit, which include the IBC of the IP₃R, are arranged like the four blades of a propeller around a hole in the middle of the domed cytoplasmic cap of the mushroom (Fig. 1B). The stalk forms the ion-conducting pore, the architecture of which is broadly similar to that of voltage-gated cation channels. The pore is lined by the tilted S6 helices from each subunit, and together the helices form a hydrophobic constriction towards the cytosolic end of the ER membrane. In the open channel, this constriction must dilate to allow Ca²⁺ to pass (10). A selectivity filter at the luminal entrance of the pore (12) and a ring of acidic residues around the cytosolic vestibule (11) contribute to the cation-selectivity of the open pore. The S6 helix of the intracellular Ca²⁺ channels is unusual in reaching well beyond the ER membrane and into the cytosol. Within IP₃R, each extended S6 helix is linked to the α -helical C-terminal domain (CTD) by a pair of short orthogonal helices. Together these domains form an almost continuous α -helical structure extending from the pore, through the core of the protein, to the cytosolic surface, where the CTD contacts the IBC of a neighbouring subunit (Fig. 1D). This arrangement, which had been presciently predicted from analyses of the effects of IP₃ and Ca²⁺ on the accessibility of C-terminal residues (2), is intriguing because it suggests a direct link between IP₃-evoked closure of the clam-like IBC and the distant pore. Other inter-subunit interfaces, notably those involving the essential SD and the IBC (5) or ARM3 domain of an adjacent subunit (11) may also facilitate communication between IP₃ binding and the pore. The idea that IP₃R activation is associated with movements at domain interfaces aligns with evidence from RyRs showing that most disease-causing mutations, which tend to cause channels to open too willingly, are located at the boundaries between domains (6). The structures beautifully illustrate how IP₃ binds to evoke conformational changes around the IBC, they reveal the structure of the pore, and they suggest how the initial conformational changes might be transmitted to the pore (Fig. 1). But where does Ca²⁺ binding sit within this sequence? The answer to that is likely to come from structures of the IP₃R in its active state.

Steep concentration-effect relationships for IP₃-evoked Ca²⁺ release suggest that IP₃ must bind to several of the four IP₃-binding sites before the channel can open (13), but it has never been clear whether every site must bind IP₃. A recent report resolves the issue by demonstrating that within a tetrameric concatamer of IP₃R subunits, each must have a functional IP₃-binding site for IP₃ to evoke Ca²⁺ release. Tying protein subunits together

with flexible linkers to provide concatenated structures of defined composition has been used for other ion channels (14), but it is challenging for subunits as large as the IP₃R, and daunting because residues near the N- and C-termini of IP₃Rs control gating. The task is made a little easier by the proximity of the N- and C-termini of adjacent subunits within a tetrameric IP₃R (Fig. 1B), allowing them to be joined with a 14-residue linker (15). Alzayady *et al.* (16) used these methods to produce a concatenated IP₃R tetramer that behaves normally, but it opens only if all four subunits can bind IP₃. Furthermore, the need for all four IP₃-binding sites could not be circumvented by high concentrations of the co-agonist, Ca²⁺, or by an allosteric regulator of gating, ATP. IP₃R can open only when all four IP₃-binding sites are occupied, and seemingly no amount of help from co-regulators can surmount that need. This is an important observation because it suggests that dissociation of any one of four bound IP₃ is enough to close an IP₃R, and it implies that regenerative propagation of Ca²⁺ signals by Ca²⁺-induced Ca²⁺ release can proceed only via IP₃R in which all four IP₃-binding sites have bound IP₃. That conclusion has some interesting consequences.

In a typical cell, expressing ~7200 tetrameric IP₃Rs (17), the cytosolic concentration of IP₃-binding sites is ~100nM (18). That concentration is similar to the affinity of IP₃R for IP₃ (equilibrium dissociation constant, $K_D = 119\text{nM}$) measured under conditions that match those used for functional assays (19). Hence, across the range of IP₃ concentrations that achieve graded occupancy of IP₃Rs, the IP₃Rs are themselves significant buffers of the free IP₃ concentration (Fig. 2A). Many years ago, an influential analysis of Ca²⁺ and IP₃ diffusion coefficients in cytoplasm concluded that whereas Ca²⁺ was heavily buffered, IP₃ diffused freely (20), leading to a view that only Ca²⁺ functions as a local messenger. However, that analysis used cytoplasm from *Xenopus* oocytes, where the concentration of IP₃Rs is likely to be substantially lower than in more typical cells. Since IP₃Rs must bind four IP₃ molecules to become active, binding events at low concentrations of IP₃ are mostly 'unproductive' because the IP₃ is too thinly spread across IP₃Rs for individual receptors to acquire the four IP₃ molecules they need. With a free concentration of IP₃ (~40nM) that occupies 25% of the IP₃-binding sites, for example, only 0.4% of IP₃Rs will have four IP₃ bound ($25\%^4$), just 28 IP₃Rs in our typical cell. But at this concentration of IP₃, equating to 12,000 IP₃ molecules per cell, some 7200 IP₃ would be bound to IP₃R (Fig. 2A). The total concentration of IP₃ would therefore need to increase to 64nM to provide a free concentration of 40nM. Hence, buffering of IP₃ by IP₃R and the need for IP₃R to bind four IP₃ conspire to ensure that substantial increases in IP₃ concentration are required to achieve appreciable IP₃R activity. *En route* to that activity most IP₃Rs will have bound some IP₃ (Fig. 2B). This distribution of IP₃ across IP₃Rs may allow local signalling to IP₃Rs from phospholipase C (PLC), and perhaps thereby explain how different PLC-coupled receptors can evoke Ca²⁺ release from different IP₃-sensitive stores within a single cell (21). We can envisage a steeply declining IP₃ concentration radiating outwards from PLC as IP₃ encounters IP₃Rs (Fig. 2C). Close to PLC, IP₃ concentrations may be sufficient to achieve IP₃R activation, but beyond that there will be larger zones within which many IP₃Rs have bound some IP₃. Although these partially occupied IP₃Rs may not open, IP₃ binding may modify their behaviour in other ways. We, for example, have suggested that partially liganded IP₃Rs may cluster and so prime IP₃R to respond to Ca²⁺ released by their

neighbours (22), although that idea has been contentious (1). Another possibility is that IP₃ might trigger dissociation of IRBIT (IP₃R-binding protein released by IP₃), an endogenous antagonist of IP₃Rs (23), from a neighbouring subunit and so prime it to bind IP₃. The key point is that under physiological conditions, opening of IP₃Rs is preceded by a wave of IP₃ binding to very many more IP₃Rs, and that may play some part in better preparing them to respond when they acquire additional IP₃.

The Ca²⁺ signals detected in intact cells after photolysis of caged IP₃, where IP₃ is uniformly delivered to the cytosol, are remarkable for two reasons that become more so in light of the evidence that IP₃Rs can open only after they bind four IP₃ molecules (16). First, IP₃ evokes local Ca²⁺ signals that reflect the almost simultaneous opening of a handful of IP₃Rs within a small cluster. Second, these Ca²⁺ release events are sparse, just a few per cell, and yet they often revisit the same site (1). Ca²⁺ is proposed to mediate the rapid recruitment of IP₃Rs within these clusters, but the new evidence from Alzayady *et al.* (16) tells us that this can occur only if the neighbours have four IP₃ bound. How, when so few IP₃Rs open (just a handful from the 7000 in a cell), does the IP₃ binding become so concentrated into a few small clusters of IP₃Rs?

After more than 30 years of waiting, cryo-electron microscopy has provided our first glimpse of the IP₃R in sufficient detail (11) to begin to see how IP₃ binding to each of its four subunits (16) leads to opening of the Ca²⁺ pore that initiates cytosolic Ca²⁺ signalling. That breakthrough comes as gene-editing and high-resolution measurements of Ca²⁺ signals in intact cells provide the techniques needed to explore the structural determinants of IP₃R behaviour in living cells.

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Fig. 1. Structure of the IP₃R

(A) The essential 4- and 5-phosphate groups of IP₃ bind to opposite sides (β- and α-domains) of the clam-like IP₃-binding core (IBC) causing the clam to close and re-position the suppressor domain (SD), which moves with the α-domain of the IBC (Protein Data Bank, PDB, 3U4J) (5). Locations of key domains within the primary sequence are shown below. (B) View from the cytosol showing the four N-terminal regions (SD and IBC) and the C-terminal domains (CTD) from each subunit (the central splayed α-helices). The coloured subunits (orange and green) show how the CTD from one subunit contacts the N-terminal

region (the β -IBC) of another. The N- (blue) and C-termini (red) of each subunit, through which Alzayady *et al.* linked subunits to form concatamers (16), are highlighted; the termini of one subunit are enclosed in the yellow box. Within each IBC, the position where IP_3 would bind is shown by a yellow circle (PDB, 3JAV) (11). **(C)** View across the ER membrane, with 2 subunits highlighted in shades of orange and green. The transmembrane region with its 24 α -helices, six from each subunit, are shown (PDB, 3JAV) (11). **(D)** One of the likely inter-subunit interactions through which IP_3R binding to the IBC is communicated to the pore. The β -domain of the IBC of one subunit contacts the C-terminal domain (CTD) of another. The CTD extends as an almost unbroken α -helix through the centre of the protein to helix S6, which lines the pore. For the pore to open and allow Ca^{2+} to pass, S6 must move to disrupt the hydrophobic constriction that occludes the closed pore (red arrow).

Fig. 2. IP₃Rs bind and buffer IP₃

(A) Within a typical cell, the concentration of IP₃-binding sites and their affinity (K_D) for IP₃ are similar (~100nM). An inescapable consequence is that within the range of IP₃ concentrations that achieve graded occupancy of the binding sites, a substantial fraction of the IP₃ is bound, thereby depleting the cytosol of free IP₃ (18). (B) Buffering of cytosolic IP₃ by IP₃R shifts the relationship between IP₃ concentration and IP₃ binding to higher concentrations of IP₃: a total concentration that exceeds the K_D is now required to occupy 50% of the binding sites. Because 4 sites must be occupied to activate an IP₃R, the IP₃

concentrations needed to activate 50% of IP_3 Rs are much higher than those needed to occupy 50% of the binding sites. In this simple scheme, a concentration of IP_3 that occupied 50% of all binding sites, would allow only 6% of all IP_3 Rs, about 450 IP_3 Rs in a typical cell, to bind the 4 IP_3 needed for activity. (C) IP_3 produced by PLC is captured by IP_3 Rs as it diffuses into the cytosol creating a radial concentration gradient. Within the gradient, IP_3 Rs very close to PLC may acquire the 4 IP_3 molecules needed for activity (green), but beyond that there is an extensive penumbra of IP_3 Rs that bind IP_3 to fewer of their subunits.