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Inositol 1,4,5-trisphosphate Receptors in the Endoplasmic Reticulum: a Single-channel Point of View

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Abstract

As an intracellular Ca^{2+} release channel at the endoplasmic reticulum membrane, the ubiquitous inositol 1,4,5-trisphosphate (InsP_3) receptor (InsP_3R) plays a crucial role in the generation, propagation and regulation of intracellular Ca^{2+} signals that regulate numerous physiological and pathophysiological processes. This review provides a concise account of the fundamental single-channel properties of the InsP_3R channel: its conductance properties and its regulation by InsP_3 and Ca^{2+} , its physiological ligands, studied using nuclear patch clamp electrophysiology.

Keywords

Intracellular calcium; inositol 1,4,5-trisphosphate receptor; ion channel; calcium signal; patch clamp electrophysiology; endoplasmic reticulum

1. Introduction

The ubiquitously expressed inositol 1,4,5-trisphosphate (InsP_3) receptor (InsP_3R) is mostly localized to the endoplasmic reticulum (ER) membrane [1], where it functions as an ion channel to release Ca^{2+} stored in the ER lumen ($\text{Ca}^{2+}_{\text{ER}}$) into the cytoplasm to raise the cytoplasmic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) when it is activated by its physiological ligand, InsP_3 , generated in the cytoplasm as part of a signal cascade resulting from activation of specific plasma membrane receptors by various extracellular stimuli [2]. InsP_3R thus plays a crucial role in the generation, propagation and regulation of cytoplasmic Ca^{2+} (Ca^{2+}_i) signals that regulate numerous physiological and pathophysiological processes. It is therefore not surprising that it has been the subject of intense study ever since its identification [3]. Many aspects of the InsP_3R channel have been extensively reviewed

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recently, including its molecular structure [4–7] and its regulation by phosphorylation [8], redox reagents [9, 10] and ATP [8]. Thus, this short review will focus on the more fundamental properties of the single InsP₃R channel: its ion conductance properties and its regulation by its physiological ligands—InsP₃ and Ca²⁺, especially those reported since our previous reviews of single InsP₃R channel properties [11, 12].

Studies of individual InsP₃R channels began with the reconstitution of isolated and purified functional InsP₃R channels in artificial planar lipid bilayers [13], which allows the reconstituted channel(s) to be studied with known and rigorously controlled ionic and ligand conditions on both its cytoplasmic and luminal sides [14]. Because of the intracellular localization of the InsP₃R, application of patch clamp electrophysiology to investigate behaviors of the InsP₃R channel in a more native membrane environment was not feasible until isolated nuclei were used as surrogate for the ER [15, 16] because of the continuity of the outer nuclear membrane with the ER [17]. Since isolated nuclei with intact outer nuclear membranes can be obtained with high success rates [18], nuclear patch clamping in the “on-nucleus” configuration (Fig. 1A) preserves the protein environment on the luminal side of the recorded InsP₃R channel(s) while maintaining rigorous control of ligand and ionic conditions on both sides of the channel(s) [19]. Combining rapid perfusion techniques with nuclear patch clamping in luminal-side-out (lum-out) (Fig. 1B) or cytoplasmic-side-out (cyto-out) (Fig. 1C) configurations allows rapid (~ ms), repeated (tens of times in one experiment) and reversible exchange of the bath solution to study dynamic responses of InsP₃R channel activity to abrupt changes in InsP₃ and Ca²⁺ concentrations on either side of the channel, as well as to compare the gating and conductance properties of the *same* channels in the *same* isolated membrane patches under different ionic environments [19]. Performing nuclear patch clamping on an isolated nucleus with its outer nuclear membrane stripped by chemical treatment [20, 21] (Fig. 1D) can achieve the nucleoplasmic-side-out (nucleo-out) configuration (Fig. 1E) to study InsP₃R localized to the inner nuclear membrane [20, 22].

Single-channel properties of the InsP₃R channel have also been studied by applying whole-cell patch clamp techniques to chicken lymphocyte DT40 cells [23–29], in which InsP₃R channels are localized to the plasma membrane at very low density (< 5 channels/cell) [24]. However, the lipid and protein environments around these InsP₃R channels in the plasma membrane are different from those around the ER channels, and conductance and ligand regulation of InsP₃R channels in the two locations were significantly different [19]. Strictly speaking, InsP₃R channels localized to the plasma membrane are acting as plasma membrane Ca²⁺ entry channels rather than intracellular Ca²⁺ release channels [23].

Ca²⁺ signals generated by InsP₃R channels located in the ER near the plasma membrane in intact mammalian cells can also be studied using total internal reflection fluorescence (TIRF) microscopy [30]. By using a fast [Ca²⁺] indicator dye and loading Ca²⁺ buffer (EGTA) into cells to rapidly sequester Ca²⁺ released by active InsP₃R channels, spatial and temporal resolution of observed Ca²⁺ signals were sufficiently improved so that Ca²⁺ release by single InsP₃R channels can be imaged. With such “optical patch-clamping”, many individual channels can be monitored in their native environment in intact cells, thereby preserving the interaction between an active InsP₃R channel with neighboring channels

mediated by Ca^{2+} released by the active channel. However, to date, the temporal resolution and signal-to-noise ratio of electrophysiological patch clamping are still substantially superior to those of optical patch clamping. Thus, optical and electrophysiological patch clamping are mutually complimentary techniques to study InsP_3R -mediated Ca^{2+} signals.

Another factor besides the intracellular location of the InsP_3R that complicates the study of InsP_3R channels is the primary amino acid sequence diversity of the channels. Vertebrates have three isoforms of InsP_3R : types 1 (InsP_3R -1), 2 (InsP_3R -2) and 3 (InsP_3R -3) that are encoded by three separate genes and are ~ 60–80% homologous. Invertebrates have only one InsP_3R that is most closely related to InsP_3R -1. Alternate splicing further enhances diversity of InsP_3R . InsP_3R -1 has three major splice regions (S1, S2 and S3), and a few minor ones. Mammalian neuronal cells express the S2+ variant while other peripheral cells express mainly the S2– form. InsP_3R -2 has at least one splice region. The invertebrate InsP_3R also has multiple splice variants. Given that most vertebrate cells express multiple InsP_3R isoforms in various levels, and InsP_3R can form homo- and heterotetrameric channels, the diversity at the InsP_3R channel level can be impressive indeed (see [11] and references therein).

To avoid possible complications arising from heterotetrameric channels, initial studies of InsP_3R channel used cells or tissues that were known to express predominantly one InsP_3R isoform [31], like cerebellum [32] for InsP_3R -1 S2+, *Xenopus* oocytes [15, 16] for InsP_3R -1 S2– [33], ventricular cardiac myocytes [34] for InsP_3R -2, RIN-5F insulinoma cells [35] for InsP_3R -3, and insect *Spodoptera frugiperda* Sf9 cells [36] for invertebrate InsP_3R . Nevertheless, the possibility that these cells may express different splice variants of the same InsP_3R isoforms cannot be ruled out. To address that concern, recombinant InsP_3R were over-expressed in cells determined to have low expression levels of endogenous InsP_3R , like *Xenopus* oocytes [37], COS-1 [38], COS-7 [39] and Sf9 cells [40]. However, all of those cells still express endogenous InsP_3R that can oligomerize with the recombinant InsP_3R to form contaminating heterotetrameric channels. A mutant cell line (DT40-3KO) derived from a chicken B cell line was finally generated with all three endogenous InsP_3R genes disrupted [41]. To date, it is still the only cell line available that has truly no endogenous InsP_3R background. This cell line has been used to generate cells stably expressing a single recombinant InsP_3R isoform (mutant or wild type) for single-channel studies of homotetrameric channels [42].

2. Conduction properties of InsP_3R

The conduction properties of a channel are one kind of information about protein ion channels that can only be obtained through single-channel studies. Such data in sufficient quantity can be used to inform the modeling of the structure of the channel pore [43, 44]. Practically, conduction characteristics, especially the channel conductance, are used to confirm the identity of the channel observed, especially in nuclear patch clamp experiments in on-nucleus or lum-out configurations, for which verifying the InsP_3 dependence of the observed channels by changing $[\text{InsP}_3]$ in the cytoplasmic (pipette) solution is difficult.

2.1. Monovalent cation conductance

The most relevant channel conductance value is that observed when the channel is in physiological ionic conditions: symmetric $[K^+]$ of ~140 mM. This was not measured in planar lipid bilayer experiments [45, 46], probably due to the presence of contaminating channels in the reconstituted preparation. In nuclear patch clamp experiments in the absence of divalent cations, InsP₃R channels have linear, ohmic current-voltage (I - V) relation over a broad range of applied voltages (± 60 mV). Conductance values given below were measured for InsP₃R channels in outer nuclear membrane in symmetric 140 mM KCl unless stated otherwise.

The endogenous InsP₃R-1 S2- channel in *Xenopus* oocytes has a conductance of 370 ± 5 pS [47]. The endogenous rat InsP₃R-1 S2+ channel in the inner nuclear membrane of Purkinje neurons has a conductance of 356 ± 4 pS in 150 mM KCl [20]. Comparable conductance values of 366 ± 5 pS and 378 ± 11 pS (in 150 mM KCl) were observed for endogenous rat InsP₃R-1 S2+ channels in the inner nuclear membrane of CA1 pyramidal neurons and dentate gyrus granule neurons, respectively [22]. The endogenous InsP₃R-1 S2+ channel in mouse embryonic cortical neurons has a conductance of 375 pS [48]. Recombinant rat InsP₃R-1 S2- and S2+ channels expressed in COS-7 cells have conductance values of 389 ± 5 pS and 369 ± 6 pS, respectively [39]. Recombinant rat InsP₃R-1 S2+ channel expressed in DT40-3KO cells exhibits a conductance of 373 ± 2 pS [49].

A conductance of 375 ± 5 pS was observed for recombinant InsP₃R-2 channel expressed in DT40-3KO cells [50], identical to that for InsP₃R-1 S2+ channel measured by the same lab [49].

A conductance of 358 ± 8 pS was observed for InsP₃R channels in *Xenopus* oocytes with low expression levels of endogenous InsP₃R-1 injected with recombinant rat InsP₃R-3 cRNA [37]

These conductance values measured by different labs from endogenous and recombinant channels of different InsP₃R isoforms in multiple cell types were in remarkable agreement, suggesting that the high degree of sequence homology in the pore-forming regions (the pore helix, the selectivity filter together with transmembrane helices 5 and 6) among the three vertebrate InsP₃R isoforms [51] plays a crucial role in determining the channel conductance.

However, the picture is not so simple. Our lab that recorded the conductance of InsP₃R-3 channel expressed by cRNA injection in *Xenopus* oocytes also reported a significantly bigger conductance of 545 ± 7 pS for recombinant rat InsP₃R-3 channel expressed in DT40-3KO cells [52]. Furthermore, another lab consistently reported two significantly lower conductance values of ~200 pS and ~120 pS for both recombinant rat InsP₃R-1 S2+ [23, 53] and rat InsP₃R-3 channels [54, 55] expressed in DT40-3KO cells. It was unlikely that the lower conductance values arose from substates occasionally observed in InsP₃R channels [15, 52] since channels in the same nuclear membrane patches always exhibited the same conductance, and transitions from one conductance to another were never observed in single-channel membrane patches [54]. Other labs studying the same homotetrameric InsP₃R channels in the same DT40-3KO system have not reported such dual conductance

values for the same kind of InsP₃R channel, and there is as yet no satisfactory explanation for the phenomenon.

Interestingly, a similar low conductance of ~ 200 pS was observed by two different labs using whole-cell patch clamping for plasma membrane-localized InsP₃R channels formed by endogenous chicken InsP₃R in wild-type DT40 cells [23]; and recombinant rat InsP₃R-1 S2+ [23, 25], rat InsP₃R-1 S2- [27] and mouse InsP₃R-2 [26] expressed in DT40-3KO cells.

These diverse conductance values for InsP₃R channels suggest that besides the primary sequence of the pore-forming regions of the InsP₃R, other factors, like the membrane environment around the channel, can also substantially affect the conductance of InsP₃R channels.

The conductance of 475 pS [48] for endogenous InsP₃R channels in human B lymphoblasts [48] is probably that of a heterotetrameric InsP₃R channel since it is different from any of the conductance values reported for homotetrameric channels.

The invertebrate InsP₃R is most closely related to InsP₃R-1 in its sequence. Endogenous invertebrate InsP₃R from Sf9 cells has a conductance of 477 ± 3 pS [36]. It is not sure if the similarity of this conductance value to that of the lymphoblast channel is coincidence or not.

2.2. Channel conductance in the presence of divalent cations

Localized to the ER membrane, InsP₃R channels are exposed to symmetric ~ mM Mg²⁺ and [Ca²⁺] of a few hundred μ M on their luminal side. In such ionic conditions, the *I-V* relation of the channel becomes nonlinear with significantly lower slope conductance values [15, 20, 22, 37, 47, 52]. This indicates that divalent cations are permeant ion blockers of the InsP₃R channels due to the presence of high affinity divalent cation binding site(s) in the channel pore. Although divalent cations can pass through the channel pore, they bind strongly to some site(s) in the pore and interfere with the flow of K⁺ through the channel.

For InsP₃R-3 channels expressed in DT40-3KO cells, divalent cations can only block the InsP₃R channel partially and Mg²⁺ blocks the channel to the same extent whether it is on the cytoplasmic side or luminal side of the channel, suggesting that the channel has one unique saturable divalent cation binding site in the ion permeation pathway that is equally accessible from either side of the channel. This site has a substantially higher affinity for Ca²⁺ over Mg²⁺ since the half-maximal blocking concentration for Mg²⁺ is about twice that for Ca²⁺. However, at saturating concentrations, both Ca²⁺ and Mg²⁺ block the channel to the same extent [52].

Permeability ratios of various ions through different InsP₃R channels can be evaluated from reversal potentials measured in asymmetric ionic conditions using the general Goldman-Hodgkin-Katz equation [52] (tabulated in Table 1). The permeability sequence of $P_{Ca} > P_{Ba} > P_{Mg} > P_K > P_{Cl}$ is generally preserved for all InsP₃R channels in nuclear envelope studied. The ratios show that even though the InsP₃R is a Ca²⁺-release channel, it is relatively non-selective for Ca²⁺ compared with plasma membrane Ca²⁺ channels. For a cation channel, it even has rather high permeability for Cl⁻. The high permeability of InsP₃R

channels for K^+ and Cl^- suggests that Ca^{2+} efflux from the ER lumen through an open $InsP_3R$ channel to the cytoplasm during an $InsP_3$ -induced Ca^{2+} release event can be sufficiently balanced by countercurrents of K^+ and Cl^- through the same open channel to maintain electrical neutrality of the ER lumen [56].

2.3. Unitary Ca^{2+} current through the $InsP_3R$ channel in physiological ionic conditions

Since the fluxes of Ca^{2+} and K^+ through the $InsP_3R$ channel are not independent, the magnitude of the Ca^{2+} flux through the channel under physiological ionic conditions cannot be evaluated using the Goldman-Hodgkin-Katz equation and must be determined experimentally. Despite the large K^+ conductance of $InsP_3R$ channels, the relatively low Ca^{2+} permeability of the channels and the low (~ 0.5 mM) $[Ca^{2+}]$ gradient across the ER membrane suggest that the Ca^{2+} current driven through a single open $InsP_3R$ channel by the ER $[Ca^{2+}]$ gradient (unitary Ca^{2+} current) is probably < 200 fA. Such signals are less than the noise (instrumental and thermal) observed in nuclear patch clamp experiments.

To overcome this obstacle to measure i_{Ca} [52], instead of trying to measure the unitary Ca^{2+} current (i_{Ca}) driven by a physiological $[Ca^{2+}]$ gradient at constant zero applied voltage (V_{app}), a voltage ramp was applied so that for part of the ramp (magenta bars at top of Fig. 2A and C), the open channel current (I_{open} , blue data points in Fig. 2A and C) could be clearly distinguished from the closed channel leak current (I_{closed} , pink data points in Fig. 2A and C). I_{open} and I_{closed} data points were manually selected and linear fitted (purple and red lines in Fig. 2). To measure i_{Ca} with high accuracy, any offset voltage in the instrument must be compensated precisely. This was done for each experiment by applying rapid perfusion techniques to excised lum-out outer nuclear membrane patches. With 140 mM KCl and no Ca^{2+} in both the pipette and bath solutions, no current would pass through the non-selective leak or the open Ca^{2+} -selective $InsP_3R$ channel when there was no electrical potential gradient across the isolated nuclear membrane. Therefore the intersection of the lines fitted to I_{open} and I_{closed} (indicated by orange arrowhead in Fig. 2B) marked the zero current level for that experiment (black dotted line in Fig. 2B). When the bath solution was changed to one containing physiological levels of free Ca^{2+} on the luminal side of the channel (Ca^{2+}_{ER}) besides 140 mM KCl, the non-selective I_{closed} should still be zero when there was no electrical potential gradient across the membrane (orange arrowhead in Fig. 2D). The current passing through the open $InsP_3R$ channel at that point was therefore i_{Ca} (green double arrow in Fig. 2D).

For homotetrameric $InsP_3R$ -3 expressed in DT40-3KO cells, i_{Ca} is linearly related to the free Ca^{2+} concentration gradient across the $InsP_3R$ channel for physiological levels of free $[Ca^{2+}]$ in the ER lumen ($[Ca^{2+}]_{ER}$), with a slope of 0.30 ± 0.02 pA/mM. The linear relation is not surprising since in the absence of electrical potential gradient, the $[Ca^{2+}]$ gradient across the ER membrane provides the only driving force to move Ca^{2+} across the channel. Interestingly, the magnitude of i_{Ca} is not affected by physiological levels of free $[Mg^{2+}]$ or free $[Cl^-]$ [52]. This may be due to the greater affinity for Ca^{2+} over Mg^{2+} of the divalent cation-binding site in the channel pore.

For a non-depleted ER Ca^{2+} store with $[Ca^{2+}]_{ER} \sim 0.5$ mM, $i_{Ca} \sim 0.15$ pA [52]. This agrees reasonably well with estimation of Ca^{2+} flux (~ 0.1 pA) observed for individual $InsP_3R$

channels in intact cells by optical patch clamping [30]. This is a substantial Ca^{2+} flux that can raise the $[\text{Ca}^{2+}]_i$ in the vicinity of an open InsP_3R channel to produce positive feed-through regulation of the gating properties of the open channel itself ([57] and section 3.3 below). Furthermore, Ca^{2+} flux through a single InsP_3R channel sufficiently activated by InsP_3 can recruit neighboring InsP_3R channels in a cluster through calcium-induced calcium release (CICR) into coordinated opening [58, 59] to generate elementary Ca^{2+} release events (puffs) [60].

3. Steady-state regulation of InsP_3R channel by InsP_3 and Ca^{2+}_i

3.1. Regulation of InsP_3R channel gating by Ca^{2+}_i

The biphasic regulation of InsP_3R channel activity (open probability, P_o) by Ca^{2+}_i was the first regulatory mechanism for the channel explored after discovery of its activation by InsP_3 [32]. Even in saturating levels of $[\text{InsP}_3]$ (see below), InsP_3R P_o is very low at resting (~ 50 nM) $[\text{Ca}^{2+}]_i$. Channel P_o increases as $[\text{Ca}^{2+}]_i$ is raised, up to a maximum activating $[\text{Ca}^{2+}]_i$. Beyond a minimum inhibitory $[\text{Ca}^{2+}]_i$, increases in $[\text{Ca}^{2+}]_i$ reduce channel P_o (Fig. 3A). The biphasic regulation by Ca^{2+}_i suggests that the InsP_3R has at least two functional cytoplasmic Ca^{2+} -binding sites: a high-affinity activating one and a low-affinity inhibitory one. If the Ca^{2+} affinity of the activating site is substantially higher than that of the inhibitory site, then the activating site can be saturated at some $[\text{Ca}^{2+}]_i$ before the inhibitory site starts to bind Ca^{2+} appreciably, and the Ca^{2+} -dependence curve of the channel has a plateau region between the maximum activating $[\text{Ca}^{2+}]_i$ and the minimum inhibitory $[\text{Ca}^{2+}]_i$. If the Ca^{2+} affinity of the activating site is similar to or lower than that of the inhibitory site, then Ca^{2+} binding to the inhibitory site can occur even at $[\text{Ca}^{2+}]_i$ that is sub-saturating for the activating site, then the maximum activating $[\text{Ca}^{2+}]_i$ and the minimum inhibitory $[\text{Ca}^{2+}]_i$ coincide, and the Ca^{2+} -dependence curve has a distinct peak.

This regulation has been studied for both endogenous and recombinant channels of all three InsP_3R isoforms in many different cell types using both reconstitution into artificial lipid bilayer and nuclear patch clamping techniques. A remarkably wide variety of Ca^{2+} dependencies were observed, with both plateau- and peak-shaped dependence curves [11, 12]. In particular, the same recombinant InsP_3R channel expressed in different cell types can display very different Ca^{2+} dependencies even when studied with the same nuclear patch clamping technique. Rat InsP_3R -3 channels expressed in DT40-3KO cells [57] are more than ten-fold less sensitive to Ca^{2+} activation than those expressed in *Xenopus* oocytes [61]. Rat InsP_3R -1 S2+ channels expressed in DT40-3KO cells [50] are activated significantly more abruptly within a narrower range of $[\text{Ca}^{2+}]_i$ (more cooperatively) than those expressed in COS-7 cells [39]. This suggests that Ca^{2+} regulation of InsP_3R channel activity can be quite labile, affected by various cytoplasmic factors and conditions besides the primary sequence of the channels, thereby allowing the magnitude and even the nature of the InsP_3R -mediated intracellular Ca^{2+} signals to be delicately tuned by multiple regulatory factors besides the primary InsP_3R ligands. The inhibitory site of the endogenous *Xenopus* InsP_3R -1 can even be rendered completely insensitive to Ca^{2+} irreversibly by temporary exposure to non-physiological low $[\text{Ca}^{2+}]_i$ (< 10 nM) [62]. Such environmental disruption of the

inhibitory Ca^{2+} site is probably the reason behind reports of lack of Ca^{2+} inhibition for some InsP_3R channels [34, 35, 46].

Another known factor that modifies Ca^{2+} sensitivities of InsP_3R is ATP^{4-} , which allosterically enhances the sensitivity to Ca^{2+} activation of endogenous *Xenopus* InsP_3R -1 channel [11], recombinant rat InsP_3R -3 channel expressed in *Xenopus* oocytes [63], and recombinant InsP_3R -1 channel expressed in DT40-3KO cells [50]. In recombinant InsP_3R -1 [50] and InsP_3R -3 [11] channels, ATP^{4-} also increases the sensitivity of the InsP_3R channels to Ca^{2+} inhibition, thus left-shifting the Ca^{2+} -dependence curve. In contrast, ATP^{4-} does not affect the sensitivities of recombinant mouse InsP_3R -2 channels expressed in DT40-3KO cells to Ca^{2+} activation or inhibition [50]. Besides sensitivity to Ca^{2+} regulation, ATP^{4-} also modifies the cooperativity of Ca^{2+} activation of InsP_3R channels, but does not affect the cooperativity of their inhibition by Ca^{2+} [50, 63]. It is somewhat paradoxical that ATP^{4-} reduces the cooperativity of Ca^{2+} activation of rat InsP_3R -1 channels expressed in DT40-3KO cells [50] and rat InsP_3R -3 channels expressed in *Xenopus* oocytes [11] in a similar way, but does not affect the Ca^{2+} activation cooperativity of native InsP_3R -1 channels in *Xenopus* oocytes [11].

To control for various factors that affect Ca^{2+} regulation of InsP_3R channels, recombinant channels in DT40-3KO cells expressing the three isoforms individually should be studied under the same conditions (especially $[\text{ATP}^{4-}]$) using the same experimental method. Although these experiments have been done [50, 57], $[\text{ATP}^{4-}]$ used in those experiments were not the same, complicating comparisons of Ca^{2+} regulation of homotetrameric channels of different InsP_3R isoforms. Fortunately, Ca^{2+} regulation of InsP_3R -2 channel is not sensitive to $[\text{ATP}^{4-}]$ [50] so it can be used as the reference to be compared with the channels of the other isoforms (Fig. 3B–D). In saturating $[\text{InsP}_3]$ (see below), InsP_3R -3 channels are over 10-fold less sensitive to Ca^{2+} activation than InsP_3R -2, and about 4-fold less sensitive to Ca^{2+} inhibition (Fig. 3C and D). Ca^{2+} activation of InsP_3R -3 is also significantly less cooperative than that of InsP_3R -2. Cooperativity of Ca^{2+} inhibition is similar for the two isoforms. In 5 mM ATP^{4-} , sensitivities to both Ca^{2+} activation and inhibition are similar for InsP_3R -2 and InsP_3R -1, but both Ca^{2+} activation and inhibition of InsP_3R -2 are more cooperative than those of InsP_3R -1 (Fig. 3B and C). In low 0.1 mM ATP^{4-} , InsP_3R -1 channel is about 4-fold less sensitive to Ca^{2+} activation and nearly 15-fold less sensitive to Ca^{2+} inhibition than InsP_3R -2. However, Ca^{2+} activation of InsP_3R -1 channel is substantially more cooperative than that of InsP_3R -2, whereas its Ca^{2+} inhibition remains less cooperative than that of InsP_3R -2.

3.2. InsP_3 regulation of InsP_3R channel gating

InsP_3 is the natural ligand of InsP_3R channels [2] that activates the channel saturably. Three different forms of InsP_3 modulation of InsP_3R channel gating have been reported.

The first form reported is through tuning the sensitivity of the channel to Ca^{2+} inhibition. This has been observed in endogenous InsP_3R -1 $\text{S}2^-$ channels in *Xenopus* oocytes, endogenous $\text{Sf}9$ InsP_3R , recombinant rat InsP_3R -3 expressed in *Xenopus* oocytes [11] and DT40-3KO cells [57] studied using nuclear patch clamping (Fig. 3D), as well as in endogenous rat InsP_3R -1 channels reconstituted into lipid bilayers [64]. Even at saturating

10 μM InsP_3 (found to be saturating in all nuclear patch clamp studies of InsP_3R channels), Ca^{2+} at high enough concentrations still inhibits InsP_3R channels. When $[\text{InsP}_3]$ is reduced to sub-saturating levels, reduction of $[\text{InsP}_3]$ increases the sensitivity of the channel to Ca^{2+} inhibition, thereby decreasing the range of $[\text{Ca}^{2+}]_i$ within which InsP_3R channel activity can be observed. At low enough $[\text{InsP}_3]$, the channel becomes more sensitive to Ca^{2+} inhibition than Ca^{2+} activation, and the channel starts to be inhibited before it is fully activated so the maximum channel P_o is decreased [11, 57]. In this kind of InsP_3 regulation, only the sensitivity of the channel to Ca^{2+} inhibition is modulated. The sensitivity of the channel to Ca^{2+} activation and the levels of cooperativity of the activation and inhibition are not affected [11, 57].

Another form of InsP_3 regulation of InsP_3R channel P_o is by changing the maximum channel activity level. This was first reported for endogenous $\text{InsP}_3\text{R-1 S2+}$ channels in the inner nuclear membrane of rat Purkinje neurons [20], and was also observed in recombinant rat $\text{InsP}_3\text{R-1}$ and mouse $\text{InsP}_3\text{R-2}$ channels expressed in DT40-3KO cells [50] (Fig. 3B and C). For these channels, as $[\text{InsP}_3]$ increases through the sub-saturating range, the maximum channel activity level is increased without altering the sensitivity or cooperativity of Ca^{2+} activation and inhibition. Thus the shape of the Ca^{2+} dependence curve remains essentially unchanged.

In the above mentioned forms of InsP_3 and Ca^{2+} regulation of InsP_3R channel activity, changes in channel P_o were brought about mainly by changes in the mean duration of channel closings (t_c), which can vary over several orders of magnitude in response to changes in $[\text{InsP}_3]$ and $[\text{Ca}^{2+}]_i$, while the mean duration of channel openings (t_o) remain relatively unaffected by ligand concentrations [36, 50, 57, 61, 65]. This suggests that once an InsP_3R channel opens, it will stay open for a stereotypical period of time (t_o), during which it remains insensitive to changes in $[\text{InsP}_3]$ and $[\text{Ca}^{2+}]_i$, before it closes. This can limit the extent of the negative feed-through effect of the Ca^{2+} fluxing through an open InsP_3R channel to terminate elementary Ca^{2+} release events.

The last form of InsP_3 regulation of InsP_3R channel P_o is a complex and indirect one. A study of recombinant rat $\text{InsP}_3\text{R-3}$ and $\text{InsP}_3\text{R-1}$ channels expressed in DT40-3KO cells using nuclear patch clamp techniques [54, 55] reported that exposure to InsP_3 induces rapid clustering of InsP_3R channels that are normally randomly distributed in the outer nuclear membrane without association with other InsP_3R channels. This clustering alters the gating behavior of the channels. Clustered channels have lower sensitivity to InsP_3 activation. In saturating $[\text{InsP}_3]$ but sub-optimal $[\text{Ca}^{2+}]_i$, the clustered channels gate independently with identical kinetics, but with P_o half that of lone channels. Furthermore, the reduction in channel P_o is mainly due to reduction in channel t_o . In contrast, under optimal ligand conditions (saturating $[\text{InsP}_3]$ and optimal $[\text{Ca}^{2+}]_i$), clustered channels gate with the same overall P_o as lone channels, but exhibit coupled gating, i.e., in membrane patches with two active channels, the probabilities that the channels were both open or both closed are higher than predicted for two identical and independent channels. This regulation is controversial. Nuclear patch clamp studies in multiple systems, including recombinant $\text{InsP}_3\text{R-3}$ channels expressed in DT40-3KO cells, have demonstrated that InsP_3R channels in the outer nuclear membrane form clusters before exposure to InsP_3 [36, 37, 66, 67]. A study of $\text{InsP}_3\text{R-}$

mediated elementary Ca^{2+} release events (Ca^{2+} puffs) in intact cells by TIRF microscopy revealed that those events arise from InsP_3R channel clusters established before exposure to InsP_3 , and that the size of the channel clusters does not increase after exposure to InsP_3 [68]. Moreover, single-molecule tracking of InsP_3R revealed no short-term change in the motility or clustering pattern of InsP_3R following activation of the InsP_3 - Ca^{2+} signaling pathway [69]. Importantly, channel gating analyses revealed no statistical difference between channel P_o in single- and multi-channel records obtained under sub-optimal ligand conditions, and no significant coupled gating behavior in two-channel records obtained under optimal or suboptimal ligand conditions for endogenous channels in *Xenopus* oocytes and Sf9 cells, and recombinant rat InsP_3R -3 channels expressed in *Xenopus* oocytes and DT40-3KO cells [67]. Thus, whether InsP_3 -induced rapid clustering of InsP_3R channels that leads to modification of channel gating is a real or universal phenomenon is very much open to question.

Now that homotetrameric channels of the three InsP_3R isoforms have been studied in the same cell system (DT40-3KO), the sensitivity of those channels to InsP_3 activation can be compared properly. Comparing the values of the half-maximal $[\text{InsP}_3]$ (EC_{50}) is complicated by the fact that those InsP_3R channels whose sensitivity to Ca^{2+} inhibition is tuned by $[\text{InsP}_3]$, like the homotetrameric recombinant InsP_3R -3 channel expressed in DT40-3KO cells (Fig. 3D), have different EC_{50} at different $[\text{Ca}^{2+}]_i$. Low $[\text{InsP}_3]$ ($< 1 \mu\text{M}$) is sufficient to activate the channel at low $[\text{Ca}^{2+}]_i$ ($< 1 \mu\text{M}$), but a much higher $[\text{InsP}_3]$ (3–10 μM) is required to activate the channel at high $[\text{Ca}^{2+}]_i$ ($> 10 \mu\text{M}$) (Fig. 3D). It is much simpler to compare the maximum P_o that can be observed at the same sub-saturating $[\text{InsP}_3]$ for those channels, as suggested in [50]. In 1 μM (sub-saturating) InsP_3 , the maximum observed P_o are 0.27, 0.51 and 0.17 for InsP_3R -1, InsP_3R -2 and InsP_3R -3 channels, respectively. Thus, the InsP_3 sensitivity sequence is InsP_3R -2 $>$ InsP_3R -1 $>$ InsP_3R -3, which agrees with the sequence reported for InsP_3 -binding affinities of the channels [70].

Like the sensitivity of the channels to $[\text{Ca}^{2+}]_i$ regulation, sensitivity of InsP_3R channels to InsP_3 activation is affected by factors other than the sequence of the InsP_3R . Although recombinant InsP_3R -3 channel has the lowest InsP_3 sensitivity among the three isoforms expressed in DT40-3KO cells, the same recombinant channel expressed in *Xenopus* oocytes has a maximum P_o of 0.77 in 33 nM InsP_3 [61], indicating a much higher InsP_3 sensitivity than any of the InsP_3R channels expressed in DT40-3KO cells. In fact, the native InsP_3R -1 channel in *Xenopus* oocytes has a similar InsP_3 sensitivity with maximum P_o of 0.77 in 33 nM InsP_3 , suggesting that the unknown factor(s) in *Xenopus* oocytes that enhances the InsP_3 sensitivity of InsP_3R channels is the dominant effect on channel InsP_3 sensitivity.

3.3. Regulation of InsP_3R channel gating by $[\text{Ca}^{2+}]_{\text{ER}}$

Whereas regulations of InsP_3R channel activities by $[\text{InsP}_3]$ and $[\text{Ca}^{2+}]_i$ have been well studied, effects of $[\text{Ca}^{2+}]_{\text{ER}}$ on InsP_3R channels remain controversial. In a recent study [57], the rapid perfusion technique was applied in nuclear patch clamp experiments to examine the behaviors of the same InsP_3R channels in the same isolated nuclear membrane patches in different $[\text{Ca}^{2+}]_{\text{ER}}$. When $[\text{Ca}^{2+}]_{\text{ER}}$ was switched from 70 nM, a level at which no Ca^{2+} flux was driven through the InsP_3R channel from the luminal side, to 0.3–1.1 mM, levels that generate significant Ca^{2+} flux, activities of the InsP_3R channels were indeed modified (Fig.

4A). However, the nature (activating or inhibitory) and magnitude of the modulation depended not only on $[Ca^{2+}]_{ER}$ (Fig. 4A vs. B), but also on cytoplasmic conditions, including $[InsP_3]$ (Fig. 4B vs. C), $[Ca^{2+}]_i$ (Fig. 4B vs. D) and Ca^{2+} buffering capacity (Fig. 4C vs. E). Inhibition and activation of $InsP_3R$ channel by $[Ca^{2+}]_{ER}$ were even completely abrogated by strong Ca^{2+} buffering on the cytoplasmic side together with V_{app} that opposed the $[Ca^{2+}]$ gradient to reduce the Ca^{2+} flux through the channel (Fig. 4F and G).

Taken together, these observations indicate that Ca^{2+} in the ER lumen modifies $InsP_3R$ channel P_o not by direct binding to some regulatory site(s) on the luminal side of the $InsP_3R$ channel, but by a feed-through effect. Ca^{2+} driven by high $[Ca^{2+}]_{ER}$ through the open $InsP_3R$ channel raises concentration of $[Ca^{2+}]_i$ in the vicinity of the channel and binds to the cytoplasmic activating and inhibitory Ca^{2+} sites to modulate the channel activity [57].

3.4. Graded recruitment of $InsP_3R$ channels by favorable ligand conditions

The amount of Ca^{2+} flux (J) through a patch of ER membrane depends on three factors: the unitary Ca^{2+} current passing through an open $InsP_3R$ channel (i_{Ca}), the activity level of the $InsP_3R$ channels (P_o), and the total number of active channels in the membrane patch (N_A). All three factors can be individually measured using nuclear patch clamp electrophysiology. Specifically, N_A in an isolated membrane patch is taken to be the maximum number of open-channel current levels observed. To avoid undercounting N_A , and therefore overestimating P_o , only current records that lasted long enough to ensure a high degree of accuracy (>95% confidence) in determining N_A were used for N_A and P_o evaluation [36, 71]. By counting N_A in tens of membrane patches for each of a dozen different combinations of $[InsP_3]$ and $[Ca^{2+}]_i$ in a nuclear patch clamp study of the endogenous Sf9 $InsP_3R$ channel, it was demonstrated that on average, N_A observed in the presence of favorable ligand conditions (optimal $[Ca^{2+}]_i$, saturating $[InsP_3]$ or both) was significantly higher than that observed in sub-optimal or inhibitory $[Ca^{2+}]_i$, or sub-saturating $[InsP_3]$ [36]. However, in that study, it was not certain whether factors other than ligand concentrations that can affect N_A , like the tip size of the patch clamp microelectrodes used and the topology of the membrane patch isolated at the tip of the microelectrodes, were sufficiently controlled. Modulation of N_A by ligand concentrations was more convincingly demonstrated in a later study that counted N_A observed in the *same* isolated nuclear membrane patch when its cytoplasmic side was exposed to various ligand concentrations. This was done by performing rapid perfusion exchange of the bath solution in nuclear patch clamp experiments in the cyto-out configuration [71]. N_A increased incrementally as $[Ca^{2+}]_i$ was increased from resting through sub-optimal to optimal levels in saturating $[InsP_3]$. Similarly, more channels were active in saturating $[InsP_3]$ than in sub-saturating $[InsP_3]$ in the same membrane patch. This was observed for the endogenous $InsP_3R$ channels in Sf9 cells, which have only one $InsP_3R$ gene. More important, this was also observed for homotetrameric recombinant $InsP_3R$ -3 channels expressed in DT40-3KO cells, suggesting that even a homogeneous population of $InsP_3R$ channels can exhibit heterogeneous sensitivity to Ca^{2+}_i and $InsP_3$ activation. Graded recruitment of $InsP_3R$ channel by ligands was completely abrogated by treatment with reducing reagents and restored by treatment with oxidizing reagents, suggesting that the heterogeneous ligand sensitivity is regulated largely by post-transcriptional redox modulation of the channels.

In a population of InsP₃R channels with heterogeneous sensitivities to ligands, those channels with higher ligand sensitivity may act as “hot spots” to initiate fundamental Ca²⁺-release events (blips) [72] as [InsP₃] starts to rise in response to extracellular stimuli. The rise in [Ca²⁺]_i not only enhances the activities of the InsP₃R channels that are already activated, but also recruits more InsP₃R channels with lower [Ca²⁺]_i sensitivities to further amplify the magnitude of the Ca²⁺-induced Ca²⁺ release to propagate the Ca²⁺ signal. Thus, graded recruitment of InsP₃R channels with favorable ligand conditions can be an important mechanism in both the generation and propagation of intracellular Ca²⁺ signals.

3.5. Ligand regulation of modal gating behaviors of InsP₃R channels

In some experimental systems for nuclear patch clamping, like the native Sf9 InsP₃R and recombinant InsP₃R expressed in DT40-3KO cells, the activity of individual InsP₃R channels can be stably recorded for extensive periods (tens of minutes) [71, 73]. Examination of such long single-channel records of Sf9 InsP₃R revealed that even under constant ligand conditions ([InsP₃] and [Ca²⁺]_i), channels exhibited relatively occasional (one to two orders of magnitude less frequent than channel gating), spontaneous and abrupt transitions among three distinct gating patterns (modes) (Fig. 5A): a low-activity (L) mode with long channel closings and brief channel openings ($P_o = 0.007$), an intermediate (I) mode with rapid channel gating kinetics ($P_o = 0.24$), and a high-activity (H) mode with prolonged bursts of channel activity ($P_o = 0.85$) [73]. These modes were observed under all ligand conditions and remarkably, channel gating kinetics within a mode were not significantly altered by [Ca²⁺]_i and [InsP₃] (Fig. 5B). Thus, the graded dependencies of InsP₃R channel P_o on [Ca²⁺]_i and [InsP₃] of various InsP₃R channels (Fig. 3B–D) is mostly the result of continuous shifting of the relative probabilities of the channel being in these modes, especially the L and H modes (Fig. 5C) as the ligand concentrations vary. Interestingly, only the mean duration of the H mode was significantly affected by ligand concentrations, while those of the L and I modes remained within a narrow range for the [InsP₃] and [Ca²⁺]_i examined. The ligand independence of the long mean duration of the L mode ($\langle\tau^L\rangle$) suggests that once a channel enters the L mode, it becomes insensitive to [InsP₃] and [Ca²⁺]_i, and will remain in the L mode with very low for a stereotypical long duration $\sim\langle\tau^L\rangle$. This may be one of the mechanisms to terminate an InsP₃R-mediated Ca²⁺ release event (puff), especially when the local cytoplasmic ligand conditions are not optimal so the channels have high probability of entering the L mode.

Subsequently, such modal behaviors have also been observed for endogenous InsP₃R channels from human lymphoblasts, mouse embryonic fibroblasts and mouse primary embryonic cortical neurons [48]. In these channels, modal switching is not only the major mechanism for physiological ligand regulation of InsP₃R channel activities, but is also the mechanism behind the pathogenic dysregulation of InsP₃R channel by familial Alzheimer's disease-linked mutant presenilins. Interaction between the mutant presenilins and InsP₃R channels greatly increases the propensity of InsP₃R channels being in the H mode, thereby causing exaggerated Ca²⁺ release through those channels [48].

Recently, modal gating was observed in recombinant rat InsP₃R-1 S2+ and mouse InsP₃R-2 expressed in DT40-3KO cells [50]. Both channels exhibit two gating modes: a “park” mode

in which the channels only open briefly and very occasionally ($P_o < 0.01$) and a “drive” mode with bursting channel activities ($P_o = 0.64$) [74]. Within a mode, channel gating kinetics are practically the same in all $[Ca^{2+}]_i$, $[InsP_3]$ and $[ATP^{4-}]$. In $InsP_3R$ -1 channel, more favorable $[Ca^{2+}]_i$ both prolongs the drive mode duration and shortens the park mode duration; more favorable $[InsP_3]$ mainly shortens the park mode duration and lengthens the drive mode to a smaller extent; and increase in $[ATP^{4-}]$ shifts the effects of $[Ca^{2+}]_i$ on the modal durations towards lower $[Ca^{2+}]_i$ levels. Ligand regulation of model behaviors of $InsP_3R$ -2 channel is more complicated. In saturating $[InsP_3]$, $[ATP^{4-}]$ does not affect durations of the modes and favorable $[Ca^{2+}]_i$ lengthens drive mode duration and shortens park mode duration as in $InsP_3R$ -1. At low $[InsP_3]$ and saturating $[ATP^{4-}]$, more favorable $[Ca^{2+}]_i$ only shorten the park mode duration but do not change the drive mode duration. When both $[InsP_3]$ and $[ATP^{4-}]$ are low, the channel has low P_o with long park mode duration and short drive mode duration over most $[Ca^{2+}]_i$; and drive mode duration is further reduced and park mode duration is further lengthened at extreme $[Ca^{2+}]_i$. The insensitivity of the mean duration of the L/park mode to ligand conditions observed in the insect Sf9 $InsP_3R$ channel was not observed in the mammalian homotetrameric $InsP_3R$ -1 or $InsP_3R$ -2 channels. The durations of both the park and drive modes are affected by $[InsP_3]$, $[Ca^{2+}]_i$ and $[ATP^{4-}]$ in most ligand conditions. Thus, unlike the case for Sf9 $InsP_3R$ channels, modal gating kinetics probably does not play a significant role in the termination of $InsP_3R$ -mediated intracellular Ca^{2+} release events involving these channels.

Because of the consistency of the P_o of the $InsP_3R$ channel within a gating mode over a broad range of ligand conditions (Fig. 5B and [50]), modal gating behavior of the $InsP_3R$ channels offers a way to greatly simplify the modeling of Ca^{2+} release through these channels. The gating kinetics (opening and closing) of the channels within a mode can be ignored. An $InsP_3R$ channel in one gating mode can be considered as a channel in a conductance substate with Ca^{2+} conductance of $(i_{Ca})(P_o^M)$, where P_o^M is the P_o of the channel in that gating mode. The kinetics of the transition of the channel among the conductance substates is the kinetics of the modal transitions of the channel, which is significantly slower than the gating kinetics of the channel and therefore simpler to simulate.

3.6. Ligand regulations of heterotetrameric $InsP_3R$ channels of known isoform composition

Although most cell types examined express, at various levels, multiple isoforms of $InsP_3R$ [31, 75], which can form heterotetrameric channels [11], there has been no experimental technique to study the properties of individual heterotetrameric $InsP_3R$ channels with known isoform composition until recently. The exciting technological break-through of expressing concatenated $InsP_3R$ dimers in DT40-3KO cells to form properly localized and functional $InsP_3R$ channels enables the study of the properties of heterotetrameric $InsP_3R$ channels of a specific isoform composition [76]. In the pioneering study, on-nucleus patch clamp experiments demonstrated that the conductance of $InsP_3R$ channels formed by concatenated $InsP_3R$ -1: $InsP_3R$ -1 (R1R1) dimers is identical to that of homotetrameric $InsP_3R$ -1 channels. Furthermore, the gating and modal behaviors, and their regulation by $[InsP_3]$ and $[ATP^{4-}]$ at 200 nM $[Ca^{2+}]_i$ of channels formed by R1R1 dimers resembled those of homotetrameric $InsP_3R$ -1 channels, and those of channels formed by concatenated R2R2 dimers resembled

those of homotetrameric InsP₃R-2 channels. Remarkably, those single-channel properties of channels formed by concatenated R1R2 dimers were indistinguishable from those of homotetrameric InsP₃R-2 channels, suggesting that InsP₃R-2 dominates over InsP₃R-1 in determining the gating properties of a heterotetrameric channel formed by the two. Characterizing the conductance and gating properties of heterotetrameric InsP₃R channels with known isoform composition can provide valuable insights into the physiological reasons behind the sequence diversity of the channel and the intricate regulation of the expression levels of InsP₃R with different sequences [11].

4. Dynamic responses of InsP₃R channel to rapid changes in ligand concentrations

Although studying InsP₃R gating properties in constant [InsP₃] and [Ca²⁺]_i has provided much insight into ligand regulation of InsP₃R channels, [Ca²⁺]_i, and to a lesser extent [InsP₃], change dynamically during Ca²⁺ signaling events. Therefore, to get a better understanding of how InsP₃R channels function and contribute to intracellular Ca²⁺ signaling, it is essential to study the behaviors of InsP₃R channels under non-steady ligand conditions.

The kinetic responses of single Sf9 InsP₃R channels to abrupt changes in ligand concentrations were examined with millisecond resolution by applying rapid perfusion techniques to cyto-out nuclear membrane patches [77]. This study provided the first and, to date, the only measurements of the lag times of InsP₃ activation and deactivation (Fig. 6A and B), Ca²⁺ activation and deactivation (Fig. 6C and D), activation and deactivation by simultaneous changes in [Ca²⁺]_i and [InsP₃] (Fig. 6E and F), Ca²⁺ inhibition and recovery from inhibition (Fig. 6G and I), and the durations of fundamental single-channel Ca²⁺-induced Ca²⁺ release events (Fig. 6J). While the majority of [Ca²⁺]_i jumps from < 10 nM to 300 μM in 10 μM InsP₃ activated Ca²⁺ release bursts (Fig. 6J), a non-trivial fraction of the jumps failed to activate any burst due to Ca²⁺ binding to the inhibitory sites of the channel before the activating sites got occupied. This showed that Ca²⁺ binding to those sites are non-sequential. Similarly, in some of the [Ca²⁺]_i drops from 300 μM to < 10 nM, the channel recovered from Ca²⁺ inhibition before Ca²⁺ unbound from its activating site, generating a brief burst of channel activity (Fig. 6K), showing Ca²⁺ unbinding from the activating and inhibitory sites are also non-sequential. The study also clearly demonstrated the absence of long-term adaptation [78] behaviors in InsP₃R channels following changes in [InsP₃] or [Ca²⁺]_i (Fig. 6L and M, respectively). The surprisingly long times InsP₃R channels took to recovery from Ca²⁺ inhibition (Fig. 6I) suggest that this may contribute significantly to the refractory interval between consecutive Ca²⁺ release from the same site in the ER [79]. The lag times for channel activation by [InsP₃] jumps from 0 to 100 μM were only modestly shorter than those for [InsP₃] jumps from 0 to 10 μM (Fig. 6H vs. A), indicating that InsP₃ binding to the channel, and the subsequent conformation changes that lead to the channel arriving at its open conformation(s) both contribute significantly to the InsP₃ activation lag times. In contrast, the lag times for channel activation by [Ca²⁺]_i jumps from < 10 nM to 300 μM were substantially shorter than those for [Ca²⁺]_i jumps from < 10 nM to 2 μM (Fig. 6J vs. C), suggesting that Ca²⁺ binding to the channel is the major

contribution to the Ca^{2+} activation lag times. Furthermore, the study revealed an unexpected cooperativity between Ca^{2+} and InsP_3 modulation of the channel: in the absence of InsP_3 , Ca^{2+} binding to an InsP_3R channel stabilizes it in a closed conformation so that a jump in $[\text{InsP}_3]$ in the constant presence of high ($2\ \mu\text{M}$) $[\text{Ca}^{2+}]_i$ takes significantly longer to activate the channel than when $[\text{InsP}_3]$ and $[\text{Ca}^{2+}]_i$ are increased simultaneously (Fig. 6A vs. E). On the other hand, in the presence of InsP_3 , Ca^{2+} binding to an InsP_3R channel stabilizes it in an open conformation so that decreases in $[\text{InsP}_3]$ in the constant presence of $2\ \mu\text{M}$ $[\text{Ca}^{2+}]_i$ take longer to deactivate the channel than when both $[\text{InsP}_3]$ and $[\text{Ca}^{2+}]_i$ are dropped simultaneously (Fig. 6B vs. F). The mean duration of the L mode of the Sf9 channel (Fig. 5D) is substantially longer than the lag times of activation of the channel by abrupt jumps in $[\text{InsP}_3]$ (Fig. 6A), or in $[\text{Ca}^{2+}]_i$ (Fig. 6C), or in both $[\text{Ca}^{2+}]_i$ and $[\text{InsP}_3]$ (Fig. 6E). This suggests that the channel has a complex kinetic gating scheme with the channel in distinct closed kinetic states depending on the occupancies of its activating Ca^{2+} and InsP_3 binding sites. These different closed kinetic states are connected to different open kinetic states, as described in [80]. Thus, when the channel is activated by jumps in $[\text{InsP}_3]$ or $[\text{Ca}^{2+}]_i$, it is exiting from closed kinetic states that are different from the closed kinetic state(s) involved when the channel transits from the L to the I or H modes, with different transition kinetics.

5. Moving forward

The InsP_3R channel is unusual in that even its fundamental properties: single-channel conductance and regulation by InsP_3 and Ca^{2+} , are apparently very mutable. Not only are the sensitivities and degrees of cooperativity of ligand regulation different among channels of different isoforms, but also the ways InsP_3 regulates those different channels are fundamentally different. Even the conductance and the sensitivity to InsP_3 activation of the same recombinant homotetrameric channel expressed in different cells can be radically different. Therefore due caution should be exercised when attempting to extrapolate single InsP_3R channel properties observed in one experimental system to another system. It is advisable to characterize the basic properties: conductance and $[\text{Ca}^{2+}]_i$ and $[\text{InsP}_3]$ dependencies, of InsP_3R channels in a new experimental system, using buffer solutions with commonly used physiological compositions ($140\text{--}150\ \text{mM}$ KCl, $0.5\ \text{mM}$ ATP^{4-} and pH 7.3) for easier comparison with previous studies.

The recent development of the capability of expressing concatenated InsP_3R dimers to form functional InsP_3R channels in DT40-3KO cells [76] offers the opportunity to study heterotetrameric InsP_3R channels with known isoform compositions. That study also demonstrated that concatenated InsP_3R tetramers could also be expressed in DT40-3KO cells, though in significantly lower levels than concatenated dimers. If the expression of concatenated tetramers can be enhanced, then properties of channels formed by all three InsP_3R isoforms, like R1R2R3R1, and channels with asymmetric isoform compositions, like R1R1R1R2, can be studied and compared with properties of native InsP_3R channels in various cells to shed light on the physiological significance of the molecular diversity of the InsP_3R .

Many computational kinetic models have been developed over the years to account for the observed InsP_3 and Ca^{2+}_i regulation of single InsP_3R channel gating ([81] and references

therein). These kinetic models were then used to simulate the generation and propagation of more complex intracellular Ca^{2+} signals (blips, puffs and waves) by organized clusters of InsP_3R channels [72]. Even though recent experiments demonstrated that modulation of modal switching is the main mechanism for steady-state ligand regulation of InsP_3R channel activities [50, 73], only a few models have been developed to account for InsP_3R modal behaviors [74, 80]. Furthermore, the roles of modal behaviors in the dynamic responses of InsP_3R channel to changes in $[\text{InsP}_3]$ and more importantly $[\text{Ca}^{2+}]_i$ have barely been investigated [80]. Much work needs to be done to develop kinetic models that can adequately describe the complex gating behaviors of single InsP_3R channels to enable proper simulation of organized gating of multiple InsP_3 channels to generate local and global Ca^{2+} release events.

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Highlights

InsP₃R single-channel properties studied by nuclear patch clamping are reviewed

We examine InsP₃R K⁺ conductance and unitary Ca²⁺ current in physiological solutions

Steady-state Ca²⁺ and InsP₃ dependencies of channels of various isoforms are compared

Ligand-regulated modal gating and graded recruitment of InsP₃R channels are examined

Responses of InsP₃R channels to abrupt ligand concentration changes are presented

Fig. 1. Different configurations for nuclear patch clamping experiments

Schematic diagrams illustrating the orientation of InsP₃R channels in nuclear membrane patches in various configurations of nuclear patch-clamp experiments. (A) On-nucleus configuration with outer nuclear membrane intact, (B) excised luminal-side-out configuration, (C) excised cytoplasmic-side-out configuration, (D) on-nucleus configuration with outer nuclear membrane removed, (E) excised nucleoplasmic-side-out configuration. (F) Diagram showing how the two aspects of the InsP₃R channel are represented in this figure. Figure modified from [19].

Fig. 2. Measurement of unitary Ca²⁺ current through a type 3 InsP₃R channel in physiological ionic conditions

(A) Linear fits (purple and red lines) to selected open-channel current (*I*_{open}) and closed-channel leak current (*I*_{closed}) vs. *V*_{app} data (blue and pink points, respectively) from a single-channel nuclear patch clamp experiment in excised lum-out configuration under symmetric ionic conditions with pipette and perfusion solutions containing 140 mM KCl, 0.5 mM MgCl₂, and 3 μM free [Ca²⁺]. Pipette solution contained 2 μM InsP₃. (B) Fitted *I*_{open}-*V*_{app} and *I*_{closed}-*V*_{app} lines in *I*-*V*_{app} region marked by the black rectangle in (a). Zero-current level (black dotted line) established at the intersection of the *I*-*V*_{app} fits at *V*_{app} = 0 (marked by orange arrowhead). (C) Linear fits to *I*_{open} and *I*_{closed} data from *V*_{app} ramps recorded for the same membrane patch under asymmetric ionic conditions with perfusion solution containing 2 mM CaCl₂. Color and symbol conventions, *I* and *V*_{app} ranges are the same as in (a). (D) Fitted *I*-*V* lines in the same *I*-*V* region and with the same zero-current level as (b). *V*_{app} = 0 (marked by orange arrowhead) at the intersection of the *I*_{closed}-*V*_{app} line and zero-current level. Unitary Ca²⁺ current is *I*_{open} at *V*_{app} = 0 (marked by green double arrow). Figure modified from [52].

Fig. 3. Ligand dependencies of gating of homotetrameric recombinant InsP₃R channels of various isoforms expressed in DT40-3KO cells

(A) Typical single-channel on-nucleus patch-clamp current traces of InsP₃R-3 channels in sub-optimal (190 nM), optimal (2 μM) and inhibitory (23 μM) [Ca²⁺]_i in the presence of saturating (10 μM) [InsP₃]. $V_{app} = -40$ mV. Arrow indicates closed channel baseline current level for these and all subsequent current traces. (B–D) [Ca²⁺]_i-dependencies of mean channel P_o in various [InsP₃] and [ATP⁴⁻] as tabulated, for rat InsP₃R-1, mouse InsP₃R-2 and rat InsP₃R-3 channels, respectively. Curves are fitted to mean P_o data points using the empirical biphasic Hill equation with 5 parameters [11]. Data in (b) and (c) are from [50]. (a) and (d) are modified from [57].

Fig. 4. Effects of $[\text{Ca}^{2+}]_{\text{ER}}$ on the gating of homotetrameric $\text{InsP}_3\text{R-3}$ channels expressed in DT40-3KO cells

Typical current traces from nuclear patch clamp experiments in lum-out configuration under experimental conditions ($[\text{InsP}_3]$, $[\text{Ca}^{2+}]_i$, concentrations of Ca^{2+} chelator in pipette solution and V_{app}) tabulated at top. Color bars at top indicate changes in $[\text{Ca}^{2+}]_{\text{ER}}$ achieved by rapid perfusion techniques. Blue bars at bottom mark the part of the current traces used to evaluate the channel P_o tabulated below. Figures are modified from [57]. For (G), V_{app} should be +70 mV.

Fig. 5. Modal gating behaviors of endogenous insect Sf9 InsP_3R

(A) Black traces are baseline-subtracted current records obtained in saturating 10 μM InsP_3 , with sub-optimal (100 nM), optimal (1 μM) and inhibitory (89 μM) $[\text{Ca}^{2+}]_i$, as tabulated. Blue and purple traces are idealized gating records before and after burst analysis, respectively, derived from the corresponding current records. Color bars at bottom indicate the gating mode the InsP_3R channel was in during the shown records (red, orange and green for L, I and H modes, respectively). (B) Channel open probability in each of the three modes (P_o^M), (C) probability of the channel being in each of the three modes (π^M), and (D) mean durations ($\langle \tau^M \rangle$) of each of the three modes observed are plotted against the ligand concentrations in which they were observed. Note the non-linear y-axis used in (B) to better show the low P_o^L values for the L mode, and the logarithmic scale used in (D). In (B-D), the

top x-axis labels indicate the cytoplasmic free calcium concentrations, the bottom labels indicate the cytoplasmic InsP_3 concentrations. Figure modified from [12] and [73].

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Fig. 6. Dynamic responses of endogenous insect Sf9 InsP₃R to abrupt changes in [Ca²⁺]_i and [InsP₃] observed in cyto-out nuclear patch clamp experiments

Color bars at top indicate changes in [Ca²⁺]_i and [InsP₃] achieved by rapid exchange of bath solutions. Times when such exchanges occurred were marked by changes in closed-channel current levels due to difference in [KCl] (100 mM vs. 70 mM) in the bath solutions used. Gray bars at bottom indicate the lag times between ligand concentration switches and the resulting changes in InsP₃R channel gating behaviors. Two arrows indicate the closed-channel current levels before and after the bath solution switches for each current traces. In (J), the dark gray bar indicates the Ca²⁺ activation lag time and the light gray bar indicates the Ca²⁺ inhibition lag time. Note that a different time scale is used for (L) and (M). Figure modified from [77].

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Table 1

Permeability ratios of InsP₃R channels in nuclear envelope

InsP ₃ R channel	Permeability ratios					references
	P_{Ca}	P_{Ba}	P_{Mg}	P_K	P_{Cl}	
<i>Xenopus</i> InsP ₃ R-1 ^a	7.6	: 4.3	: 3.2	: 1	: 0.23	[11]
Rat InsP ₃ R-1 ^a	6.6	: 6.5	:	1		[22]
Sf9 InsP ₃ R ^a	10	:	6.8	: 1	: 0.22	[36]
Rat InsP ₃ R-1 ^b	4.3	:		1	: 0.07	[39]
Rat InsP ₃ R-3 ^b	15.2	: 11.8	: 10.2	: 1	: 0.27	[52]

^a endogenous channels

^b recombinant channels