Spontaneous Channel Activity of the Inositol 1,4,5-Trisphosphate (InsP$_3$) Receptor (InsP$_3$R). Application of Allosteric Modeling to Calcium and InsP$_3$ Regulation of InsP$_3$R Single-channel Gating

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ABSTRACT The InsP$_3$R Ca$^{2+}$ release channel has a biphasic dependence on cytoplasmic free Ca$^{2+}$ concentration ([Ca$^{2+}$]). InsP$_3$ activates gating primarily by reducing the sensitivity of the channel to inhibition by high [Ca$^{2+}$]. To determine if relieving Ca$^{2+}$ inhibition is sufficient for channel activation, we examined single-channel activities in low [Ca$^{2+}$], in the absence of InsP$_3$, by patch clamping isolated Xenopus oocyte nuclei. For both endogenous Xenopus type 1 and recombinant rat type 3 InsP$_3$R channels, spontaneous InsP$_3$-independent channel activities with low open probability $P_o$ (~0.03) were observed in [Ca$^{2+}$]$_o$ < 5 nM with the same frequency as in the presence of InsP$_3$, whereas no activities were observed in 25 nM Ca$^{2+}$. These results establish the half-maximal inhibitory [Ca$^{2+}$]$_i$ of the channel to be 1.2–4.0 nM in the absence of InsP$_3$, and demonstrate that the channel can be active when all of its ligand-binding sites (including InsP$_3$) are unoccupied. In the simplest allosteric model that fits all observations in nuclear patch-clamp studies of [Ca$^{2+}$]i and InsP$_3$ regulation of steady-state channel gating behavior of types 1 and 3 InsP$_3$R isoforms, including spontaneous InsP$_3$-independent channel activities, the tetrameric channel can adopt six different conformations, the equilibria among which are controlled by two inhibitory and one activating Ca$^{2+}$-binding and one InsP$_3$-binding sites in a manner outlined in the Monod-Wyman-Changeux model. InsP$_3$ binding activates gating by affecting the Ca$^{2+}$ affinities of the high-affinity inhibitory sites in different conformations, transforming it into an activating site. Ca$^{2+}$ inhibition of InsP$_3$-liganded channels is mediated by an InsP$_3$-independent low-affinity inhibitory site. The model also suggests that besides the ligand-regulated gating mechanism, the channel has a ligand-independent gating mechanism responsible for maximum channel $P_o$, being less than unity. The validity of this model was established by its successful quantitative prediction of channel behavior after it had been exposed to ultra-low bath [Ca$^{2+}$].

KEY WORDS: single-channel electrophysiology • patch clamp • calcium • Xenopus oocyte • nucleus

INTRODUCTION

In many cell types, the second messenger inositol 1,4,5-trisphosphate (InsP$_3$) is generated in the cytoplasm in response to the binding of extracellular ligands to plasma membrane receptors. InsP$_3$ binds to its receptor, the InsP$_3$R, in the ER and activates it as a Ca$^{2+}$ channel to liberate stored Ca$^{2+}$ from the ER lumen into the cytoplasm. This rapid release of Ca$^{2+}$ modulates the cytoplasmic free Ca$^{2+}$ concentration ([Ca$^{2+}$]$_i$), which serves as a ubiquitous cellular signal that can be manifested temporally as repetitive spikes or oscillations, and spatially as propagating waves or highly localized events (Meyer and Stryer, 1991; Berridge, 1993; Toescu, 1995). The temporal and spatial complexity of this signaling system involves sophisticated regulation of the activity of the InsP$_3$R by various mechanisms, including cooperative activation by InsP$_3$ (Meyer et al., 1988; Mak et al., 1998) and biphasic feedback from the permeant Ca$^{2+}$ ion (Iino, 1990; Bezprozvanny et al., 1991; Mak et al., 1998).

A family of three InsP$_3$ receptor isoforms has been identified—types 1, 2, and 3, with different primary sequences derived from different genes (Patel et al., 1999). Recent studies have demonstrated that channel $P_o$ of both the types 1 and 3 InsP$_3$R isoforms is modulated with biphasic dependencies on cytoplasmic free Ca$^{2+}$ concentration ([Ca$^{2+}$]$_i$), suggesting that the channels have two distinct types of functional Ca$^{2+}$-binding sites: activating and inhibitory (Mak et al., 1998, 2001b). InsP$_3$ activates the InsP$_3$R by tuning the sensitivity of the channel to Ca$^{2+}$ inhibition, with increases in the cytoplasmic concentration of InsP$_3$ ([InsP$_3$]) causing a decrease in the apparent Ca$^{2+}$ affinity of the inhibitory binding sites of the channel. Nevertheless, the fully InsP$_3$-liganded channel can still be inhibited

Abbreviations used in this paper: InsP$_{3o}$, inositol 1,4,5-trisphosphate; InsP$_3$R, InsP$_3$ receptor; $X$InsP$_3$R-1, Xenopus type 1 InsP$_3$R; r-InsP$_3$R-3, rat type 3 InsP$_3$R; $P_o$, open probability.
by Ca\(^{2+}\), albeit at sufficiently high concentrations (Mak et al., 1998, 2001b). Importantly, InsP\(_3\) has little apparent effect on activation parameters (half-maximal activation [Ca\(^{2+}\)]\(_i\), \(K_{act}\), and activation Hill coefficient, \(H_{act}\)) of the biphasic Hill equation that describes the Ca\(^{2+}\) response of the channel, nor does it affect the robust maximum open probability exhibited by either InsP\(_3\)R isoform under optimal activating conditions.

Whereas previous studies provided estimates of the affinity of the inhibitory Ca\(^{2+}\)-binding sites in subsaturating and saturating concentrations of InsP\(_3\) (Mak et al., 1998, 2001b), the apparent affinity of the inhibitory Ca\(^{2+}\)-binding sites of an InsP\(_3\)R channel in the absence of InsP\(_3\) has not been determined. The effects of InsP\(_3\) have been modeled empirically assuming infinitely high affinity of the Ca\(^{2+}\) inhibition sites in a channel not bound to InsP\(_3\) (Mak et al., 1998, 2001b), but it is more reasonable to expect that the inhibitory Ca\(^{2+}\)-binding sites adopt a finite maximal Ca\(^{2+}\) affinity in the absence of InsP\(_3\).

Here, we examined activities of the types 1 and 3 InsP\(_3\)R channels in the absence of InsP\(_3\) to characterize the apparent affinity of the inhibitory Ca\(^{2+}\)-binding site of the InsP\(_3\)R not bound to InsP\(_3\). We reasoned that since InsP\(_3\) activates the channel by preventing Ca\(^{2+}\) from inhibiting it, it might be possible to activate the channel in the absence of InsP\(_3\) by removing Ca\(^{2+}\) from the inhibitory site by simply reducing [Ca\(^{2+}\)]\(_i\) to very low levels. We demonstrate that the InsP\(_3\)R channel opens spontaneously in the absence of InsP\(_3\) when the channel is exposed to [Ca\(^{2+}\)]\(_i\) < 5 nM, but not when [Ca\(^{2+}\)]\(_i\) is elevated to 25 nM. These observations establish the apparent affinity of the Ca\(^{2+}\) inhibition sites of an InsP\(_3\)R channel not bound to InsP\(_3\), and they support an allosteric model of InsP\(_3\)R activation by Ca\(^{2+}\).

Many models have been developed to account for InsP\(_3\)R-mediated [Ca\(^{2+}\)]\(_i\) signals, but all previously proposed models of InsP\(_3\)R single-channel gating (De Young and Keizer, 1992; Swillens et al., 1994; Kaftan et al., 1997; Marchant and Taylor, 1997; Swillens et al., 1998; Adkins and Taylor, 1999; Moraru et al., 1999) assumed that only the InsP\(_3\)-bound state(s) of the receptor is active. Thus, they fail to account for the spontaneous, InsP\(_3\)-independent activities of the InsP\(_3\)R observed in our study. To provide insights into the mechanisms underlying ligand regulation of InsP\(_3\)R channel activity, we have developed an allosteric molecular model that can quantitatively account for not only the spontaneous, InsP\(_3\)-independent channel activities in low [Ca\(^{2+}\)]\(_i\), but all other characteristics of InsP\(_3\) and [Ca\(^{2+}\)]\(_i\), regulation of both types 1 and 3 InsP\(_3\)R isoforms observed in nuclear patch clamp experiments (Mak et al., 1998, 2001b, 2003).

Materials and Methods

Selection and Microinjection of Xenopus Oocytes

Maintenance of Xenopus laevis and surgical extraction of ovaries were performed as previously described (Jiang et al., 1998). The level of endogenous InsP\(_3\)R channel activity was determined for each new batch of oocytes by patch clamping at least 3 isolated nuclei, obtaining 4–6 patches from each (Mak et al., 2000, 2001b). Rat type 3 InsP\(_3\)R (r-InsP\(_3\)R-3) channels were expressed by cRNA injection into oocytes ascertained to have extremely low level of endogenous InsP\(_3\)R activities. In these studies, one endogenous Xenopus oocyte type 1 InsP\(_3\)R (X-InsP\(_3\)R-1) channel was observed in 100 patches from 5 batches of oocytes used for r-InsP\(_3\)R-3 cRNA injection. In contrast, 544 channels were detected in 330 membrane patches, with 108 patches containing multiple InsP\(_3\)R channels, from nuclei of r-InsP\(_3\)R-3-expressing oocytes 4–5 days after cRNA injection. Assuming that the types 1 and 3 InsP\(_3\)R associate randomly to form tetrameric channels, 97.6% of InsP\(_3\)R channels detected in these experiments were contributed by type 3 homotetramers (Mak et al., 2000).

The endogenous X-InsP\(_3\)R-1 was studied using batches of oocytes with high level of endogenous InsP\(_3\)R activities, up to four days after ovary extraction (Mak and Foskett, 1994, 1997, 1998).

Patch Clamp Data Acquisition and Analysis

Patch clamp electrophysiology of isolated nuclei was performed as described (Mak and Foskett, 1994, 1997, 1998; Mak et al., 2000) in “on-nucleus” configuration at room temperature with the pipette electrode at +20 mV (unless stated otherwise) relative to the reference bath electrode. Transmembrane currents were amplified, filtered at 1 kHz, digitized at 5 kHz and recorded directly onto hard disk.

Channel opening and closing events were identified with a 50% threshold, and channel open probabilities and mean open and closed durations, were evaluated using MacTac software (Bruxton). The number of channels in the membrane patch was assumed to be the maximum number of open channel current levels observed throughout the current record (Mak et al., 2001b). When low channel open probability (\(P_o < 0.1\)) was observed, generally only current records lasting >30 s and exhibiting only one open channel current level were used in our analyses to avoid under-estimating the total number of active InsP\(_3\)R channels present in the membrane patch, which would lead to over-estimation of channel \(P_o\).

The data points shown for each set of experimental conditions are the means of results from at least four separate patch-clamp experiments performed under the same conditions. Error bars indicate the SEM.

Iterative fitting of the experimentally obtained channel \(P_o\) in various [InsP\(_3\)] and [Ca\(^{2+}\)]\(_i\) by the different molecular models were performed using Igor Pro software (WaveMetrics) with a nonlinear least-square fit (Levenberg-Marquardt) algorithm.

Solutions for Patch Clamp Experiments

All pipette solutions used in patch clamp experiments contained 140 mM KCl and 10 mM HEPES, except the low KCl solutions, which contained 14 mM KCl and 1 mM HEPES. The pipette solutions were pH adjusted to 7.3 with KOH.

By using KCl as the current carrier and appropriate quantities of the high-affinity Ca\(^{2+}\) chelator, BAPTA (1,2-bis(O-aminophenoxy) ethane-N,N',N''-tetraacetic acid; Molecular Probes) (500–1,000 \(\mu\)M), Ca\(^{2+}\) concentrations in our experimental solutions were tightly controlled (Mak et al., 2003). For solutions...
with free [Ca\(^{2+}\)] > 10 nM, free [Ca\(^{2+}\)] was directly measured using Ca\(^{2+}\)-selective minielectrodes (Baudet et al., 1994). For experimental solutions with [Ca\(^{2+}\)] < 10 nM, the total [Ca\(^{2+}\)] was determined by induction-coupled plasma mass spectrometry (Mayo Medical Laboratory) to be 6–10 nM. In the presence of 1 mM BAPTA in 140 mM KCl, 10 mM HEPES and 0.5 mM ATP at pH 7.3, the [Ca\(^{2+}\)] was calculated to be 0.9–1.5 nM using the Maxchelator software (C. Patton, Stanford University, Stanford, CA). Direct measurement by Ca\(^{2+}\)-selective electrode confirmed the free [Ca\(^{2+}\)] to be < 5 nM, but the accuracy of this measurement was limited by the nonlinearity of the calibration curve of the electrode in such low free [Ca\(^{2+}\)].

Pipette solutions contained various concentrations of Na\(^{2+}\)ATP, either 0 or 10 \(\mu\)M of InsP\(_3\) (Molecular Probes) and either 0 or 100 \(\mu\)g/ml heparin (Sigma-Aldrich) as stated.

The bath solutions used in all experiment had 140 mM KCl, 10 mM HEPES, 300 \(\mu\)M CaCl\(_2\), 500 \(\mu\)M BAPTA (measured [Ca\(^{2+}\)] = 400–500 nM), and pH 7.3.

**Online Supplemental Material**

The online supplemental material provides details, descriptions, and derivations of the allosteric models (both Monod-Wyman-Changeux [MWC] based and non-MWC-based models) that were considered to describe the ligand regulation of the InsP\(_3\)R channel gating. The mathematical derivations from first principles of the equations used to calculate the theoretical InsP\(_3\)R channel Po for each and every of those models are presented, and comparisons between the calculated channel Po and experimental data under selected conditions are discussed. Online supplemental material is available at http://www.jgp.org/cgi/content/full/jgp.200508809/DC1.

**RESULTS**

**Regulation of Types 1 and 3 InsP\(_3\)R Channel Po by Cytoplasmic Ca\(^{2+}\), InsP\(_3\) and ATP**

Single X-InsP\(_3\)R-1 and r-InsP\(_3\)R-3 channels in the same nuclear membrane system exhibit biphasic regulation by [Ca\(^{2+}\)], with open probabilities (Po) well described by the empirical biphasic Hill equation (Mak et al., 1998, 2001b):

\[
P_o = P_{\text{max}} \left\{ 1 + \left( \frac{K_{\text{inh}}}{[\text{Ca}^{2+}]_{\text{i}}} \right)^{H_{\text{inh}}} \right\}^{-1} + \left( \frac{[\text{Ca}^{2+}]_{\text{i}}}{K_{\text{act}}} \right)^{H_{\text{act}}} \left\{ 1 + \left( \frac{[\text{Ca}^{2+}]_{\text{i}}}{K_{\text{act}}} \right)^{H_{\text{act}}} \right\}^{-1}
\]

(1)

where \(P_{\text{max}}\) is the maximum channel open probability that can be achieved by the InsP\(_3\)R channel under optimal [Ca\(^{2+}\)], and saturating [InsP\(_3\)], \(K_{\text{act}}\) is the half-maximal activating [Ca\(^{2+}\)], \(H_{\text{act}}\) is the activation Hill coefficient, \(K_{\text{inh}}\) is the half-maximal inhibitory [Ca\(^{2+}\)], and \(H_{\text{inh}}\) is the inhibition Hill coefficient.

In nuclear patch clamp experiments, both InsP\(_3\)R isoforms achieve a robust \(P_{\text{max}}\) of 0.8 under optimal conditions. X-InsP\(_3\)R-1 and r-InsP\(_3\)R-3 channels both exhibit similar inhibition by Ca\(^{2+}\): \(K_{\text{inh}}\) in the presence of saturating [InsP\(_3\)] is \(~40–50\) \(\mu\)M, and \(H_{\text{inh}}\) is \(~3–4\), indicating that the Ca\(^{2+}\) inhibition process is highly cooperative. InsP\(_3\) activates both channel isoforms by increasing \(K_{\text{inh}}\), i.e., decreasing the sensitivity of the channel to Ca\(^{2+}\) inhibition, with no effect on the other Hill equation parameters \((P_{\text{max}}, H_{\text{act}}, H_{\text{inh}})\) (Mak et al., 1998, 2001b). In the presence of 0.5 mM free ATP, the InsP\(_3\)-concentration dependence of \(K_{\text{inh}}\) of each InsP\(_3\)R isoform can be described empirically by a simple Hill equation (Mak et al., 1998, 2001b):

\[
K_{\text{inh}} = K_{\text{inh}}^0 \left\{ 1 + \left( \frac{[\text{InsP}_3]}{K_{\text{inh}}^0} \right)^{H_{\text{inh}}} \right\}^{-1}
\]

(2)

with similar parameters: the half-maximal activating [InsP\(_3\)] \((K_{\text{inh}})\sim50\) nM, the Hill coefficient \((H_{\text{inh}})\sim4\), and the maximum half-maximal inhibitory [Ca\(^{2+}\)], at saturating [InsP\(_3\)] \((K_{\text{inh}}^0)\sim45\) \(\mu\)M.

Since the affinity of the inhibitory Ca\(^{2+}\)-binding site must be finite even in the absence of InsP\(_3\), we hypothesized that the InsP\(_3\) requirement for channel activities could be waived if Ca\(^{2+}\) could be dissociated from the inhibitory Ca\(^{2+}\)-binding site by an InsP\(_3\)-independent method. Although one straightforward method to accomplish this would be by lowering [Ca\(^{2+}\)], channel opening requires Ca\(^{2+}\) binding to Ca\(^{2+}\)-activation sites. We speculated that InsP\(_3\)-independent channel activities should occur in low [Ca\(^{2+}\)], conditions in which Ca\(^{2+}\) would dissociate only from the inhibitory sites but not from the activating sites. Thus, we attempted to determine if simply dissociating Ca\(^{2+}\) from the inhibitory sites would be sufficient to activate channel opening, by using experimental conditions in which the affinity of the activating Ca\(^{2+}\)-binding site was as high as possible. It was previously demonstrated that cytoplasmic free ATP acid (ATP\(^{2-}\) and ATP\(^{4-}\)) markedly enhances the Ca\(^{2+}\) affinity of the activation sites in both isoforms (Mak et al., 1998, 2001b). In saturating \(10 \mu\)M InsP\(_3\), the r-InsP\(_3\)R-3 in 0.5 mM ATP and the X-InsP\(_3\)R-1 in 9.5 mM ATP both exhibit a moderate \(P_o\) of 0.2–0.4 in the presence of very low \(25 (\mu\)M) [Ca\(^{2+}\)] (Mak et al., 1999, 2001a). Thus, the requirement for Ca\(^{2+}\) binding to the Ca\(^{2+}\) activation site is satisfied at this Ca\(^{2+}\) concentration under these conditions. We reasoned that if the minimum value of \(K_{\text{inh}}\) of the channel is not too low (for example, \(>20\) nM), then InsP\(_3\)R channel activity should be observable at 25 nM Ca\(^{2+}\) in appropriate [ATP] even in the absence of InsP\(_3\).

**Lack of Channel Activity at 25 nM Ca\(^{2+}\) for InsP\(_3\)R Not Bound to InsP\(_3\)**

A series of experiments was performed with membrane patches obtained from the same areas (\(\pm 2 \mu\)m) of isolated nuclei from un.injected oocytes, where clustering of endogenous X-InsP\(_3\)R-1 channels gave an exceptionally high probability of observing channel activity in membrane patches (Mak and Fossett, 1997). The pipette solutions alternately contained either 25 nM Ca\(^{2+}\), no InsP\(_3\), and 9.5 mM free ATP; or 1,150 nM
Ca\(^{2+}\), 10 \(\mu\)M InsP\(_3\), and 0.5 mM ATP. The latter solution is one that maximizes the \(P_o\) of the channel (Mak et al., 1998), and was therefore used to ensure that lack of channel activities in the former solution was not due to absence of InsP\(_3\)R in the patched membranes. Whereas X-InsP\(_3\)R-1 channel activities were detected in all eight patches with pipette solutions containing 10 \(\mu\)M InsP\(_3\), no channel activity was observed in any of the 26 patches with pipette solutions lacking InsP\(_3\). Thus, the type 1 channel cannot open in the absence of InsP\(_3\) in 25 nM Ca\(^{2+}\). In a parallel series of experiments using nuclei from r-InsP\(_3\)R-3–expressing oocytes, in which the expressed recombinant channels exhibit similar clustering (Mak et al., 2000), no r-InsP\(_3\)R-3 channel activity was detected in any of the eight patches with pipette solutions lacking InsP\(_3\), even though r-InsP\(_3\)R-3 channel activities were observed in all seven patches with pipette solutions that contained 10 \(\mu\)M InsP\(_3\). These results therefore suggested that the apparent \(K_{inh}\) in the absence of InsP\(_3\) (\(K_{inh}^{0}\)) for both the X-InsP\(_3\)R-1 and r-InsP\(_3\)R-3 channel isoforms is lower than 25 nM.

**InsP\(_3\)-independent Activity of X-InsP\(_3\)R-1 at Ultra-low [Ca\(^{2+}\)]**

When [Ca\(^{2+}\)] was further decreased to <5 nM (calculated to be 0.9–1.5 nM, see materials and methods), with no InsP\(_3\) and 0.5 mM ATP in the pipette solution, channel activities with low open probability of \(\sim 0.03\), and with conduction and gating properties very similar to those of the InsP\(_3\)R were observed in nuclei from un-injected oocytes (Fig. 1A). Even though these channel activities were observed in the absence of InsP\(_3\), several characteristics identified them as being contributed by the endogenous XInsP\(_3\)R-1. First, the most frequently observed (>90%) channel conductance was 330 ± 15 pS (Fig. 2A), indistinguishable from that of the InsP\(_3\)-activated XInsP\(_3\)R-1 channels observed in the same system (Mak and Foskett, 1998). Importantly, no channel activities with conductances between 100 and 450 pS have been observed previously in the absence of InsP\(_3\) in thousands of nuclear patch clamp recordings on isolated oocyte nuclei (Mak and Foskett, 1998; Mak et al.,...
The observations of InsP$_3$-independent channel activities support our working hypothesis that ligand-independent channel activity can be achieved under conditions that dissociate Ca$^{2+}$ from the inhibitory Ca$^{2+}$-binding site. A further prediction of this hypothesis is that not only is InsP$_3$ not necessary for channel activities under ultra-low [Ca$^{2+}$], conditions, but that channel activities will in fact be insensitive to InsP$_3$. To investigate the dependence on InsP$_3$ of InsP$_3$R-1 channel activity in <5 nM [Ca$^{2+}$], we used pipette solutions containing either 10 μM InsP$_3$, or no InsP$_3$. To rule out effects of possible contaminating InsP$_3$ present in our system, 100 μg/ml heparin, a competitive inhibitor of InsP$_3$ binding to the InsP$_3$R (Worley et al., 1987; Cullen et al., 1988), was used in the pipette solution with no InsP$_3$. Similar channel activities were observed (Fig. 1, B and C) with comparable $P_o$ as in the absence of InsP$_3$ (Fig. 3 A). In addition, there was no systematic or statistically significant difference in the single-channel $P_o$ ($\sim 0.03$) in the presence or absence of InsP$_3$ and heparin (Fig. 3 B). Thus, the X-InsP$_3$R-1 has a low but non-zero $P_o$ at < 5 nM [Ca$^{2+}$], regardless of whether the InsP$_3$-binding site is occupied or not. This result suggests that the inhibitory Ca$^{2+}$-binding sites of the channel was mostly unoccupied at [Ca$^{2+}$], < 5 nM regardless of the [InsP$_3$].

Of note, because $K_{act} = 190$ nM in 0.5 mM ATP (Mak et al., 1998), the activating Ca$^{2+}$-binding site of the X-InsP$_3$R-1 channel was also effectively unoccupied when [Ca$^{2+}$], < 5 nM. This result suggests that the spontaneous channel activity can occur when both the activating as well as the inhibitory Ca$^{2+}$ sites are un-ligated. Because ATP stimulates channel activities by enhancing the functional affinity of the activating Ca$^{2+}$-binding sites (Mak et al., 1999), the fact that the activating Ca$^{2+}$-binding sites remain effectively unoccupied in <5 nM Ca$^{2+}$ predicts that the InsP$_3$-independent channel activities should be unaffected by ATP. In agreement, channel activities with similar conductances were observed regardless of [ATP] (0–9.5 mM; Fig. 1, A, D, and E). Neither $P_d$ nor $P_o$ of the X-InsP$_3$R-1 channel in the absence of InsP$_3$ were significantly affected by [ATP] (Fig. 3, P > 0.05). Together, these results demonstrate that the X-InsP$_3$R-1 channel has an intrinsic, low $P_o$ even when its InsP$_3$-binding sites and activating Ca$^{2+}$-binding sites are not occupied, as long as its inhibitory Ca$^{2+}$-binding sites are unoccupied.

**InsP$_3$-independent Activity of r-InsP$_3$R-3 at Ultra-low [Ca$^{2+}$]**

Similar results were obtained for the recombinant r-InsP$_3$R-3 channels. In <5 nM [Ca$^{2+}$], channel activities with conductances very similar to those of the X-InsP$_3$R-1 were also observed in nuclei from r-InsP$_3$R-3 cRNA-injected oocytes, independent of [InsP$_3$] (0 or 10 μM), [ATP] (0 or 0.5 mM), or the presence of heparin (100 μg/ml) in the pipette solution.
**Finite Affinity of the Inhibitory Ca\(^{2+}\)-binding Sites in InsP\(_3\)R Channels**

This study has revealed that InsP\(_3\)R channels can be active spontaneously in the absence of InsP\(_3\) when [Ca\(^{2+}\)]\(_i\) is lowered to <5 nM, but not when it is lowered only to 25 nM. These observations suggest that the inhibitory Ca\(^{2+}\) sites were mostly unoccupied at <5 nM [Ca\(^{2+}\)]\(_i\), whereas they were occupied when [Ca\(^{2+}\)]\(_i\) was 25 nM. The lack of occupancy of the inhibitory Ca\(^{2+}\) sites at <5 nM [Ca\(^{2+}\)]\(_i\) obviated the requirement for InsP\(_3\) binding, enabling the channel to open in the absence of the physiological ligand. These results support a model in which InsP\(_3\) binding activates InsP\(_3\)R channel by reducing the apparent affinity of inhibitory Ca\(^{2+}\)-binding sites (Mak et al., 1998), and they have implications for our understanding of the molecular mechanisms that regulate channel activity.

To provide a better empirical description of the tuning by [InsP\(_3\)] of the channel sensitivity to Ca\(^{2+}\) inhibition that incorporates our present observations, the simple Hill equation describing the effects of InsP\(_3\) (Eq. 2) has to be modified to:

\[
K_{\text{inh}} = K_{\text{inh}}^0 + (K_{\text{inh}}^0 - K_{\text{inh}}^0)\left\{\frac{1}{1 + ([\text{InsP}_3]/K_{\text{inh}}^0)^{H_{\text{inh}}^{-1}}}ight\}
\]  (3)

where \(K_{\text{inh}}^0\) is the nonzero minimum \(K_{\text{inh}}\) in the absence of InsP\(_3\). The empirical biphasic Hill equation describing the Ca\(^{2+}\) dependence of the \(P_o\) of the InsP\(_3\)R (Eq. 1) also has to be modified to:

\[
P_o = \left\{\frac{P_{\text{max}}^o}{1 + ([\text{Ca}^{2+}\_i]/K_{\text{act}}^0)^{H_{\text{act}}^{-1}}}\right\}\left\{1 + ([\text{Ca}^{2+}\_i]/K_{\text{inh}})^{H_{\text{inh}}^{-1}}\right\}
\]  (4)

with \(P_{\text{max}}^o\) and \(P_{\text{max}}\) being the maximum \(P_o\) when the activating Ca\(^{2+}\) sites are unoccupied or fully occupied, respectively. Because the values of \(P_{\text{max}}^o\), \(H_{\text{act}}\), \(H_{\text{inh}}\), and \(K_{\text{act}}\) in the presence of various [ATP] have already been obtained in our previous studies for X-InsP\(_3\)R-1 (Mak et al., 1998, 1999) and r-InsP\(_3\)R-3 (Mak et al., 2001a,b), the channel \(P_o\) for various [Ca\(^{2+}\)]\(_i\), [InsP\(_3\)] and [ATP] can be evaluated using Eqs. 3 and 4. Therefore, even
3.8 nM. The observed channel
the dashed curves are calculated with
and
X
activity was detected at 25 nM [Ca
0.9–1.5 nM [Ca2++]) in various InsP3 and ATP concentrations
with P
as shown in Figs. 3 B and 5 B. The ob-
erved X-InsP3R-1 channel P
are consistent with those
calculated from Eqs. 3 and 4 using P
max = 0.02–0.07, and
inh = 1.2–5.5 nM (Fig. 6 A). Similarly, experimental r-InsP3R-3 channel P
agree with those calculated from Eqs. 3 and 4 using P
max = 0.005–0.018, and
inh = 1.2–3.8 nM (Fig. 6 B).
If the InsP3R channel can be active in the absence of InsP3 binding, how can high fidelity Ca2++ release responses be achieved during cellular signaling? Although InsP3R channels can exhibit InsP3-independent activities, such spontaneous activities only occur in the presence of ultra-low [Ca2++] (<25 nM), levels unlikely to be achieved under physiological conditions. Therefore, although the detection of the InsP3-independent spontaneous channel activities provides insights into the molecular bases for the complex regulation of the channel by Ca2++ and InsP3 (discussed below), such spontaneous activities by themselves probably have limited physiological implications in intracellular Ca2++ signaling. However, the regulation of the channel can now be viewed as a complex strategy designed to prevent spontaneous Ca2++ release while satisfying competing requirements of the channel. First, the channel requires Ca2++-induced Ca2++ release (CICR) properties to enable it to amplify and propagate [Ca2++] signals. Conversely, the activity of the channel must be highly controlled to enable it to provide signals with high temporal and spatial specificity and fidelity. By using Ca2++ as a high-affinity inhibitor of channel activity, the channel is provided with a mechanism to prevent spontaneous channel activity from triggering inappropriate CICR. By using InsP3 as a negative regulator of Ca2++ inhibition, the channel is provided with a mechanism to ensure graded Ca2++ release activity with high temporal specificity in response to cellular signals.

**Figure 6.** Estimating 
inh and 
max from channel activities at low [Ca2++] for (A) X-InsP3R-1 and (B) r-InsP3R-3. Different colors correspond to different InsP3 and ATP concentrations as tabulated in the graphs. InsP3R channel P
in calculated in studied [Ca2++] of
0.9–1.5 nM are plotted as data points at [Ca2++] = 1.5 nM (cf. Figs. 3 B and 5 B). InsP3R channel P
at various [Ca2++] can be calculated with Eq. 4 using the values of P
max, K
act, H
act, and H
inh obtained in our previous studies for X-InsP3R-1 (Mak et al., 1998, 1999) and r-InsP3R-3 (Mak et al., 2001a,b). The values of parameters K
inh and 
max in Eq. 4, which were not determined in previous experiments, must be constrained so that: (a) the calculated channel P
at various InsP3 and ATP concentrations agree with experimental observations (i.e., lie within the error limits of the data points at 1.5 nM [Ca2++]), and (b) the calculated channel P
in the absence of InsP3 is <0.001 at 25 nM [Ca2++], so that no channel activity was detected at 25 nM [Ca2++] and InsP3. The continuous and dashed curves represent channel P
calculated using two extreme sets of values for K
inh and 
max that satisfy those requirements. For X-InsP3R-1, the continuous curves are calculated with 
max = 0.07 and K
inh = 1.2 nM; and the dashed curves are calculated with 
max = 0.02 and K
inh = 5.5 nM. For r-InsP3R-3, the continuous curves are calculated with 
max = 0.018 and K
inh = 1.2 nM; and the dashed curves are calculated with 
max = 0.005 and K
inh = 3.8 nM. The observed channel P
data points in both graphs and the continuous curves in A are slightly offset along the [Ca2++] axis for easier visualization.

Although Eqs. 3 and 4 can describe the regulation of InsP3R channel P
by its ligands Ca2++ and InsP3, enabling the channel P
at any [Ca2++], and [InsP3] to be evaluated in terms of a set of parameters (P
max, 
max, K
act, H
act, K
inh, K
inh, K
inh, K
P3, and H
P3) that are deduced from experimental data, the equations are empirical and they do not provide insights into the specific molecular mechanisms underlying ligand regulation of InsP3R activity. Therefore, it is desirable to develop a molecular model for ligand regulation of
InsP₃R activity that can, in terms of simple molecular mechanisms, account for all the features of the regulation of InsP₃R channels (both types 1 and 3 isoforms) by [Ca²⁺], and [InsP₃] observed in extensive nuclear patch-clamp studies (Mak et al., 1998, 2001b, 2003; Boehning et al., 2001; and this study), as well as satisfy constraints imposed by the known structure of the InsP₃R molecule and channel.

The observations, for both types 1 and 3 isoforms, that must be accounted for in such a molecular model are as follows:

(i) The InsP₃R channel can be active when none of its ligand-binding sites are occupied ([InsP₃] = 0 and [Ca²⁺] = 1.5–2 nM << Kₘₐₓ and Kₐₜₜ), Spontaneous activities of the InsP₃R channel in the absence of all ligands observed in the present study are reminiscent of the spontaneous activities observed in the acetylcholine receptor channel (Jackson, 1984) and cyclic nucleotide–gated channels (Picones and Korenbrot, 1995). In those channels, ligand-independent gating suggested that allosteric models, in which the channel has a nonzero probability of being open even when its ligand-binding sites are unoccupied, were more appropriate than schemes that assume ligand binding to be necessary for channel opening. The ligand-independent opening of the InsP₃R channels observed here cannot be accounted for by previously proposed models of InsP₃R single-channel gating (De Young and Keizer, 1992; Swillens et al., 1994; Kaftan et al., 1997; Marchant and Taylor, 1997; Swillens et al., 1998; Adkins and Taylor, 1999; Moraru et al., 1999), in which only the InsP₃ₐₜₜ-bound state(s) of the receptor is assumed to be active. Instead, our new observations suggest that an allosteric model in which the InsP₃R channel has a finite probability of being open even when its activating Ca²⁺ and InsP₃ binding sites are unoccupied (Monod et al., 1965) probably offers a better molecular picture for the ligand activation of the InsP₃R. Furthermore, the model must also account for the absence of any spontaneous InsP₃ₐₜₜ-independent channel activities in [Ca²⁺] = 25 nM.

(ii) When the channel is studied in regular bath [Ca²⁺] (400–500 nM), InsP₃ has no effect on Ca²⁺ activation parameters (specifically Kₘₐₓ and Hₐₜₜ) in the empirical Hill equation (Eq. 2 or 4) of the channel (both isoforms). At a low [Ca²⁺], (for example, 100 nM), the channel Pₒ remains unchanged at either sub-saturating (33 nM) or saturating (10 µM) concentrations of InsP₃. InsP₃ activates the InsP₃R by reducing the sensitivity of the channel to high [Ca²⁺], inhibition (i.e., increasing Kₐₜₜ in Eq. 2 or 4) (Mak et al., 1998, 2001b). This lack of effect of InsP₃ on Kₘₐₓ and Hₐₜₜ cannot be accounted for by any previously proposed model for the InsP₃R channel (De Young and Keizer, 1992; Swillens et al., 1994; Kaftan et al., 1997; Marchant and Taylor, 1997; Swillens et al., 1998; Adkins and Taylor, 1999; Moraru et al., 1999), in which InsP₃ binding to the channel affects Ca²⁺ binding to the activating site, and vice versa.

(iii) When studied in the presence of regular bath [Ca²⁺] (400–500 nM), InsP₃R channel Pₒ exhibits biphasic regulation by [Ca²⁺], in the presence of both saturating (10 µM) as well as subsaturating (≤100 nM) [InsP₃] (Mak et al., 1998, 2001b).

(iv) Ca²⁺ inhibition of InsP₃R channel activity is extremely sensitive to small changes in [InsP₃] when 10 nM < [InsP₃] < 100 nM. When the [InsP₃] is raised from 10 to 100 nM, the Kₘₐₓ value for InsP₃ₐₜₜ-R1 increases by over two orders of magnitude (Mak et al., 1998). Fitting the experimentally derived Kₐₜₜ for types 1 and 3 isoforms by Eq. 3 indicates that the empirical Hill coefficient for the InsP₃ dependence of Kₐₜₜ is ~2.4.

(v) The response of InsP₃R channel activity to InsP₃ saturates very abruptly. InsP₃ₐₜₜ-R1 channel activity is already maximal when InsP₃ = 100 nM, so that the sensitivity of the channel to Ca²⁺ inhibition exhibits no discernible change when [InsP₃] is further increased by over three orders of magnitude from 100 nM to 180 µM. Despite the effect of InsP₃ on the apparent affinity of the inhibitory Ca²⁺ sites of the InsP₃R, once the InsP₃R is fully activated by InsP₃ (i.e., [InsP₃] > 100 nM), the presence of a higher [InsP₃] does not necessitate a higher [Ca²⁺], to inhibit the channel. Instead, the Pₒ of InsP₃ₐₜₜ-R1 is equally low at 60 µM [Ca²⁺], in the presence of 180 µM or 10 µM InsP₃ (Mak et al., 1998).

(vi) The maximum channel Pₒ (Pₒₐₜₜ) attained when the InsP₃R is optimally activated is ~0.8, less than 1 (Mak et al., 1998, 2001b).

(vii) The regulation of the InsP₃R channel Pₒ by Ca²⁺ and InsP₃ mainly affects the mean closed channel duration <τₜₛ>, which correlates inversely with the channel Pₒ, decreasing when the channel is activated and increasing when the channel is inhibited (Mak et al., 1998, 2001b,c). On the other hand, <τₜₛ> remains within a narrow range (5–15 ms) over all [Ca²⁺], and [InsP₃] until the channel Pₒ drops to <0.1 (Mak et al., 1998, 2001b,c).

(viii) In addition to the observed properties of the ligand regulation of single-channel InsP₃R activity, a molecular model of the regulation of the InsP₃R channel must also take into consideration the molecular structure of the channel. It is well established that a functional InsP₃R channel is a tetrameric unit (Mikoshiya et al., 1993). Although different isoforms of InsP₃R can assemble to form heterotetramers (Joseph et al., 1995), the InsP₃R channels (both types 1 and 3 isoforms) studied in our nuclear patch clamp experiments were overwhelmingly homotetrameric (Mak and Foskett, 1994; Mak et al., 2000), made up of four identical InsP₃R molecules. Thus, the molecular model for InsP₃R channel should exhibit either a fourfold sym-
Allosteric Models Considered for Describing the Ligand Regulation of the \( \text{InsP}_3R \) Channel

Because previously proposed models of \( \text{InsP}_3R \) gating, in which only the \( \text{InsP}_3 \)-bound state(s) of the receptor can be active, fail to account for the spontaneous, \( \text{InsP}_3 \)-independent channel activities of the \( \text{InsP}_3R \), we systematically examined a series of allosteric models in increasing levels of complexity to find the simplest molecular model that can account for all the characteristics of the regulation by \([\text{InsP}_3]\) and \([\text{Ca}^{2+}\)] of the \( \text{InsP}_3R \) channel tabulated in the previous section. We started with allosteric schemes based on the Monod-Wyman-Changeux (MWC) model. As outlined in (Monod et al., 1965), the four identical \( \text{InsP}_3R \) molecules in the homotetrameric channel occupy equivalent positions (condition viii) with an axis of rotational symmetry along the axis of the pore of the channel (as depicted in Mikoshiba et al., 1993), and the four monomers in the channel always adopt the same conformation, changing from one conformation to another concerted. The \( \text{InsP}_3R \) channel can change from one conformation with any number of ligands bound to its ligand-binding sites to another conformation with the same number of ligands bound. The equivalent ligand-binding sites of all the identical monomers in an \( \text{InsP}_3R \) channel have the same affinity. Furthermore, whereas the affinities of the ligand-binding sites can differ in different conformations of the channel, they are not affected by the state of occupation of any other ligand-binding site (Monod et al., 1965; Changeux and Edelstein, 1998). The following MWC-based models were examined:

(a) MWC models in which the \( \text{InsP}_3R \) tetramer can assume two conformations (one open and one closed), and each \( \text{InsP}_3R \) monomer has two or more \( \text{Ca}^{2+} \)-binding sites (at least one activating and one inhibitory);

(b) MWC-based models in which the \( \text{InsP}_3R \) tetramer has three conformations (one open and two closed conformations, or two open and one closed conformations), and each \( \text{InsP}_3R \) monomer has two \( \text{Ca}^{2+} \)-binding sites;

(c) an MWC-based model in which the \( \text{InsP}_3R \) tetramer has four conformations (two open and two closed conformations), and each \( \text{InsP}_3R \) monomer has two \( \text{Ca}^{2+} \)-binding sites;

(d) an MWC-based model in which the \( \text{InsP}_3R \) tetramer has four conformations (two open and two closed conformations), and each \( \text{InsP}_3R \) monomer has three \( \text{Ca}^{2+} \)-binding sites;

(e) a variation of model (d) in which the \( \text{InsP}_3R \) tetramer has two extra closed conformations.

Besides MWC-based models, we also examined allosteric models in which the constraints assumed in the MWC-based models were relaxed to various extents to allow more degrees of freedom to describe the gating behaviors of the \( \text{InsP}_3R \) channel. In those non-MWC models we considered, the constraint that all the \( \text{InsP}_3R \) monomers in the tetrameric channel change conformation concertedly is retained. However, the constraints that the equivalent ligand-binding sites of all the monomers in an \( \text{InsP}_3R \) channel have the same affinity, and that the affinities of the ligand-binding sites are not affected by the state of occupation of any other ligand-binding site, are selectively relaxed. We examined the following non-MWC models:

(f) a “type I” non-MWC model—an allosteric model in which the affinity of the inhibitory \( \text{Ca}^{2+} \)-binding site is affected by the binding status of the \( \text{InsP}_3 \)-binding site on the same \( \text{InsP}_3R \) monomer—with the \( \text{InsP}_3R \) tetramer having two conformations, and each \( \text{InsP}_3R \) monomer having one activating \( \text{Ca}^{2+} \)-binding site and one inhibitory \( \text{Ca}^{2+} \)-binding site;

(g) a type I non-MWC model with the \( \text{InsP}_3R \) tetramer having two conformations, and each \( \text{InsP}_3R \) monomer having one activating and two inhibitory \( \text{Ca}^{2+} \)-binding sites, with only one of the inhibitory \( \text{Ca}^{2+} \)-binding sites affected by \( \text{InsP}_3 \) binding;

(h) a “type II” non-MWC model—an allosteric model in which \( \text{InsP}_3 \)-binding to the \( \text{InsP}_3 \)-binding sites in the tetramer affects the affinities of all the inhibitory \( \text{Ca}^{2+} \)-binding sites and \( \text{InsP}_3 \)-binding sites in the tetramer—with the \( \text{InsP}_3R \) tetramer having two conformations, and each \( \text{InsP}_3R \) monomer having one activating and one inhibitory \( \text{Ca}^{2+} \)-binding sites;

(i) a type II non-MWC model with the \( \text{InsP}_3R \) tetramer having two conformations, and each \( \text{InsP}_3R \) monomer having one activating and two inhibitory \( \text{Ca}^{2+} \)-binding sites;

(j) a variation of model (i) in which the \( \text{InsP}_3R \) tetramer has three conformations.

In all the models considered, each \( \text{InsP}_3R \) monomer has only one \( \text{InsP}_3 \)-binding site because of condition (ix). Detailed descriptions of all the models considered, mathematical derivation of analytical formulas to calculate the \( \text{InsP}_3R \) channel \( P_i \) at various \([\text{InsP}_3]\) and \([\text{Ca}^{2+}]\), the rationales for selecting those models to be studied and not considering other possible allosteric models, and comparisons of experimental \( \text{InsP}_3R \) chan-
Basic Features of the Simplest Allosteric Model That Can Describe the Ligand Regulation of InsP₃R Channel Activity

Among all the models considered, the simplest model, defined as the one involving the fewest number of free parameters (Jones, 1999), that can account for all our observations of the regulation by [Ca²⁺] and [InsP₃] of InsP₃R channel activity, and can satisfy the constraints imposed by the structure of the InsP₃R channel, is the MWC-based, four-plus-two-conformation model (model e above). This model postulates that the InsP₃R monomers, and therefore the InsP₃R tetrameric channel as a whole, can adopt six different conformations (Fig. 7). The channel is open when it is in the A* and C* conformations. The B, D, A, and C' conformations are closed. The equilibria between A*, B, C*, and D conformations are dependent on InsP₃ and Ca²⁺ binding to the channel, which confers regulation of channel activity by [InsP₃] and [Ca²⁺], i.e., the affinities of the InsP₃ and Ca²⁺ sites of the InsP₃R channel are the same in A* and A'. The ratio of the total durations an InsP₃R channel spends in the A* conformation and in the A' conformation is the same regardless of [InsP₃] and [Ca²⁺]. Thus, the A* and A' conformations can be grouped together as the "active" A conformation (a conformation in which the channel can open, denoted by a green box in Fig. 7) when we consider the effects of InsP₃ and Ca²⁺ on channel conformations. Similarly, C' and C* are grouped together as the active C conformation (denoted by a green box with dashed border in Fig. 7) because the equilibrium C*↔C' is likewise not affected by [InsP₃] or [Ca²⁺]. Thus, even in the presence of optimal [InsP₃] and [Ca²⁺], when the InsP₃R channel hardly exists in the closed B and D conformations, the maximum observed channel Pₒ is <1 because the channel exists a fraction of the time in the closed A' and C' conformations. This accounts for the observation that the maximum InsP₃R channel Pₒ in saturating [InsP₃] (10 μM) and optimal [Ca²⁺], is only ~0.8 (<1) (Mak et al., 1998). The model also postulates that each of the four InsP₃R monomers has one InsP₃-binding site (Q) and three different functional Ca²⁺-binding sites (F, G, and H) on the cytoplasmic side of the channel. Because of its tetrameric structure, an InsP₃R channel can bind a maximum of four InsP₃ molecules in its Q sites and four Ca²⁺ in each of the three types (F, G, and H) of Ca²⁺ sites. The affinities of these ligand-binding sites are different in the different channel conformations (A, B, C, and D). InsP₃ and Ca²⁺ regulate channel activity because binding of InsP₃ or Ca²⁺ to these sites will stabilize those conformations in which the sites have higher affinities, thereby affecting the equilibria among the A, B, C, and D conformations, as outlined in Monod et al. (1965).

An important feature of a MWC-based model of ligand regulation is that the effect of ligand binding on the equilibrium between two channel conformations is determined by the affinities of the site in the two conformations. At ligand concentrations << the lower dissociation constant of the site, there is not enough ligand binding to the site to shift the equilibrium position. At ligand concentrations >> the higher dissociation constant, the ligand binding site is saturated and

![Figure 7](https://www.jgp.org/content/full/jgp.200308809/DC1)
ligand concentration is no longer relevant to the equilibrium position since the ligand will bind to the site regardless of what conformation the channel is in. Thus, the difference between the higher and lower dissociation constants of the site corresponds approximately to the range of ligand concentrations over which the effects of the site can be felt. Importantly, the magnitude of the difference between the two affinities of the site determines the full extent of the effect of the site, i.e., how much activation or inhibition the site produces between zero and saturating ligand concentrations. (Of course, this cannot be the case if the difference between the dissociation constants is so large that the equilibrium position is already totally shifted to the favorable conformation before the ligand concentration becomes >> higher dissociation constant."

The mechanisms for $\text{Ca}^{2+}$ and InsP$_3$ regulation are mostly segregated in this model (see the APPENDIX for more detailed reasoning behind this assertion), allowing further reduction in the number of free parameters involved. This means that in our model, InsP$_3$ binding to the Q sites only affects the equilibria A$\leftrightarrow$C, and B$\leftrightarrow$D (red double arrows in Fig. 7). In the absence of InsP$_3$, the equilibria overwhelmingly favor the A and B conformations. InsP$_3$ regulates the InsP$_3$R channel solely by stabilizing the C conformation relative to the A conformation; and stabilizing the D conformation relative to the B conformation (indicated by the pink arrows in Fig. 7). Thus, in saturating [InsP$_3$], the channel exists mostly in the C and D conformations. The equilibria A$\leftrightarrow$B and C$\leftrightarrow$D (brown double arrows in Fig. 7) are InsP$_3$-independent, i.e., the affinities of the Q sites in A and B conformations are the same, and so are those of the Q sites in C and D conformations.

The F sites are responsible for the InsP$_3$-independent $\text{Ca}^{2+}$ activation of the channel. $\text{Ca}^{2+}$ binding to the F sites only affects the InsP$_3$-independent A$\leftrightarrow$B and C$\leftrightarrow$D equilibria (brown double arrows in Fig. 7), stabilizing the active A and C conformations (indicated by the yellow arrows in Fig. 7). The affinities of the F sites are the same in A and C conformations, and so are the affinities of those in B and D conformations. Thus, $\text{Ca}^{2+}$ binding to the F sites does not affect the InsP$_3$-independent A$\leftrightarrow$C, or B$\leftrightarrow$D equilibria (red double arrows in Fig. 7).

The H sites are responsible for inhibition of the channel by high $[\text{Ca}^{2+}]_i$. The affinities of the H sites are the same in the A and C conformations and are the same in the B and D conformations. Thus, InsP$_3$-induced shifts (pink arrows in Fig. 7) in the A$\leftrightarrow$C and B$\leftrightarrow$D equilibria (red double arrows in Fig. 7) do not affect $\text{Ca}^{2+}$ binding to the H sites. $\text{Ca}^{2+}$ binding to the H sites only affects the InsP$_3$-independent equilibria A$\leftrightarrow$B and C$\leftrightarrow$D (brown double arrows in Fig. 7), stabilizing the closed B and D conformations relative to the active A and C conformations (indicated by the yellow arrows).

Regulation of the InsP$_3$R by the G sites is more complex because the G sites have different affinities (Table I) in the four conformations (A, B, C, and D). The G sites in the closed B conformation have higher $\text{Ca}^{2+}$ affinity than those in the active A conformation, so that the G sites are inhibitory $\text{Ca}^{2+}$-binding sites (as indicated by the top yellow arrow in Fig. 7) in the A$\leftrightarrow$B equilibrium, which is the dominating equilibrium in the absence of InsP$_3$. Most interestingly, however, the G sites in the active C conformation have higher $\text{Ca}^{2+}$ affinity than those in the closed D conformation, so in the C$\leftrightarrow$D equilibrium, which is the dominating equilibrium under saturating [InsP$_3$], the G sites are activating $\text{Ca}^{2+}$-binding sites (as indicated by the yellow arrow in the lower half of Fig. 7). Between zero and saturating [InsP$_3$], InsP$_3$ binding to the channel shifts it from the A and B conformations toward the C and D channel. Thus, in subsaturating [InsP$_3$], the “effective” dissociation constant of the G sites in the closed channel lies between those in the B and D conformations, according to the equilibrium position of the channel among the conformations as dictated by [InsP$_3$]. Similarly, the “effective” dissociation constant of the G sites in the active conformations lies between those in the A and C.
conformations. As [InsP₃] increases, not only do the G sites change from being inhibitory to activating, the difference between the effective affinities of the G sites in the closed and active channel also changes. As discussed earlier, the InsP₃-induced change in the magnitude of the affinity difference of the G sites alters the full extent of the effect of the G sites on the channel, i.e., how much activation (or inhibition) the G sites produce between zero and saturating [Ca²⁺]. It should be noted that since the F and H sites are both InsP₃ independent, the G site is the only one modulated by InsP₃ binding to the channel. Thus, all InsP₃ regulation of the InsP₃R stems from the effect of InsP₃ binding on the properties of the G site.

Since InsP₃ binding to the channel affects Ca²⁺ binding to G sites, microreversibility dictates that Ca²⁺ binding to G sites should affect the InsP₃-dependent equilibria A→C and B→D (as indicated by the vertical yellow arrows in Fig. 7). However, this effect is much weaker than the effect of InsP₃ binding to the Q sites and so is not noticeable in our experiments.

Considering the InsP₃-independent equilibria A↔B and C↔D, the affinities of the Ca²⁺-binding sites are in the order G > F > H. For the C↔D equilibrium, Ca²⁺ will tend to bind first to the G sites and the F sites, both stabilizing the open C conformation, and then to the H sites, stabilizing the closed D conformation. For the A↔B equilibrium, as [Ca²⁺] increases, Ca²⁺ will tend to first bind to the G sites, stabilizing the closed B conformation, and to the F sites, stabilizing the open A conformation. However, Ca²⁺ binding to the F sites cannot overcome the inhibitory effects of the G sites, so the channel remains mostly in the closed conformation. This is because the magnitude of the difference between the affinities of the G site in the closed B and active A conformations is greater than that of the F sites (Table I). Thus, the F site is less effective at activating the channel than the G site is at inhibiting it.

It should be noted that this molecular model does not take into consideration the kinetically abrupt termination of the InsP₃R channel activities that causes the channel activities observed in our patch clamp experiments to disappear over time under constant [InsP₃] and [Ca²⁺] (Mak and Foskett, 1997). Therefore, it also does not account for any possible Ca²⁺ dependence of the termination of the channel activities (Boehning et al., 2001). Furthermore, this model does not consider other ligands that bind at or near the InsP₃ binding site and activate channel gating, including the fungal metabolite adenophostin (Takahashi et al., 1994; Marchant et al., 1997; Mak et al., 2001c) and the neuronal CaBP1 protein (Yang et al., 2002). We have restricted our analyses to InsP₃ because the dataset is much more extensive. To a first approximation, however, we believe that our conclusions regarding the effects of InsP₃ can likely be generalized to these other ligands as well.

**Figure 8.** Fitting of the InsP₃R channel $P_o$ in various [Ca²⁺] and [InsP₃] by the MWC-based four-plus-two-conformation model. (A) InsP₃R-1 in regular bath (300 nM [Ca²⁺]), (B) InsP₃R-3 in regular bath (300 nM [Ca²⁺]), and (C) InsP₃R-1 in bath containing <5 nM Ca²⁺. The symbols represent the experimental $P_o$ in the tabulated [InsP₃]. The continuous curves are the theoretical $P_o$ calculated from the model. The dashed curves indicate the range of calculated $P_o$ for ±10% of the tabulated [InsP₃]. Parameters used for the $P_o$ calculations are tabulated in Table I.

**Agreement between the Selected Simplest Allosteric Model and Features of InsP₃ and Ca²⁺ Regulation of InsP₃R Channel Observed in Regular Bath Solution**

We extended the mathematical treatment for the MWC allosteric model outlined in (Monod et al., 1965) to de-
rive analytical equations to evaluate the channel $P_o$ in the presence of various $[\text{InsP}_3]$ and $[\text{Ca}^{2+}]$, according to the MWC-based four-plus-two-conformation model (see appendix for derivation of the equations). The theoretical channel $P_o$ values calculated from these equations (Fig. 8) with the optimized set of physical parameters (association constants of the various ligand-binding sites in different channel conformations, and the equilibrium constants of the transitions between different channel conformations in the absence of any ligands, as listed in Table I) fit reasonably well the experimental channel $P_o$ observed in extensive nuclear patch-clamp studies for both types 1 and 3 isoforms (Mak et al., 1998, 2001b, 2003; and this study).

It should be noted that the agreement between theoretical and experimental channel $P_o$ is remarkable considering the multitude of distinctive features of ligand regulation of $\text{InsP}_3R$ channel activities the model had to account for, and the wide range of $[\text{InsP}_3]$ and $[\text{Ca}^{2+}]$, examined for two distinct channel isoforms from two species.

Specifically, the model accounts for the following experimentally observed features.

The $\text{InsP}_3R$ Channel Can Be Active When None of its Ligand Binding Sites Is Occupied (Condition i)

The spontaneous $\text{InsP}_3R$ channel activities observed in this study are accounted for in the model as they are in the standard MWC model. In the absence of any ligand binding ($[\text{Ca}^{2+}] < 5$ nM and 0 $\text{InsP}_3$), the channel is mostly in the closed B conformation. However, there is a nonzero probability for the channel to adopt the open $A^*$ conformation, giving rise to the spontaneous channel activities observed.

Why does raising the $[\text{Ca}^{2+}]$ inhibit spontaneous opening? In the absence of $\text{InsP}_3$, the channel exists overwhelmingly in the A and B conformations. In these conformations the $\text{Ca}^{2+}$-binding G sites are inhibitory and they are more effective than the activating $\text{Ca}^{2+}$-binding F sites, as discussed above. Therefore, no channel activity is observed at $[\text{Ca}^{2+}] = 25$ nM because, at that concentration, cytoplasmic $\text{Ca}^{2+}$ will bind to the G sites and stabilize the closed B conformation strongly, thus inhibiting channel activity. $\text{Ca}^{2+}$-binding to the activating F sites also occurs, but F site occupancy is insufficient to counter the inhibitory effect of the G sites.

$\text{InsP}_3$ has No Effect on $\text{Ca}^{2+}$ Activation Parameters ($K_{\text{act}}$ and $H_{\text{act}}$) of the $\text{InsP}_3R$ Channel (Condition ii)

In our model, $\text{Ca}^{2+}$-binding to the F sites activates the $\text{InsP}_3R$ channel by stabilizing the active A and C conformations relative to the closed B and D conformations. $\text{InsP}_3$ has no effect on this $\text{Ca}^{2+}$ activation of channel activity because:

1. the $A \leftrightarrow B$ and $C \leftrightarrow D$ (active$\leftrightarrow$closed) equilibria (brown double arrows in Fig. 7) that are driven by $\text{Ca}^{2+}$ binding are $\text{InsP}_3$ independent; and
2. in the $\text{InsP}_3$-dependent $A \leftrightarrow C$ and $B \leftrightarrow D$ equilibria (red double arrows in Fig. 7), $\text{Ca}^{2+}$ affinities of the F sites are not affected by $[\text{InsP}_3]$ because the affinities of F sites are the same in the A and C conformations, and also the same in the B and D conformations (Table I).

Nevertheless, a novel insight emerges from our model: there is another, distinct contribution to $\text{Ca}^{2+}$ activation that is indeed provided by $\text{InsP}_3$. This $\text{InsP}_3$-dependent contribution to the $\text{Ca}^{2+}$ activation of the channel arises because $\text{InsP}_3$ changes the G sites from inhibitory to activating. However, empirically, this effect is not clearly distinguishable from the activation of the channel by the F sites because of the similar affinities of the activating F and G sites in the C conformation of the $\text{InsP}_3R$ channel (i.e., $K_{\text{FC}} = K_{\text{GC}}$ in Table I). This can account for why just one set of $\text{Ca}^{2+}$ activation parameters ($K_{\text{act}}$ and $H_{\text{act}}$) in the empirical Hill equation was required to fit the experimental observations, and why those parameters exhibited no $\text{InsP}_3$ dependence (Mak et al., 1998, 2001b).

Biphasic $[\text{Ca}^{2+}]$, Regulation of $\text{InsP}_3R$ Channel Activity Is Observed at all $[\text{InsP}_3]$ (Condition iii)

A distinguishing feature of our allosteric model is that a third type of $\text{Ca}^{2+}$-binding site, the H site, is postulated to exist. Besides the $\text{InsP}_3$-independent activating F sites, and the G sites that are entirely responsible for the $\text{InsP}_3$ dependence of the channel, inclusion of this novel $\text{InsP}_3$-insensitive inhibitory site in the model was necessary to account for $\text{Ca}^{2+}$ inhibition of the channel. The H sites are independent of $[\text{InsP}_3]$ because their affinities are the same in the B and D channel conformations, and in the A and C conformations (Table I).

In low $[\text{InsP}_3]$ at which the G sites are inhibitory, the activating F sites and the inhibitory G sites together produce the biphasic $\text{Ca}^{2+}$ regulation observed. The inhibitory effect of the H sites is not observable. As $[\text{InsP}_3]$ increases, the G sites become activating. Then the $\text{InsP}_3$-independent H sites are the only inhibitory $\text{Ca}^{2+}$-binding sites. The activating F and G sites, together with the inhibitory H sites, produce the biphasic $\text{Ca}^{2+}$ dependence of the channel $P_o$. Thus, the biphasic $\text{Ca}^{2+}$ regulation is observed at all $[\text{InsP}_3]$.

$\text{Ca}^{2+}$ Inhibition of $\text{InsP}_3R$ Channel Activity Is Sensitive to Small Changes in $[\text{InsP}_3]$ (Condition iv)

Several factors contribute to the exquisite sensitivity of the channel $P_o$ to small changes in $[\text{InsP}_3]$ at low $[\text{InsP}_3]$. The affinity of the Q sites for $\text{InsP}_3$ in the C and D channel conformations is extremely high (Table I) so that even at very low concentrations, $\text{InsP}_3$ starts to
bind to the channel. Furthermore, as [InsP₃] increases, the strong binding of InsP₃ to the Q sites rapidly shifts the equilibrium toward the C and D conformations. As discussed above, this shift changes the effective affinities of the G sites in the closed and active channel, thereby changing the nature of the G sites from inhibitory to activating. Consequently, the mechanism of Ca²⁺ inhibition of the channel changes from being mediated by Ca²⁺ binding to the high-affinity G sites to being mediated by Ca²⁺ binding to the low-affinity H sites. This switch results in a substantial change in the ability of Ca²⁺ to inhibit the channel. Consequently, the apparent half-maximal inhibitory [Ca²⁺]i, \( K_{inh} \), of the type 1 InsP₃R changes >300-fold when [InsP₃] increases just 10-fold (Mak et al., 1998), even though each InsP₃R monomer has only one InsP₃-binding site.

Response of InsP₃R to InsP₃ Saturates Rapidly and Abruptly by [InsP₃] = 100 nM so That Higher [InsP₃] Does Not Require Higher [Ca²⁺]i for Inhibition (Condition vi)

The abrupt saturation of the response of the InsP₃R to InsP₃ cannot be due to saturation of the InsP₃ binding site because the apparent affinity of Ca²⁺ to inhibit the type 1 InsP₃R channel \( K_{inh} \) is still highly sensitive to changes in [InsP₃] near 100 nM where the response saturates. The G sites are activating at 100 nM InsP₃, so the only mechanism available for Ca²⁺ inhibition of the channel is that mediated by Ca²⁺ binding to the H sites. The abrupt saturation of the response to InsP₃ is due to the fact that properties of the H sites are InsP₃ independent. Even as [InsP₃] is further increased over three orders of magnitude, the same [Ca²⁺]i, is required to inhibit the InsP₃R channel (Mak et al., 1998).

The Maximum Channel \( P_o \) Is Always \(< 0.8 \) (Condition vii)

Even when the experimental conditions are optimized to bias the equilibria among the A, B, C, and D channel conformations in favor of the active conformations, the observed channel \( P_o \) is still limited by the fact that the InsP₃R channel in the active A (or C) conformation spends only a fraction of its time being open (in the A* or C* conformation), resulting in channel \( P_{max} < 1 \). Furthermore, because the ligand-independent equilibria A* ↔ A’ and C* ↔ C’ have the same equilibrium constant in our model, the theoretical channel \( P_{max} \) is not affected by any of the experimental conditions that may shift the equilibria among the A, B, C, and D conformations. This feature accounts for the observation that the channel \( P_{max} \) remains the same in all experiments. It is possible that the ligand-independent conformation transitions A* ↔ A’ and C* ↔ C’ are controlled by a gating mechanism different from that controlling the ligand-dependent conformation transitions among A*, C*, B, and D.

\[ \text{The Mean Channel Open Duration } <\tau_o> \text{ Is Ligand Independent over a Wide Range of } [\text{Ca}^{2+}], \text{ and } [\text{InsP}_3], \text{ Whereas the Mean Channel Closed Duration Is Ligand Dependent (Condition vii)} \]

According to the model, an open channel in the A* or C* conformations can close either through a ligand-dependent transition into the B or D conformations, or through a ligand-independent transition into the A’ or C’ conformations (Fig. 7). The observed mean channel open duration \(<\tau_o>\) is determined by the fastest one of the transitions out of the open A* and C* conformations. Our model postulates that the rates of the ligand-independent conformation transitions, A* ↔ A’ and C* ↔ C’, are substantially higher than the rates of the ligand-dependent transitions among the A*, C*, B, and D conformations under most [InsP₃] and [Ca²⁺], examined. Thus, once the channel opens into the A* (or C*) conformation from the B (or D) conformation, it undergoes many ligand-independent A* ↔ A’ (or C* ↔ C’) transitions before it closes via a ligand-dependent transition back to the B or D conformations. This limits the open channel duration. The rates of channel closing via the ligand-dependent transitions (A* → B, A* → D, C* → B and C* → D) become comparable to the ligand-independent transitions (A* → A’ and C* → C’) only in conditions ([Ca²⁺], \(< K_{inh} \), or [Ca²⁺], \( > K_{inh} \)) when channel activity is significantly inhibited (\( P_o < 0.1 \)). Therefore, the observed \(<\tau_o>\) of InsP₃R channel remain within a narrow range even under various conditions of [Ca²⁺] and [InsP₃] in which the channel \( P_o \) changes dramatically (Mak et al., 1998, 2001b,c). \(<\tau_o>\) only decreases when channel activity is substantially inhibited (\( P_o < 0.1 \)), when one of the ligand-dependent channel-closing transitions becomes more frequent than the ligand-independent transitions.

We rejected the possibility that the channel conformations are connected such that A* ↔ A’ ↔ B because in this case, the channel can only exit the open A* and C* conformations by entering the closed A’ and C’ conformations, respectively, through ligand-independent conformation transitions. In that case, \(<\tau_o>\) would not be affected by [Ca²⁺], or [InsP₃] at all, contrary to observations.

On the other hand, a closed channel in the B and D conformations opens only through ligand-dependent transitions, whereas a closed channel in the A’ and C’ conformations opens only through ligand-independent transitions. The mean channel closed duration \(<\tau_o>\), the mean of the durations of the channel being in the B, D, A’, and C’ conformations, is dominated by the slowest of the channel opening transition rates, which is ligand dependent in our model. Hence, \(<\tau_o>\) exhibits ligand dependence with a trend opposite to that of the channel \( P_o \), i.e., \(<\tau_o>\) decreases as channel \( P_o \) increases.
Ligand Regulation of the InsP3R Channel after Exposure to Ultra-low Bath \([\text{Ca}^{2+}]\) Can be Accounted for by the MWC-based Four-Plus-Two-Conformation Allosteric Model

The MWC-based four-plus-two-conformation allosteric model postulates that the InsP3R channel has three types of regulatory \(\text{Ca}^{2+}\)-binding sites that are mutually independent. The model predicts, therefore, that it could be theoretically possible, by mutagenesis or other experimental or physiological means, to specifically modify any one of the \(\text{Ca}^{2+}\)-binding sites without affecting the other ligand binding sites. Furthermore, the model enables quantitative predictions to be made about the behavior of a channel with any specific \(\text{Ca}^{2+}\) site so modified. We therefore considered whether the novel InsP3R-I channel behaviors observed following exposure of nuclei to an ultra-low bath \([\text{Ca}^{2+}]\) (Mak et al., 2003) could be predicted from our model by assuming that the experimental treatment specifically rendered the H site nonfunctional, because this site is responsible for high \(\text{Ca}^{2+}\) inhibition. In other words, we simply assumed that the only effect of exposure to ultra-low bath \([\text{Ca}^{2+}]\) is to make the affinities of the H sites the same in the A, B, C, and D conformations. Remarkably, the observed channel behaviors are well-predicted by this assumption.

First, with the inhibitory H sites rendered nonfunctional by exposure to ultra-low bath \([\text{Ca}^{2+}]\), the model predicts that the channel will exhibit no \(\text{Ca}^{2+}\) inhibition in \([\text{InsP}_3]\) that is high enough (\(\approx 10 \text{nM}\)), such that the combined effect of \(\text{Ca}^{2+}\) binding to the F and G sites is activating. Indeed, in all \([\text{InsP}_3]\) used (10 nM, 20 nM, and 10 \(\mu\)M), the channel \(P_o\) observed in our experiments after the nuclei were exposed to ultra-low bath \([\text{Ca}^{2+}]\) increased as \([\text{Ca}^{2+}]\) was raised from 100 nM to 2 \(\mu\)M due to the combined activating effect of the F and G sites. Then the channel \(P_o\) remained at the same plateau value for all \([\text{Ca}^{2+}]\), > 2 \(\mu\)M (up to 1.5 mM) with no detectable inhibition by \([\text{Ca}^{2+}]\), (Mak et al., 2003). The model predicts this because with the F and G sites being activating and no functional H sites, there is no more \(\text{Ca}^{2+}\)-binding sites in the InsP3R channel to generate any inhibitory effect.

Second, the model predicts that rendering the H sites nonfunctional by exposure to ultra-low bath \([\text{Ca}^{2+}]\) should not affect the function of the F and G sites because the \(\text{Ca}^{2+}\)-binding sites are independent in our model. Thus, the model predicts that exposure to ultra-low bath \([\text{Ca}^{2+}]\) should have no effect on the \(\text{Ca}^{2+}\) activation properties of the channel in saturating \([\text{InsP}_3]\). Indeed, in 10 \(\mu\)M \([\text{InsP}_3]\), \(\text{Ca}^{2+}\) activation (100 nM < \([\text{Ca}^{2+}]\) < 1 \(\mu\)M) of the channel exposed to ultra-low bath \([\text{Ca}^{2+}]\) was very similar to that of channels exposed to regular bath \([\text{Ca}^{2+}]\) (400–500 nM) (Mak et al., 2003).

Third, the model predicts that even with the H sites nonfunctional, the InsP3R channel activity should nevertheless remain \(\text{InsP}_3\) dependent because the G sites remain inhibitory in the absence of \(\text{InsP}_3\). This is indeed what was observed. Even though the major apparent effect of \(\text{InsP}_3\) is to relieve high \([\text{Ca}^{2+}]\), inhibition of the channel exposed to regular bath \([\text{Ca}^{2+}]\) (Mak et al., 1998), and exposure of the channel to the ultra-low bath \([\text{Ca}^{2+}]\) eliminates high \([\text{Ca}^{2+}]\), inhibition in the presence of saturating \(\text{InsP}_3\), \(\text{InsP}_3\) nevertheless is still required to activate channel activity (Mak et al., 2003).

Fourth, the model predicts that even with the H site nonfunctional, and with the experimental conditions overwhelmingly favoring the channel being in the active C conformation, the channel will still exist for a ligand-independent fraction of time in the closed C’ conformation. Indeed, even after exposure to ultra-low bath \([\text{Ca}^{2+}]\), the channel still exhibited a \(P_{\text{max}}\) of \(\approx 0.8\), (<1) in saturating \([\text{InsP}_3]\) and high \([\text{Ca}^{2+}]\), (Mak et al., 2003).

More importantly, the model predicts that distinct and novel channel behavior should be observed in sub-saturating \([\text{InsP}_3]\) after exposure to an ultra-low bath \([\text{Ca}^{2+}]\) renders the H sites nonfunctional. Specifically, in the absence of functional H sites, the model indicates that the effect of \(\text{InsP}_3\) on the channel should be manifested as a change in the maximum channel \(P_o\), a behavior distinguished from the behavior of the channel with the H site functional, where the effect of \(\text{InsP}_3\) is manifested as a change in the apparent \(K_{\text{inh}}\), with no effect on the parameter \(P_{\text{max}}\) used in the biphasic Hill equation (Eq. 1). The observed behavior of the channel in various \([\text{InsP}_3]\) after exposure to an ultra-low bath \([\text{Ca}^{2+}]\) (Mak et al., 2003) is in very good agreement with this prediction. Understanding this novel behavior requires consideration of the effects of \(\text{InsP}_3\) on the properties of the G site. In sub-saturating \([\text{InsP}_3]\), increases in \([\text{InsP}_3]\) shift the channel toward the C and D conformations. This not only changes the nature of the G site from being inhibitory to activating, but also changes the difference between the effective affinities of the G site in the closed and active channel, thereby alters how much activation or inhibition the G site produces. At \(\approx 10 \text{nM}\), the equilibria of the X-InsP3R-I is shifted sufficiently toward the C and D conformation that the G sites become activating (Fig. 8). Thus, as \([\text{Ca}^{2+}]\) increases from 0.1 to 2 \(\mu\)M, \(\text{Ca}^{2+}\) binding to the F and G sites activates the channel and raises the channel \(P_o\) (Fig. 8 C). However, the extent of this activation is limited because at 10 nM \([\text{InsP}_3]\), the difference between the effective affinities of the G sites in the closed and active channel is small. Thus, the channel \(P_o\) is only increased to a maximum of 0.2 (Mak et al., 2003), sub-
stantially lower than $P_{\text{max}} \approx 0.8$. With no functional H sites, there is no Ca$^{2+}$ inhibition so the channel $P_o$ remains at that maximum level even as [Ca$^{2+}$] increases. Further increases in [InsP$_3$] further favor the C and D conformations, increasing the difference between the effective affinities of the G sites, thereby enhancing the extent of activation of the channel. This enhancement is manifested as an increase in the maximum $P_o$ the channel exhibits. Increases in [InsP$_3$] continue to raise the maximum channel $P_o$ until it reaches $0.8 - P_{\text{max}}$, which is dictated by the $C'\leftarrow C^*$ equilibrium.

Thus, with a single simple assumption that the exposure of the InsP$_3$R channel to ultra-low bath [Ca$^{2+}$] renders the H sites in the channel nonfunctional, the MWC-based, four-plus-two-conformation allosteric model can quantitatively account for the ligand regulation of the channel exposed to ultra-low bath [Ca$^{2+}$] observed in (Mak et al., 2003), without involving any additional free parameters. This is significant, because the model we have developed here was devised to account for the regulation by [Ca$^{2+}$], and [InsP$_3$] of the InsP$_3$R channel in regular bath [Ca$^{2+}$] (400–500 nM). The fact that it successfully quantitatively predicts independent and distinct experimental data (regulation by [Ca$^{2+}$], and [InsP$_3$]) of the channel after exposure to ultra-low bath [Ca$^{2+}$]) provides strong support for its validity.

We would like to point out that in the extension of our model described above, we use our model to separately account for the behaviors of the InsP$_3$R channel when it is exposed to regular Ca$^{2+}$ bath, and when it has been exposed to low Ca$^{2+}$ bath. Thus, we limit the description of the sensing mechanism that detects the exposure of the channel to ultra-low bath [Ca$^{2+}$] to a qualitative one, as a switch that turns on and off the inhibition of channel gating mediated by the H sites, depending on the bath [Ca$^{2+}$] the channel has been exposed to. We did not attempt to quantitatively incorporate the sensing mechanism into our model for the following reasons. First, our allosteric model is derived based on the behavior of the InsP$_3$R channel in steady-state conditions. Thus, it cannot, in its present form, provide a quantitative description for the kinetic behavior of InsP$_3$R channels in response to changes in [InsP$_3$] and [Ca$^{2+}$], including the time course of the disruption of high-[Ca$^{2+}$], inhibition of the channel after it was exposed to ultra-low bath [Ca$^{2+}$], or the reversal of the disruption when the nucleus was returned to regular bath [Ca$^{2+}$]. Second, because we do not know the physical location (cytoplasmic or luminal) of the sensing mechanism in the InsP$_3$R channel, we cannot be sure of the exact experimental conditions (luminal or cytoplasmic free [Ca$^{2+}$]) that trigger the disruption of the high [Ca$^{2+}$], inhibition of the channel. Trying to describe this Ca$^{2+}$ sensing mechanism quantitatively will entail developing two alternative models, one for each possible scenario, which is premature at this point. Third, the application of our model to the understanding of the physiological regulation of InsP$_3$R by [Ca$^{2+}$], and [InsP$_3$], the main reason for developing the model, is not significantly limited by our qualitative description of the sensing mechanism. This is because at present, disruption of the high [Ca$^{2+}$], inhibition of InsP$_3$R channels was only observed when the channels were exposed to a very low [Ca$^{2+}$] (nM), in either cytoplasmic or luminal sides. Neither of these cases is likely to occur under physiologically relevant situations. The model can be modified later to better incorporate the Ca$^{2+}$ sensing mechanism when further information about the mechanism becomes available, and if physiological conditions are found to disrupt the high [Ca$^{2+}$], inhibition of InsP$_3$R channel activity.

**Conclusions**

Examination of InsP$_3$R channel activity (both *Xenopus* type 1 and rat type 3) in extremely low [Ca$^{2+}$], revealed that InsP$_3$ is not necessary for InsP$_3$R channel opening. Spontaneous InsP$_3$R channel activity was observed because the inhibitory Ca$^{2+}$-binding sites of the channel have a finite affinity even in the absence of InsP$_3$, so that in [Ca$^{2+}$]$_i$, inhibitory Ca$^{2+}$-binding sites are not occupied and there is no Ca$^{2+}$ inhibition of the channel. The observation of spontaneous, ligand-independent activity suggested that the Ca$^{2+}$ and InsP$_3$ regulation of the InsP$_3$R channel could be described by an allosteric model for channel gating in which a channel that is not bound to Ca$^{2+}$ or InsP$_3$, nevertheless has a finite, nonzero, probability of adopting an open conformation. In contrast, all previous models have assumed that channel opening has a strict requirement for InsP$_3$ binding. Thus, our modeling effort is the first one to incorporate this spontaneous activity into an allosteric model to describe the InsP$_3$R channel. Furthermore, it is the first quantitative model that takes into consideration the tetrameric structure of the InsP$_3$R channel, and thus addresses fully and quantitatively the cooperative nature of the activation and inhibition of InsP$_3$R channel gating by [Ca$^{2+}$], and the cooperative nature of InsP$_3$R channel regulation by InsP$_3$.

We examined various allosteric models to find one that could describe channel-gating characteristics observed in extensive electrophysiological studies of the InsP$_3$R in native endoplasmic reticulum membrane. The MWC-based four-plus-two-conformation model with one InsP$_3$- and three different Ca$^{2+}$-binding sites in each InsP$_3$R monomer in a tetrameric channel can account for the nine distinct observations that we explicitly defined, including the spontaneous activities observed here, for both the types 1 and 3 InsP$_3$R, over a wide observed range of [Ca$^{2+}$] ($\sim$3 nM to 200 µM).
and [InsP₃] (0 to 180 µM). This model can account for the experimental observations with the minimum number of free parameters (14), and is therefore considered most likely. Importantly, the model derived from these data can also account for independent observations regarding the lack of Ca²⁺ inhibition (up to 1.5 mM) of channel activity and the InsP₃ regulation of the maximum channel Pₑ, exhibited by X-InsP₃-R-1 exposed to ultra-low bath [Ca²⁺⁺] (< 5 nM) described in the preceding paper (Mak et al., 2003). Of note, it quantitatively did so, and without involving more parameters, by simply assuming that the exposure to ultra-low bath [Ca²⁺⁺] specifically renders one of the Ca²⁺⁺-binding sites nonfunctional. The ability of the model to predict this complex behavior strongly validates it, and suggests that it will be useful for interpreting the molecular basis for other channel behaviors observed in future studies.

The model provides insights into the possible molecular mechanisms that enable the InsP₃₉ channel to be so precisely regulated by InsP₃ and Ca²⁺⁺. It has remained difficult to understand how the InsP₃₉ channel can be regulated so exquisitely by InsP₃. Small changes in [InsP₃] over a narrow range (10–100 nM) cause the apparent Kₐₚ to change by over 2 orders of magnitude (from 160 nM to 60 µM), even though it is well established that there are only four InsP₃₉-binding sites in each InsP₃₉ tetrameric channel. Furthermore, the mechanisms that can account for the total saturation of the channel response to [InsP₃] once [InsP₃] goes beyond 100 nM have also been unclear. Insights into these properties of InsP₃₉ regulation are highly relevant for understanding the mechanisms that generate rapid and well-controlled Ca²⁺⁺ signals in cells. These properties can now be accounted for in our model, by positing three different functional Ca²⁺⁺-binding sites in each InsP₃₉ monomer that directly affect the equilibria among active and closed conformations of the channel. One of these sites is activating, whereas another is inhibitory, but both are independent of InsP₃. In contrast, a third Ca²⁺⁺-binding site is affected by InsP₃, being inhibitory in the absence of InsP₃ but becoming activating as [InsP₃] increases. All previous models of Ca²⁺⁺ regulation of InsP₃₉ function have assumed that each channel monomer possessed a single inhibitory Ca²⁺⁺-binding site, including our previous empirical description of the effects of InsP₃ on channel gating (Mak et al., 1998, 2001b). Our previous description assumed a single inhibitory Ca²⁺⁺-binding site whose apparent affinity was allosterically reduced by InsP₃ binding. The model derived here now suggests that two Ca²⁺⁺-binding sites present in each monomer contribute to Ca²⁺⁺ inhibition. Ca²⁺⁺ binding to an InsP₃-dependent G site inhibits InsP₃₉ activity in the absence of InsP₃, which is responsible for the lack of channel activity in the absence of InsP₃ in normal [Ca²⁺⁺]. InsP₃ binding to the channel changes the effective affinities of this site, and in so doing transforms it into an activating site. This InsP₃₉-mediated transformation of the nature of this Ca²⁺⁺-binding site is responsible for all the InsP₃₉-dependence of the channel, accounting for the extremely high sensitivity of Ca²⁺⁺ inhibition of InsP₃₉ channel gating to small changes in [InsP₃]. The second Ca²⁺⁺-binding site (H site) is strictly inhibitory with a lower Ca²⁺⁺ affinity (10–30 µM) that is not modulated by InsP₃ binding. The InsP₃ independence of this site is responsible for the lack of further effect of InsP₃ on the channel once [InsP₃] > 100 nM, accounting for the observation that the effects of InsP₃ abruptly saturate around this concentration. Furthermore, Ca²⁺⁺ binding to the H site is responsible for the observed inhibition of the channel even at lower [Ca²⁺⁺] (,<10–30 µM), when [InsP₃] is <100 nM. Whereas the properties of the H sites are insensitive to InsP₃ binding, they are rendered nonfunctional by a nonphysiological protocol: exposure to an ultra-low bath [Ca²⁺⁺]. Nevertheless, the channel exposed to ultra-low bath [Ca²⁺⁺] remains dependent on InsP₃ because the other Ca²⁺⁺-binding sites, specifically the InsP₃₉-dependent G site, are not affected by the low bath [Ca²⁺⁺]. Ca²⁺⁺ binding to the G site inhibits InsP₃₉ activity in the absence of InsP₃.

Our molecular model suggests that not all conformation transitions of the InsP₃₉ that affect the channel opening are regulated by InsP₃ and [Ca²⁺⁺]. In our model, Pₚₓₙ of the InsP₃₉ channel is limited to ~0.8 (<1) by conformation transitions that affect channel opening but are independent of [Ca²⁺⁺] and [InsP₃]. This can account for the observed constancy of the mean InsP₃₉ channel open durations over a wide range of [Ca²⁺⁺] and [InsP₃]. Such conformation transitions probably arise from a channel gating mechanism different from the one regulated by ligands (InsP₃ and Ca²⁺⁺).

A critical insight that has emerged from analysis of the behavior of the model is that the major effect of InsP₃ in regulating the activity of the InsP₃₉ channel is to tune the nature of the G sites. In contrast, we previously interpreted the effect of InsP₃ as tuning the sensitivity of the channel to Ca²⁺⁺ inhibition (Mak et al., 1998, 2001b). How can we reconcile the empirical observation that Kᵢₖₐₜ is tuned by [InsP₃] with this insight from the model? Normally, the functional H site has a dissociation constant in the closed B and D conformations of 20–30 µM (Table I). However, the inhibitory effect of the H sites is not only manifested at such high [Ca²⁺⁺]. The [Ca²⁺⁺], at which H site-mediated Ca²⁺⁺ inhibition is manifested depends on the properties of the G sites. In low [InsP₃] at which the G sites have just become activating, the difference between the effective affinities of the G sites in the closed and active channel
is small so that the extent of G site-mediated activation is limited. On the other hand, there is finite Ca\(^{2+}\) binding to the H site even at [Ca\(^{2+}\)]_i < K_H^o (200–300 nM), which strongly stabilizes the closed conformations. This inhibitory effect can be sufficient to counter the activating effect of the F and G sites. Therefore, in the presence of low [InsP\(_3\)] with weak G site activation, the H site inhibition is manifested even at low [Ca\(^{2+}\)]. This results in a narrow bell-shape dependence of channel \(P_o\) on [Ca\(^{2+}\)], with the channel achieving a low maximum \(P_o\), as observed (Mak et al., 1998). As [InsP\(_3\)] increases, G site activation is enhanced, so Ca\(^{2+}\) binding to the G sites stabilizes the active conformations more strongly. The inhibitory effect of H-site binding is then only manifested at higher [Ca\(^{2+}\)]. This generates a wider bell-shape dependence of channel \(P_o\) on [Ca\(^{2+}\)], with the channel exhibiting a higher maximum \(P_o\) centered at higher [Ca\(^{2+}\)].

The maximum channel \(P_o\) reaches 0.8 (\(P_{\text{max}}\)) at [InsP\(_3\)] ~30 nM. Further increases in [InsP\(_3\)] beyond this cause no further increase in the maximum channel \(P_o\). However, higher [InsP\(_3\)] continues to shift the channel equilibria toward the C and D conformations, resulting in stronger G site activation. This delays the onset of observable H site-mediated inhibition to even higher [Ca\(^{2+}\)], broadening the biphasic dependence of channel \(P_o\) on [Ca\(^{2+}\)], into a plateau shape (Mak et al., 1998). In this manner, InsP\(_3\) tuning of the extent of G site activation is empirically manifested as an apparent InsP\(_3\)-dependent shift in the ability of Ca\(^{2+}\) to inhibit the channel.

This model developed here will be useful in guiding future experimental investigations as well as providing insights for understanding existing InsP\(_3\)R channel data. First, it will be important for providing a quantitative framework for understanding the roles of other channel regulators. For example, insights into the mechanisms of Mg\(^{2+}\) effects on BK channels were greatly facilitated by having available the previously developed complex allosteric schemes that account for Ca\(^{2+}\) and voltage regulation of the channel (see Magleby, 2001). In the case of InsP\(_3\)R, the allosteric model may provide a framework for modeling the effect of ATP, phosphorylation, and other modulators on channel gating. Second, use of the model will be important as mutagenesis is applied to this channel in attempts to discover the molecular bases for ligand regulation. Because effects of mutagenesis may be allosterically coupled to the ligand-binding sites through long-range effects, the model will be important for analyzing mutant channel behavior to discriminate mutations that are truly at the binding sites from those that are allosterically coupled to the binding sites. For example, if a mutation is observed to change the properties of InsP\(_3\) activation of channel gating, the target of the mutation could be the InsP\(_3\)-binding Q site itself, but our model suggests that the mutation could possibly modify the InsP\(_3\)-dependent, Ca\(^{2+}\)-binding G sites instead. With our model, changes in channel behavior resulting from any kind of modulation of the properties of the ligand-binding sites can now be interpreted within the context of the model to make inferences regarding the molecular mechanisms involved, as we have done in our analysis of the effects of ultra-low bath [Ca\(^{2+}\)] exposure. For instance, experimental or physiological modulation of the affinity of the G sites in just the C conformation of the channel can affect all the parameters (\(P_{\text{max}}, K_{\text{act}}, K_{\text{inh}}, H_{\text{act}}, H_{\text{inh}}\)) in the empirical Hill equation (Eq. 1). Without a molecular model, it would be extremely difficult to understand the underlying mechanisms just from the effects of the modulation. Indeed, a study of the effects of a point mutation on the gating of the type 1 channel (Tu et al., 2003) was limited to phenomenological description because of a lack of a model by which to quantitatively account for the results. Third, although our model is a general one based on observations made under steady-state conditions, with all the ligand-binding reactions assumed to be possible in any sequential order, it can nevertheless incorporate sequential binding models in which certain ligand-binding sequences are “forbidden”, like that proposed in (Marchant and Taylor, 1997; Adkins and Taylor, 1999) in which Ca\(^{2+}\) cannot bind to the activating sites before InsP\(_3\) binds to the InsP\(_3\)-binding sites. Our model can incorporate sequential schemes because it only explicitly involves the equilibrium constants of ligand binding and conformation transitions. Thus, “forbidden” ligand binding sequences can be incorporated simply by assuming that certain ligand binding and conformation transitions have much slower reaction rates than other reactions in the ligand binding scheme. The model may also be useful in predicting which transitions are “forbidden”. Fourth, when transient kinetic responses of channels are measured in response to ligand concentration changes, our model with its specified equilibrium constants may help to constrain the set of possible schemes and values of reaction rate constants that need to be considered. Fifth, application of the model to datasets obtained from various InsP\(_3\)R isoforms may prove useful in identifying the properties that distinguish them and account for any observed distinct behaviors. For example, the type 2 InsP\(_3\)R channel was reported to be distinct from the type 1 channel in its relative lack of high Ca\(^{2+}\) inhibition (Ramos-Franco et al., 2000). Our model suggests that this difference could be accounted for by a less effective inhibitory H site in the type 2 channel.

Finally, besides its application to enhance our understanding of the regulation of InsP\(_3\)R channel gating, the modeling effort described here has extended sub-
stantially the basic MWC model upon which it is based. Our systematic mathematical treatment of not only the MWC-based four-plus-two-conformation model, but also of the other MWC-based and non-MWC models (presented in the online supplemental material section) may be useful in future modeling of other allosteric processes involving multiple ligands.

**APPENDIX**

**MWC-based Four-Plus-Two-Conformation Model—Model e**

In this allosteric model, the homotetrameric InsP₃R channel can assume six conformations: four (A, B, C, and D) that are connected by ligand dependent transitions, plus two (A' and C') that are connected to the others by ligand independent transitions (hence the name). The channel is open in two conformations (A* and C*), and closed in the others (B, D, A', and C'). However, since the transitions A*→A' and C*→C' are ligand independent, we consider the conformations A* and A' as one active conformation A; and C* and C' as one active conformation C when we examine the regulation of InsP₃R channel activity by ligands InsP₃ and Ca²⁺ (Fig. 7).

Each InsP₃R monomer has three Ca²⁺-binding sites (F, G, and H) and one InsP₃-binding site (Q). The ligand-binding status of an InsP₃R tetrameric channel can be represented using the convention in which A represents the channel in the A conformation with Ca²⁺ bound to the F sites, gCa²⁺ bound to the G sites, hCa²⁺ bound to the H sites, and qInsP₃ molecules bound to the Q sites (0 ≤ f, g, h, q ≤ 4).

Based on the simplifications assumed in the MWC model (Monod et al., 1965), all the F sites in an InsP₃R channel in conformation A have the same dissociation constant for Ca²⁺ binding (represented as KFA in this discussion), regardless of the ligand-binding status of the channel. Other dissociation constants are represented similarly, like KGA and KSC. After the derivation in Monod et al. (1965), these dissociation constants (a total of 16 for 4 sites in 4 conformations) together with the three independent equilibrium constants (I₀A, I₀C, and I₀H) for conformation transitions between unliganded channels (A₀F₀ ↔ A₀H₀, A₀C₀ ↔ A₀H₀, and G₀H₀ ↔ G₀D₀ respectively) constitute the full set of parameters that completely describes the regulation by InsP₃ and Ca²⁺ of the conformation changes of the InsP₃R channel. Using the symbol conventions described above, the concentrations of the InsP₃R channel in any specific ligand-binding state, [1A], can be expressed in terms of [InsP₃], [Ca²⁺], [I₀A], and the set of parameters:

\[
\begin{align*}
[1A] & = \frac{4!}{f!(4-f)!} \left[ \frac{[\text{Ca}^{2+}]}{K_{FA}} \right]^f \frac{4!}{g!(4-g)!} \left[ \frac{[\text{Ca}^{2+}]}{K_{GA}} \right]^g \\
& \times \frac{4!}{h!(4-h)!} \left[ \frac{[\text{insP}_3]}{K_{HA}} \right]^h \frac{4!}{q!(4-q)!} \left[ \frac{[\text{insP}_3]}{K_{QA}} \right]^q [1A]_0
\end{align*}
\]

Similar equations can be derived to express ligand-binding states of other conformations.

For our model of ligand regulation of InsP₃R gating, we postulate that [Ca²⁺], mainly regulates the equilibrium A→B and C→D. Furthermore, we postulate that F is an activating Ca²⁺-binding site, whereas H is an inhibitory Ca²⁺-binding site. This means that KFA < KFB and KFD, so that Ca²⁺ binding to the activating F sites stabilizes the active conformations. In contrast, KFA and KFC > KFB and KFD, so that Ca²⁺ binding to the inhibitory H sites stabilizes the closed conformations.

On the other hand, [InsP₃] regulates the A↔C and B↔D equilibria, modulating channel behavior by changing the C and D channel conformations relative to the A and B conformations (KQA and KQB >> KQC and KQD).

The observed independence of the Ca²⁺ activation of the channel on [Inf₃P] (condition ii) constrains the number of free parameters involved in this model. Because K⁴A = K⁴B and K⁴C = K⁴D, the InsP₃ affinity of the Q sites must be the same for the A and B conformations (KQA = KQB), and for the C and D conformations (KQC = KQD) so that InsP₃ binding to the channel does not affect the equilibria A↔B and C↔D. This prevents all the channels from shifting the equilibria due to InsP₃ binding, since InsP₃ binding to the inhibitory H sites stabilizes the closed conformations.

On the other hand, [InsP₃] regulates the A↔C and B↔D equilibria, modulating channel behavior by changing the C and D channel conformations relative to the A and B conformations (KQA = KQB), and for the C and D conformations (KQC = KQD) so that InsP₃ binding to the channel does not affect the equilibria A↔B and C↔D. This prevents all the channels from shifting the equilibria due to InsP₃ binding, since InsP₃ binding to the inhibitory H sites stabilizes the closed conformations.

Even though Ca²⁺ inhibition of InsP₃R is very sensitive to change in [InsP₃] from 10 to 100 nM, Ca²⁺ inhibition does not change any more once [InsP₃] reaches 100 nM despite a further three orders of magnitude increase in [InsP₃] (from 100 nM to 180 μM). Thus, higher [InsP₃] beyond 100 nM does not require higher [Ca²⁺] for inhibition (condition v). This indicates that Ca²⁺ binding to the H sites is not affected by [InsP₃]. Therefore, much like the relation between the dissociation constants of the F sites (KFA = KFC = KFB and KFD = KFD), the dissociation constants of the H sites in the A and C channel conformations are the same (KHA = KHC = KHF), and those in the B and D conformations are the same (KHB = KHD = KHF).

The concentrations of the InsP₃R channel in one conformation regardless of its ligand-binding status is shown in Eq. 6.
The open probability $P_o$ of a single InsP$_3$R channel is the fraction of time the channel spends in the open $A^*$ and $C^*$ conformations. In a stationary system, this is the same as the relative abundance of channels that adopt the open conformations in an ensemble of many (ideally infinite) channels, i.e.,

$$P_o = ([A^*] + [C^*])/([A] + [B] + [C] + [D]). \hspace{1cm} (7)$$

Whereas the equilibria between the B, D, A*, and C* conformations are ligand dependent, the equilibrium constants $R_A$ and $R_C$ for the transitions $A^* \leftrightarrow A'$ and $C^* \leftrightarrow C'$, respectively, are independent of [InsP$_3$] and [Ca$^{2+}$]. Because the experimental parameter $P_{max}$ is not affected by [InsP$_3$] (Mak et al., 1998, 2001b), $R_A = R_C = R$. Thus, we have

$$[A^*] = [A][R/(1 + R)] \hspace{1cm} \text{and} \hspace{1cm} [C^*] = [C][R/(1 + R)]. \hspace{1cm} (8)$$

This means that the channel is open for only a fraction $[R/(1 + R)]$ of the time it is in the A or C conformation. This allows the model an extra degree of freedom to fit the experimentally observed channel $P_{max}$ found to be <1 (condition ix). Together, Eqs. 6–8 describe the channel $P_o$ under all [Ca$^{2+}$], and [InsP$_3$] with 14 free parameters (Table I).

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In this section, we describe several allosteric molecular models in ascending order of complexity in our systematic search for the simplest one that can account for the many characteristics of the regulation by [InsP₃] and [Ca²⁺], of the activity of the homotetrameric InsP₃R channel. For each molecular model, we derived, from first principles, analytical formulas to calculate the channel \( P_o \) at various [InsP₃] and [Ca²⁺], in terms of physical parameters defined in that model. The number of free parameters involved was minimized using specific observed characteristics of ligand regulation of the InsP₃R channel. An optimal set of parameters was obtained using a nonlinear least-square fitting procedure to match the theoretical channel \( P_o \) calculated using the parameters to the experimental channel \( P_o \) at various [InsP₃] and [Ca²⁺]. A fit with a separate set of parameters was performed for each one of the following: (a) channel \( P_o \) of \( \lambda \text{InsP}_3\text{R}-1 \) exposed to regular (400–500 nM) bath [Ca²⁺] (Mak et al., 1998), including the spontaneous channel \( P_o \) observed in this study in the absence of InsP₃; (b) channel \( P_o \) of \( \lambda \text{InsP}_3\text{R}-3 \) exposed to regular bath [Ca²⁺] (Mak et al., 2001), also including the spontaneous channel \( P_o \) observed in this study; and (c) channel \( P_o \) of \( \lambda \text{InsP}_3\text{R}-1 \) exposed to ultra-low bath [Ca²⁺] (<5 nM) (Mak et al., 2003). A model was rejected if it failed to generate a reasonable fit for any one of these three series of observed channel \( P_o \). The best model was one that gave a good fit to all three series of experimental channel \( P_o \) with the fewest free parameters involved.

*Conventions for Symbols Used in Discussion of Molecular Models*

For clarity and easy reference, the conventions for symbols used in our discussion of various molecular models are described here. For simplicity, [Ca²⁺], is represented as [Ca²⁺] in all equations in this supplemental section.

*Conformations of the InsP₃R Channel*

The InsP₃R channel can adopt different conformations denoted as A and B (and C and D, depending on the model). \([A] \) is the total concentration of the InsP₃R channels in the A conformation regardless of their ligand binding status. In models involving two Ca²⁺-binding sites per monomer, the symbol \( A^f_0 \) represents the channel in A channel conformation with \( j \text{Ca}^{2+} \) bound to the F sites, \( g \text{Ca}^{2+} \) bound to the G sites, and \( q \text{InsP}_3 \) molecules bound to the Q sites, so,

\[
[A] = \sum_{f, g, h, q = 0}^{4} [A^f_0].
\]

In models involving three Ca²⁺-binding sites per monomer, the symbol \( A^f_0 \) represents the channel in A conformation with \( j \text{Ca}^{2+} \) bound to the activating F sites, \( g \text{Ca}^{2+} \) bound to the G sites, \( h \text{Ca}^{2+} \) bound to the H sites, and \( q \text{InsP}_3 \) molecules bound to the Q sites, so

\[
[A] = \sum_{f, g, h, q = 0}^{4} [A^f_0].
\]

\( 0 \leq f, g, h, q \leq 4 \) because of the tetrameric structure of the InsP₃R channel. The same convention is used for the other channel conformations.

*Conformation Transition Equilibrium Constants*

In steady-state conditions, the concentration of the channel in any conformation in any state of ligand binding can be expressed in terms of the dissociation constants of the ligand-binding sites and the conformation transition equilibrium constants of the unliganded channel (Monod et al., 1965). In a model postulating \( n \) conformations,
there are \((n - 1)\) independent conformation transition equilibrium constants for the unliganded channel. \(L_{AA} (= [A_B^0]/[A_A^0])\) or \([A_B^0]/[A_A^0]\) represents the equilibrium constant for transitions between unliganded channels in the A and B conformations. Similarly, \(L_{AB} \) and \(L_{DA} \) represent the equilibrium constants for transitions between unliganded channel in the C and A conformations, and in the D and B conformations, respectively.

**Dissociation Constants of Ligand-binding Sites in MWC-based Models**

According to the MWC model (Monod et al., 1965), the affinities of the ligand binding sites are independent of the state of occupation of any other ligand-binding sites. Thus, the symbol \(K_{fA}^A\) represents the dissociation constant for Ca\(^{2+}\) binding to the F sites of a channel in the A conformation. The same convention is used for the dissociation constants of all ligand-binding sites of channels in different conformations.

**Dissociation Constants of Ligand-binding Sites in Type I Non-MWC Models**

In the type I non-MWC models, the condition that the ligand-binding site affinities are invariant is relaxed, so that the affinities of the ligand-binding sites of an InsP\(_3\)R monomer is affected by the state of occupation of other ligand-binding sites of the same InsP\(_3\)R monomer through a coupling factor. Since the Ca\(^{2+}\) activation of the InsP\(_3\)R channel is not affected by [InsP\(_3\)], there is no coupling between the activating Ca\(^{2+}\)-binding F site and the InsP\(_3\)-binding Q site. However, Ca\(^{2+}\)-inhibition of InsP\(_3\)R is affected by [InsP\(_3\)]. The symbol \(S_{fQ}^{QA}\) represents the coupling factor between the inhibitory Ca\(^{2+}\)-binding G site and the InsP\(_3\)-binding Q site in the same InsP\(_3\)R monomer in a tetramer in the A conformation. If \(K_{fA}^A\) is the dissociation constant for Ca\(^{2+}\) binding to the G site of an InsP\(_3\)R monomer whose Q site is unoccupied, in an InsP\(_3\)R channel tetramer in the A conformation, then \(K_{fA}^{QA}\) \(S_{fQ}^{QA}\) is the dissociation constant for Ca\(^{2+}\) binding to the G site of an InsP\(_3\)R monomer whose Q site is occupied. This convention is also used for coupling between ligand-binding sites in channel conformation B.

**Dissociation Constants of Ligand-binding Sites in Type II Non-MWC Models**

In the type II non-MWC models, the condition of the invariance of the ligand-binding site affinities is relaxed so that the affinities of the ligand-binding sites in all InsP\(_3\)R monomers in the same channel tetramer is always the same in the type II non-MWC models. In the type II non-MWC model involving two Ca\(^{2+}\)-binding sites per monomer, the symbol \(K_{fA}^{QA}\) represents specifically the dissociation constant for the reaction \(A_{f(A-1)}^A + \text{Ca}^{2+} \leftrightarrow \text{Q}^f A^x \) (1 ≤ \(f\) ≤ 4, 0 ≤ \(g\) ≤ 4, 0 ≤ \(q\) ≤ 4), \(K_{fA}^{QA}\) is the dissociation constant for the reaction \(\text{A}^f_{A(f-1)} + \text{Ca}^{2+} \leftrightarrow \text{Q}^f A^x \) (0 ≤ \(f\) ≤ 4, 1 ≤ \(g\) ≤ 4, 0 ≤ \(q\) ≤ 4), and \(K_{fA}^{QA}\) is the dissociation constant for the reaction \(\text{A}^f_{A(f-1)} + \text{Ca}^{2+} \leftrightarrow \text{Q}^f A^x \) (0 ≤ \(f\) ≤ 4, 0 ≤ \(g\) ≤ 4, 1 ≤ \(q\) ≤ 4). Following a similar convention, in the model involving three Ca\(^{2+}\)-binding sites per monomer, the symbol \(K_{fA}^{QA}\) represents the dissociation constant for the reaction \(\text{A}^f_{A(f-1)} + \text{Ca}^{2+} \leftrightarrow \text{Q}^f A^x \) (1 ≤ \(f\) ≤ 4, 0 ≤ \(g\) ≤ 4, 0 ≤ \(h\) ≤ 4, 0 ≤ \(q\) ≤ 4), and so on for other ligand-binding sites. This convention is also used for channel conformation B also.

**Two-conformation MWC Models—Models (a)**

(As listed in discussion, “Allosteric models considered for describing the ligand regulation of the InsP\(_3\)R channel activity” subsection in the main text.)

First, we examine the simplest allosteric model for the homotetrameric InsP\(_3\)R channel: a MWC model in which the channel can adopt two conformations, the open A conformation and the closed B conformation, with two Ca\(^{2+}\)-binding sites, the activating F site and the inhibitory G site, and one InsP\(_3\)-binding Q site for each InsP\(_3\)R monomer in the tetrameric channel.

The open probability \(P_o\) of a single InsP\(_3\)R channel is the fraction of time the channel spends in the open A conformation. In a stationary system, this is the same as the relative abundance of channels that adopt the open conformation(s) in an ensemble of many (ideally infinite) channels, i.e.,

\[
P_o = \frac{[A]}{[A] + [B]}.
\]

Extending the derivation in (Monod et al., 1965) and using the symbol conventions described above, \([A_f^A]\) can be expressed in terms of [InsP\(_3\)] and [Ca\(^{2+}\)]:

\[
[A_f^A] = \left[\frac{4!}{f!(4-f)!}\right] \left\{ \left[\frac{\text{Ca}^{2+}}{K_f^A}\right]^f \right\} \left(\frac{4!}{g!(4-g)!}\right) \left\{ \left[\frac{\text{Ca}^{2+}}{K_g^A}\right]^g \right\} \left(\frac{4!}{q!(4-q)!}\right) \left\{ \left[\frac{\text{InsP}_{3}}{K_q^{QA}}\right]^q \right\}
\]

(S2)
Then, using the binomial theorem, the channel concentrations \([A]\) and \([B]\) are

\[
[A] = \left[ \alpha A_0 \right] (1 + [Ca^{2+}] / K^{FA})^4 (1 + [Ca^{2+}] / K^{GA})^4 (1 + [InsP_3] / K^{QA})^4 \\
[B] = L_B[A] \left[ \alpha A_0 \right] (1 + [Ca^{2+}] / K^{FB})^4 (1 + [Ca^{2+}] / K^{GB})^4 (1 + [InsP_3] / K^{QB})^4.
\]

(S3)

Ca^{2+} binding to the F sites is activating because this stabilizes the open A channel conformation preferentially. Therefore, \(K^{FA} < K^{GB}\). By the same token, Ca^{2+} binding to the inhibitory G sites stabilizes the closed B conformation so \(K^{GB} > K^{CA}\).

Eqs. S1 and S3 reveal that, in this model, InsP_3 stabilizes the open A channel conformation because the InsP_3-binding sites in the A conformation have a higher affinity \((K^{QA} < K^{GB})\). Thus, InsP_3 and Ca^{2+} (when binding to the F sites) activate the channel in the same manner as heterotropic ligands (Monod et al., 1965) of the InsP_3R. An important prediction of this is that InsP_3 binding to the Q sites will enhance Ca^{2+} activation of the channel, because InsP_3-binding stabilizes the A conformation, whose activating F sites have higher Ca^{2+} affinity. However, this prediction is not supported by experimental studies that have shown InsP_3 has no effect on the Ca^{2+} activation properties of the channel (Mak et al., 1998, 2001). This problem cannot be resolved by increasing the number of Ca^{2+}-binding sites (activating or inhibitory) postulated in the model. Therefore, two-conformation MWC models cannot account for the Ca^{2+} and InsP_3 regulation of the InsP_3R channel.

### Three-conformation MWC-based Models—Models (b)

Since the MWC model involving only two conformations obviously cannot account for the observed ligand regulation of InsP_3R channel, we examined an allosteric model at the next level of complexity: MWC-based models in which the InsP_3R channel can adopt three conformations (A, B, and C) with two Ca^{2+}-binding sites (F and G) and one InsP_3-binding site (Q) per InsP_3R monomer.

First, we consider the model in which only the A conformation is open, so that following our symbol conventions,

\[
P_a = [A] / ([A] + [B] + [C]).
\]

(S4)

\([A]\) and \([B]\) are given by Eq. S3, and \([C]\) is given by

\[
[C] = L_A[A] \left[ \alpha A_0 \right] (1 + [Ca^{2+}] / K^{QA})^4 (1 + [Ca^{2+}] / K^{QA})^4 (1 + [InsP_3] / K^{QB})^4.
\]

(S5)

The channel \(P_a\) calculated from the model using Eqs. S3, S4, and S5 can fit reasonably well the experimental \(P_a\) data for both types 1 and 3 InsP_3R exposed to regular bath [Ca^{2+}] (Fig. S1, A and B), with a set of 11 independent parameters—2 conformation transition equilibrium constants and 9 dissociation constants for the ligand-binding sites for each isoform (Table S1). The various experimentally observed characteristics of the InsP_3 dependence of channel activity—the spontaneous activity of the channel in extremely low [Ca^{2+}], even in the absence of InsP_3, the high sensitivity of the channel to low [InsP_3], the rapid saturation of the InsP_3 response of the channel, and the absence of InsP_3 dependence of Ca^{2+} activation—are all well described by the model.

The fitting parameters for channels in regular bath [Ca^{2+}] reveal that the high affinity of the inhibitory G sites in the closed C channel conformation is mainly responsible for strong Ca^{2+} inhibition in the absence of InsP_3. The high affinities of the Q sites in the A and B conformations and the small value of \(L_A\) indicate that even low [InsP_3] can push the channel from the C conformation into the A and B conformations, providing the channel with a high sensitivity to low [InsP_3]. Between the A and B channel conformations, Ca^{2+} binding to F sites activates the channel by stabilizing the open A conformation \((K^{QA} < K^{GB})\), and Ca^{2+} binding to G sites inhibits the channel by stabilizing the closed B conformation \((K^{GB} > K^{CA})\).
The $P_o$ data for the X-InsP$_3$R-1 exposed to ultra-low bath [Ca$^{2+}$] that exhibited no high [Ca$^{2+}$] inhibition (Mak et al., 2003) can also be described by the three-conformation MWC-based model using Eqs. S3, S4, and S5 (Fig. S1 C). However, the parameters needed to fit the $P_o$ of channels exposed to ultra-low bath [Ca$^{2+}$] are very different from those used to fit the $P_o$ of channels in regular bath [Ca$^{2+}$]. This means that the model actually takes 22 free parameters to fit all of the experimental channel $P_o$ data for X-InsP$_3$R-1. Furthermore, it implies that exposure to different bath [Ca$^{2+}$] causes a dramatic change in the channel structure that affects the affinities of most of the ligand-binding sites in the three different channel conformations, despite the fact that abrogation of high [Ca$^{2+}$] inhibition is completely reversible (Mak et al., 2003). Most importantly, the model suggests that the similarity in the Ca$^{2+}$/H$^{11001}$ activation properties of the channel exposed to different bath [Ca$^{2+}$] (Mak et al., 2003) is merely coincidental, with the exposure to different bath [Ca$^{2+}$] causing the different parameters to change in such a way that their effects on Ca$^{2+}$/H$^{11001}$ activation of the channel just happen to balance out one another. For these reasons, we rejected this model.

Besides the three-conformation MWC-based model with only one open conformation, we also examined the three-conformation model in which two of the conformations are open. It failed to generate the appropriate InsP$_3$ dependence of the channel $P_o$ (not shown).

Four-conformation MWC-based Model with Two Ca$^{2+}$-binding Sites per InsP$_3$R Monomer—Model (c)

Although the three-conformation MWC-based model with one open conformation can fit the multiple sets of experimental channel $P_o$ reasonably well, it does not give a satisfactory explanation for the loss of Ca$^{2+}$ inhibition in channels exposed to ultra-low [Ca$^{2+}$]. Therefore, we explored other molecular models to find one that is able to not only fit the experimental channel $P_o$ but also provide insights into the molecular mechanisms underlying this regulation of Ca$^{2+}$ inhibition. Because the three-conformation MWC-based model with one open conformation can fit the observed channel $P_o$ dependencies on [Ca$^{2+}$], and [InsP$_3$], it is not necessary to examine a four-conformation MWC-based model with one open conformation because it will definitely fit the experimental $P_o$ with many redundant parameters. Therefore, we examined the four-conformation MWC-based model with two open conformations.

In this model, the InsP$_3$R channel can adopt four conformations A, B, C, and D, and two of them, A and C, are open. First, we postulate that each InsP$_3$R monomer has two Ca$^{2+}$-binding sites and one InsP$_3$-binding site, as before. The channel $P_o$ is given by

$$P_o = \frac{([A]+[C])}{([A]+[B]+[C]+[D])}. \quad (S6)$$

Following our convention for symbols, the dissociation constants for F sites of the open conformations, $K^A$ and $K^C$, must be smaller than those of the closed conformations, $K^B$ and $K^D$, so that Ca$^{2+}$ binding to the activating F sites stabilizes the open conformations. In contrast, the dissociation constants for G sites of the open conformations, $K^{A\alpha}$ and
$K_{GC}$, must be larger than those of the closed conformations, $K_{GB}$ and $K_{GD}$, so that Ca$^{2+}$ binding to the inhibitory G sites stabilizes the closed conformations.

InsP$_3$ regulates channel $P_o$ by stabilizing the C and D channel conformations relative to the A and B conformations ($K_{CA}^C$ and $K_{CB}^C$ > $K_{DC}$ and $K_{DD}$). In the absence of InsP$_3$, the channel exists mostly in the A and B conformations ($L_{CA}$ and $L_{CB}$ << 1). Ca$^{2+}$ inhibition occurs at very low [Ca$^{2+}$]$_i$, because the G sites in the closed B conformation have a high affinity for Ca$^{2+}$, i.e., $K_{GB}$ is very small. In saturating [InsP$_3$], the channel is mostly in the C and D conformations. Under such conditions, the inhibition of the channel by high [Ca$^{2+}$]$_i$, is caused by the stabilization of the closed D conformation relative to the C conformation due to Ca$^{2+}$ binding to the G sites. The observed half maximal inhibitory [Ca$^{2+}$]$_i$ in saturating [InsP$_3$] (Mak et al., 1998) therefore reflects the value of $K_{GD}$ ~50 μM, which is << $K_{GC}$. The reduction of Ca$^{2+}$ inhibition as [InsP$_3$] increases is a manifestation of the InsP$_3$-induced shift in the equilibrium B$\leftrightarrow$D from the closed B conformation to the closed D conformation, which has G sites with a substantially lower Ca$^{2+}$ affinity ($K_{GD}$ >> $K_{GB}$).

The observed independence of the Ca$^{2+}$ activation of the channel on [InsP$_3$] constrains the number of free parameters involved in this model. Because $K_{CA}$ + $K_{CB}$ and $K_{CA}$ + $K_{CD}$, shifting the equilibria A$\leftrightarrow$B, or C$\leftrightarrow$D will affect Ca$^{2+}$ activation of the channel. Therefore, the InsP$_3$ affinity of the Q sites must be the same for the A and B conformations ($K_{QA}^A$ = $K_{QB}^B$), and for the C and D conformations ($K_{QA}^C$ = $K_{QB}^D$) so that InsP$_3$ binding to the channel does not affect the equilibria A$\leftrightarrow$B and C$\leftrightarrow$D. To emphasize this constraint on the dissociation constants, we define $K_{QA}^Q$ = $K_{QB}^Q$, and $K_{QA}^Q$ = $K_{QB}^Q$. Furthermore, because InsP$_3$ binding to the Q sites does affect the equilibria A$\leftrightarrow$C and B$\leftrightarrow$D (that is how InsP$_3$ regulates the channel), the affinity of F sites must be the same in the A and C conformations ($K_{FA}^A$ = $K_{FC}^A$), and the same in the B and D conformations ($K_{FB}^B$ = $K_{FD}^B$), so that shifts in the equilibria A$\leftrightarrow$C and B$\leftrightarrow$D due to InsP$_3$ binding will not affect Ca$^{2+}$ activation. Again, to emphasize the constraint on these dissociation constants, we define $K_{QA}^Q$ = $K_{QB}^Q$ and $K_{QA}^Q$ = $K_{QB}^Q$.

$[A] = [\alpha_\theta^A] (1 + [Ca^{2+}] / K_{FA}^A)^4 (1 + [Ca^{2+}] / K_{GA}^A)^4 (1 + [InsP_3] / K_{QA}^A)^4$

$[B] = L_{BA}[\alpha_\theta^A] (1 + [Ca^{2+}] / K_{FA}^B)^4 (1 + [Ca^{2+}] / K_{GB}^B)^4 (1 + [InsP_3] / K_{QB}^B)^4$

$[C] = L_{CA}[\alpha_\theta^A] (1 + [Ca^{2+}] / K_{FA}^C)^4 (1 + [Ca^{2+}] / K_{GC}^C)^4 (1 + [InsP_3] / K_{QA}^C)^4$

$[D] = L_{BA}L_{DB}[\alpha_\theta^A] (1 + [Ca^{2+}] / K_{FA}^D)^4 (1 + [Ca^{2+}] / K_{GD}^D)^4 (1 + [InsP_3] / K_{QB}^D)^4$

and the channel $P_o$ can be evaluated using Eqs. S6 and S7. This model involves 11 independent parameters. However, it cannot adequately describe the high sensitivity of the InsP$_3$R $P_o$ to low [InsP$_3$] or the rapid saturation of the InsP$_3$ response of the channel (Fig. S2 A). With only two Ca$^{2+}$-binding sites and the F site being activating, the G site is the only inhibitory Ca$^{2+}$-binding site in this model. Thus, InsP$_3$ regulates channel activity solely by inducing a change in the effective affinity of the G sites through shifting of the equilibria from A and B channel conformations to C and D conformations. Failure of the model to fit experimental $P_o$ reveals that the InsP$_3$-induced change in G site affinity cannot be both very large, when [InsP$_3$] varies over subsaturating range (10–100 nM) to generate the high sensitivity of the InsP$_3$R to [InsP$_3$], and saturate abruptly, once [InsP$_3$] exceeds 100 nM.

**Four-conformation MWC-based Model with Three Ca$^{2+}$-binding Sites per InsP$_3$R Monomer—Model (d)**

Because InsP$_3$-induced changes in G site Ca$^{2+}$ affinity alone cannot account for both the sensitivity to low [InsP$_3$] and the rapidity of the saturation of the InsP$_3$ response of the channel, we considered the possibility that these features might be captured in a different model, in which each channel monomer has three Ca$^{2+}$-binding sites (F, G, and H) instead of two. Whereas only one InsP$_3$-binding region has been identified in the InsP$_3$R sequence (Yoshikawa et al., 1996), multiple (>2) Ca$^{2+}$-binding regions that can possibly regulate InsP$_3$R channel activities have been identified experimentally (Sienaert et al., 1996, 1997). Thus, each InsP$_3$R monomer could conceivably have more than two functional Ca$^{2+}$-binding sites.

With the F sites still being activating, the sensitivity of the channel to low [InsP$_3$] can be accomplished in this model by a large InsP$_3$-induced change in Ca$^{2+}$ affinity of the G site while the H sites can be responsible for the saturation of the InsP$_3$ effect on Ca$^{2+}$ inhibition of channel activity. Ca$^{2+}$ binding to the H sites is therefore inhibitory ($K_{HA}^H$ and $K_{HC}^H$ > $K_{HB}^H$ and $K_{HD}$). InsP$_3$ regulation of Ca$^{2+}$ inhibition is caused by its modulation of the G sites. In contrast, Ca$^{2+}$-binding to the H sites is not affected by [InsP$_3$]. Thus, much like the relation between the dissociation constants of the F sites ($K_{FA}^F$ = $K_{FB}^F$ = $K_{FD}^F$ = $K_{F2}$), the affinity of the H sites in the A and C channel conformations is the same ($K_{HA}^H$ = $K_{HC}^H$ = $K_{H1}$), and the affinity in the B and D conformations is the same ($K_{HB}^H$ = $K_{HD}^H$ = $K_{H2}$).
With three Ca\(^{2+}\)-binding sites, we further extend the derivation in (Monod et al., 1965) to calculate the channel concentrations:

\[
[A] = [A_0^0](1 + [Ca^{2+}]/K^{A_0} \text{ }^A)^4(1 + [Ca^{2+}]/K^{A_0} \text{ }^B)^4(1 + [Ca^{2+}]/K^{A_0} \text{ }^C)^4(1 + [InsP_3]/K^{Q_1})^4
\]

\[
[B] = L_{BA}A_0^0(1 + [Ca^{2+}]/K^{A_0} \text{ }^B)^4(1 + [Ca^{2+}]/K^{A_0} \text{ }^C)^4(1 + [Ca^{2+}]/K^{A_0} \text{ }^D)^4(1 + [InsP_3]/K^{Q_1})^4
\]

\[
[C] = L_{CA}A_0^0(1 + [Ca^{2+}]/K^{A_0} \text{ }^C)^4(1 + [Ca^{2+}]/K^{A_0} \text{ }^D)^4(1 + [Ca^{2+}]/K^{A_0} \text{ }^E)^4(1 + [InsP_3]/K^{Q_2})^4
\]

\[
[D] = L_{DB}L_{DB}A_0^0(1 + [Ca^{2+}]/K^{F_1})^4(1 + [Ca^{2+}]/K^{F_2})^4(1 + [Ca^{2+}]/K^{F_3})^4(1 + [InsP_3]/K^{Q_3})^4
\]

(S8)

The theoretical channel \( P_o \) calculated from Eqs. S6 and S8 fits the experimental data from X\text{InsP}_3R-1 exposed to regular bath reasonably well (Fig. S2 B) with 13 independent parameters. Like the three-conformation MWC-based model, the observed characteristics of the InsP_3 dependence of the InsP_3R-1 channel are also well described by this model.

The major strength of this model is that it can account for the observed abolition of Ca\(^{2+}\) inhibition of the X\text{InsP}_3R-1 channel exposed to ultra-low bath [Ca\(^{2+}\)] (Fig. S2 C) by changing just one of its 13 independent parameters (\( K^{H_1} \) or \( K^{H_2} \)). Since Ca\(^{2+}\) inhibition of InsP,R exposed to regular bath [Ca\(^{2+}\)] in saturating [InsP_3] is caused solely by Ca\(^{2+}\) binding to H sites, Ca\(^{2+}\) inhibition of channel activity will be abrogated in channels exposed to ultra-low bath [Ca\(^{2+}\)] by the loss of functionality of the H sites, with the affinity of the H sites becoming the same in all conformations. This model still allows the channel activity to be dependent on InsP_3, because in the absence of InsP_3, the channel is mostly in the A and B conformations and the G sites are inhibitory. Ca\(^{2+}\) binding to these G sites stabilizes the closed B conformations and prevents InsP,R channel opening.

However, channel \( P_o \) calculated from the model deviates significantly from the experimental values in the “plateau” region of the biphasic Ca\(^{2+}\) response curve (1 μM< [Ca\(^{2+}\)] < 10 μM) in saturating [InsP_3] (10 μM) for channels in regular bath [Ca\(^{2+}\)] (Fig. S2 B). More seriously, whereas the experimentally observed maximum \( P_o \) in high [Ca\(^{2+}\)] (>30 μM) and saturating [InsP_3] (10 μM) for channels exposed to ultra-low bath [Ca\(^{2+}\)] is ~0.8, the calculated \( P_{o_{\text{max}}} \) in the model is unity (Fig. S2 C) when [Ca\(^{2+}\)] \( >> \) \( K^{G_1} \) and \( K^{G_2} \).

**MWC-based Four-Plus-Two-Conformation Model—Model (e)**

See appendix in main text.

**Type I Non-MWC Allosteric Model with Two Ca\(^{2+}\)-binding Sites per InsP,R Monomer—Model (f)**

To construct non-MWC allosteric models, we relax some of the constraints imposed in the MWC model. Namely, we allow the
equivalent ligand-binding sites in the same InsP₃R tetramer to have different ligand affinities. Furthermore, the affinities of ligand-binding sites can vary according to the state of occupation of other ligand-binding sites of the InsP₃R channel. However, for simplicity, we retain the constraint that when the tetrameric channel is open, all the monomers must adopt an open conformation, and similarly, all monomers in a closed channel must adopt a closed conformation. We start with the simplest non-MWC models in which each of the InsP₃R monomer has two Ca²⁺-binding sites (F and G). In a more intuitive model that deviates from the MWC model—the type I non-MWC model—we assume that the affinities of the ligand-binding sites of an InsP₃R monomer is affected by the state of occupation of other ligand-binding sites of the same InsP₃R monomer through a coupling factor (Fig. S3). Since [InsP₃] does not affect Ca²⁺ activation of the InsP₃R channel, there is no coupling between the activating Ca²⁺-binding F site and the InsP₃-binding Q site. Therefore, the only coupling in this simple model is between the InsP₃-binding Q site and the inhibitory Ca²⁺-binding G site in the same InsP₃R monomer.

First we consider the binding of ligands to the coupled Q and G sites in an InsP₃R monomer in the open conformation, \( \rho_q \), where \( q, f, \) and \( g (= 0 \text{ or } 1) \) represent the state of occupancy of the InsP₃, activating and inhibitory Ca²⁺-binding sites of the monomer, respectively. The dissociation constants \( K^{QA} \) and \( K^{GB} \), and the coupling factor \( S^{QA} = S^A \) are defined by

\[
K^{QA} = [\rho_q^0] [Ca^{2+}] / [\rho_q^1]
\]

\[
K^{QB} = [\rho_q^0] [InsP_3] / [\rho_q^1]
\]

\[
K^{GA} S^A = [\rho_q^0] [Ca^{2+}] / [\rho_q^1]
\]  

(S9)

Then

\[
[a] = \sum_{f=0}^{1} \sum_{q=0}^{1} \sum_{g=0}^{1} \rho_q^f = [\rho_q^0] \left( 1 + \frac{[Ca^{2+}]}{K^{QA}} + \frac{[InsP_3]}{K^{QB}} + \frac{[Ca^{2+}] [InsP_3]}{S^A K^{QA} K^{QB}} \right) \left( 1 + \frac{[Ca^{2+}]}{K^{FA}} \right).
\]  

(S10)

In this model, there is no coupling between the ligand-binding sites in different InsP₃R monomers in the same tetrameric InsP₃R channel. Similar equations can be derived for ligand binding to channel monomer in the closed conformation. Because InsP₃ binding to the InsP₃R channel does not affect the Ca²⁺ activation of the channel, \( K^{QA} = K^{QB} = K^0 \). Thus, the concentrations of the channel in the open ([A]) conformation and closed ([B]) conformation are:

\[
[A] = [\rho_q^0] \left( 1 + \frac{[Ca^{2+}]}{K^{QA}} + \frac{[InsP_3]}{K^{QB}} + \frac{[Ca^{2+}] [InsP_3]}{S^A K^{QA} K^{QB}} \right) \left( 1 + \frac{[Ca^{2+}]}{K^{FA}} \right) \left( 1 + \frac{[Ca^{2+}]}{K^{FB}} \right).
\]  

(S10)

The channel \( P_o \) is evaluated from Eqs. S1 and S11, with eight parameters: \( L_{BA}, K^2, K^{FA}, K^{GB}, K^{GA}, K^{GB}, S^A, \) and \( S^B \).

The channel \( P_o \) calculated from this model cannot fit the experimental \( P_o \) data obtained in regular bath \([Ca^{2+}]\) (400–500 nM). Specifically, this model fails to describe the high sensitivity of the InsP3R to low \([InsP_3]\) (10–100 nM) and the rapid saturation of the InsP3R response of the channel (Fig. S4 A). This is similar to the failure of the four-conformation MWC model with two \( Ca^{2+} \)-binding sites per InsP3R monomer.

**Type I Non-MWC Allosteric Model with Three \( Ca^{2+} \)-binding Sites per InsP3R Monomer—Model (g)**

The four-conformation MWC model with two \( Ca^{2+} \)-binding sites per InsP3R monomer fails to describe the experimental InsP3R channel \( P_o \) data, whereas the four-conformation MWC model with three \( Ca^{2+} \)-binding sites per InsP3R monomer is able to simultaneously describe the high sensitivity of the channel to \([InsP_3]\) between 10 and 100 nM and the saturation of the response of the channel to \([InsP_3]\) in \([InsP_3]\) > 100 nM. Therefore, we investigate if a type I non-MWC model with three \( Ca^{2+} \)-binding sites per monomer may be able to describe the channel \( P_o \), adequately.

By analogy to the four-conformation MWC model with three \( Ca^{2+} \)-binding sites, we assume that the third \( Ca^{2+} \)-binding site, \( H \), is inhibitory and \( InsP_3 \)-independent, with dissociation constants \( K_{11A} \) and \( K_{11B} \) in open and closed conformations, respectively. Then, the channel \( P_o \) is calculated from Eq. S1 and

\[
[A] = [A_0^0] + \frac{[Ca^{2+}]}{K_{1A}} + \frac{[InsP_3]}{K_{2}} + \frac{[Ca^{2+}][InsP_3]}{S^3 K_{1A} K_{2}} \left( 1 + \frac{[Ca^{2+}]}{K_{FA}^2} + \frac{[Ca^{2+}]}{K_{FB}^2} \right) \left( 1 + \frac{[Ca^{2+}]}{K_{1A}^2} + \frac{[Ca^{2+}]}{K_{1B}^2} \right) \tag{S12}
\]

Although the channel \( P_o \) calculated according to this type I non-MWC model with three \( Ca^{2+} \)-binding sites per InsP3R monomer agree with the experimental \( P_o \) a little better than that calculated from the two \( Ca^{2+} \)-binding sites model, it still fails to describe the experimental InsP3R channel \( P_o \) adequately (Fig. S4 B).

**Type II Non-MWC Allosteric Model with Two \( Ca^{2+} \)-binding Sites per InsP3R Monomer—Model (h)**

Next, we consider a different way to relax the constraint in the MWC-based model that the affinities of the ligand sites are not affected by the ligand binding status of the channel. Again we first consider a model in which the InsP3R channel has two \( Ca^{2+} \)-binding (F and G) sites per monomer. In this type II non-MWC model, \( InsP_3 \) binding to the Q sites regulates the affinities of ligand-binding sites in all the InsP3R monomers in the tetrameric channel (Fig. S3). Whereas the affinity of the activating F sites is \( InsP_3 \)-independent, as dictated by the observation that \( Ca^{2+} \) activation of the channel is not affected by \([InsP_3]\), \( InsP_3 \) binding to the Q sites in this model affects both the affinity of the inhibitory G sites, and thereby \( Ca^{2+} \) inhibition of the channel, as well as the affinity of the unoccupied \( InsP_3 \)-binding Q sites, so that the channel activity can be extremely sensitive to regulation by low \([InsP_3]\). For simplicity, we retain the constraint that the affinity of the same type of ligand binding sites in the whole tetrameric channel is the same for one conformation.

The tetrameric structure of the InsP3R channel dictates that there are 125 ligand-binding states for the open (A) conformation (\( \chi^A_i \) with \( 0 \leq i \leq 4 \), \( 0 \leq g \leq 4 \), \( 0 \leq q \leq 4 \)). Of the 300 possible dissociation constants (\( K_{11A}^{FA} \), \( K_{11A}^{GB} \), and \( K_{11A}^{QA} \), see conventions for symbols described above), only 124

**Figure S4.** Fitting of the InsP3R-1 channel \( P_o \) in regular bath \([Ca^{2+}]\) by the type I non-MWC model (A) with two \( Ca^{2+} \)-binding sites, and (B) with three \( Ca^{2+} \)-binding sites. The symbols represent the experimental \( P_o \) and the continuous curves are the calculated \( P_o \). The dashed curves indicate the range of calculated \( P_o \) for \( \pm 10 \% \) of the tabulated \([InsP_3]\).
of them are truly independent. We select $K_{qg}^{FA}$ for $1 \leq f \leq 4$, $0 \leq g \leq 4$, $0 \leq q \leq 4$ (total of 100), $K_{qg}^{GA} = K_{qg}^{GA}$ for $1 \leq g \leq 4$, $0 \leq q \leq 4$ (total of 20), and $K_{qg}^{QA} = K_{qg}^{QA}$ for $1 \leq q \leq 4$ (total of 4) to be the independent dissociation constants. The other dissociation constants can be expressed in terms of these 124 independent ones. There are the same number of independent dissociation constants for the closed (B) conformation.

According to this model, the Ca$^{2+}$ affinity of the F sites is independent of Ca$^{2+}$ or InsP$_3$. Thus, $K_{qg}^{FA} = K_{qg}^{FA}$ and $K_{qg}^{FB} = K_{qg}^{FB}$ for all $1 \leq f \leq 4$, $0 \leq g \leq 4$, $0 \leq q \leq 4$. The Ca$^{2+}$ affinity of the G sites is also independent of Ca$^{2+}$, but regulated by InsP$_3$. Thus, $K_{qg}^{GA}$ for the same $q$ are the same, i.e., $K_{qg}^{GA} = K_{qg}^{GA}$ for all $0 \leq q \leq 4$, $1 \leq g \leq 4$. Similarly, $K_{qg}^{FB} = K_{qg}^{FB}$. Finally, InsP$_3$ affinity of the Q sites is independent of Ca$^{2+}$, and therefore is only affected by InsP$_3$ binding to the channel. Furthermore, affinity of Q sites in the A and B channel conformations must be the same so that InsP$_3$ binding to the channel does not affect the Ca$^{2+}$ activation of the channel. Thus, $K_{qg}^{QA} = K_{qg}^{QB} = K_{qg}^{QA}$ for all $1 \leq q \leq 4$.

This model therefore involves 17 free parameters: $L_{BA}$, $K_{A}^{FA}$, $K_{B}^{FB}$, $K_{A}^{QA}$ ($0 \leq q \leq 4$), $K_{q}^{QB} (0 \leq q \leq 4)$ and $K_{q}^{QA}$ ($1 \leq q \leq 4$, $K_{q}^{QA} = 1$). Using a derivation similar to that for Eq. S2, $[A]_f$ can be expressed as:

$$[A]_f = [\omega^0_0]_f \left( \frac{4!}{f(4-f)!} \left( \frac{[\text{Ca}^{2+}]}{K_{FA}^{FA}} \right)^f \left( \frac{4!}{q!(4-q)!} \left( \frac{[\text{Ca}^{2+}]}{K_{QA}^{QA}} \right)^q \sum_{z=0}^{q} \frac{[\text{InsP}_3]^q}{q!(4-q)!} \prod_{z=0}^{q} K_{zg}^{QA} \right) \right)$$

$$[B]_f = L_{BA} [\omega^0_0]_f \left( \frac{4!}{f(4-f)!} \left( \frac{[\text{Ca}^{2+}]}{K_{FB}^{FB}} \right)^f \left( \frac{4!}{q!(4-q)!} \left( \frac{[\text{Ca}^{2+}]}{K_{QB}^{QB}} \right)^q \sum_{z=0}^{q} \frac{[\text{InsP}_3]^q}{q!(4-q)!} \prod_{z=0}^{q} K_{zg}^{QB} \right) \right)$$

Despite the large number of parameters (17) involved, this non-MWC model, like the four-conformation MWC-based model with two Ca$^{2+}$-binding sites, also fails to adequately describe the high sensitivity of the channel to low [InsP$_3$] and the rapid saturation of the InsP$_3$ response of the channel exposed to regular bath [Ca$^{2+}$] (Fig. S5 A).

**Type II Non-MWC Allosteric Models with Three Ca$^{2+}$-binding Sites per InsP$_3$R Monomer—Models (i) and (j)**

Since the type II non-MWC model with two Ca$^{2+}$-binding sites fails to fit the experimental channel $P_o$ data in a similar manner as the four-conformation MWC-based model with two Ca$^{2+}$-binding sites, we reasoned that a type II non-MWC model with three Ca$^{2+}$-binding sites for each InsP$_3$R monomer, model (i), may be able to fit the channel $P_o$ data better, just like the four-conformation MWC-based model with three Ca$^{2+}$-binding sites.

By analogy to the type II non-MWC model with two Ca$^{2+}$-binding sites, the dissociation constants of the ligand-binding sites in this model are: $K_{qg}^{FA} = K_{qg}^{FA}$, $K_{qg}^{FB} = K_{qg}^{FB}$, $K_{qg}^{QA} = K_{qg}^{QA}$, $K_{qg}^{QB} = K_{qg}^{QB}$, and $K_{qg}^{QA} = K_{qg}^{QB} = K_{qg}^{QA}$. Furthermore, as in the case of the four-conformation MWC-based model, with InsP$_3$ binding regulating the affinity of the G sites, it is not necessary for the Ca$^{2+}$ affinity of the H sites to be dependent on [InsP$_3$] in this model. Therefore, $K_{qg}^{HB} = K_{qg}^{HB}$ and $K_{qg}^{HA} = K_{qg}^{HA}$. Following the same derivation as in the type II non-MWC model with two Ca$^{2+}$-binding sites, the channel $P_o$ is calculated from Eq. S1 and

$$[A] = [\omega^0_0] \left( \frac{4!}{f(4-f)!} \left( \frac{[\text{Ca}^{2+}]}{K_{FA}^{FA}} \right)^f \left( \frac{4!}{q!(4-q)!} \left( \frac{[\text{Ca}^{2+}]}{K_{QA}^{QA}} \right)^q \sum_{z=0}^{q} \frac{[\text{InsP}_3]^q}{q!(4-q)!} \prod_{z=0}^{q} K_{zg}^{QA} \right) \right)$$

$$[B] = L_{BA} [\omega^0_0] \left( \frac{4!}{f(4-f)!} \left( \frac{[\text{Ca}^{2+}]}{K_{FB}^{FB}} \right)^f \left( \frac{4!}{q!(4-q)!} \left( \frac{[\text{Ca}^{2+}]}{K_{QB}^{QB}} \right)^q \sum_{z=0}^{q} \frac{[\text{InsP}_3]^q}{q!(4-q)!} \prod_{z=0}^{q} K_{zg}^{QB} \right) \right)$$

With 19 free parameters, $L_{BA}$, $K_{A}^{FA}$, $K_{B}^{FB}$, $K_{A}^{QA}$, $K_{B}^{QB}$, $K_{q}^{QA} (0 \leq q \leq 4)$, $K_{q}^{QB} (0 \leq q \leq 4)$, and $K_{q}^{HA}$ (1 \leq q \leq 4), the channel $P_o$ calculated from this model can fit the experimental $P_o$ data reasonably well (Fig. S5 B). Furthermore, the $P_o$ of the InsP$_3$R-1 channel exposed to ultra-low bath [Ca$^{2+}$] can also be fitted by Eqs. S1 and S15 using the same set of parameters, but with $K_{q}^{HA} = K_{q}^{HB}$ (Fig. S5 C). However, the theoretical channel $P_{max}$ for channels in saturating

9 http://www.jgp.org/cgi/doi/10.1085/jgp.200308809
[InsP₃] (10 μM) deviates significantly from the observed values, especially for channels exposed to ultra-low bath [Ca²⁺] (Fig. S5 C).

This discrepancy can be addressed with a modification similar to that used for the four-plus-two-conformation MWC-based model, i.e., besides the conformation transitions regulated by [Ca²⁺] and [InsP₃], there is another conformation transition independent of [Ca²⁺] and [InsP₃] that affects channel opening, A’↔A*. Channel \( P_{\text{max}} \) for this modified two-plus-one-conformation non-MWC model, model (j), is then limited by the equilibrium constant (\( R \)) for A’↔A*, and given by

\[
P_o = R(1 + R)^{-1} \frac{[A]}{([A] + [B])},
\]

where [A] and [B] are evaluated from Eq. S15. This model can describe reasonably well the observed channel \( P_o \) of both \( X\)-InsP₃R-1 and \( r\)-InsP₃R-3 in regular bath [Ca²⁺] (Fig. S6, A and B) with 20 independent parameters for each isoform (Table S2), as well as the channel \( P_o \) of \( X\)-InsP₃R-1 exposed to low bath [Ca²⁺] with the same set of parameters except \( K_{H1} \) (Fig. S6 C).

**What about other Non-MWC Models?**

Other non-MWC models can also be generated by further relaxing the constraints imposed in the MWC-based model; for example, allowing some monomers in the same tetrameric channel to be in open conformation and others in closed conformation (Changeux and Edelstein, 1998). However, such models will involve at least as many, if not many more parameters.

---

**Table S2**

Parameters used to calculate the \( P_o \) for InsP₃R in Fig. S6A and B according to the modified type II non-MWC model with three Ca²⁺-binding sites per monomer

<table>
<thead>
<tr>
<th>Parameters</th>
<th>( \text{InsP₃R-1}^a )</th>
<th>( \text{InsP₃R-3} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( L_{BA} )</td>
<td>30</td>
<td>15</td>
</tr>
<tr>
<td>( K^A )</td>
<td>150 nM</td>
<td>30 nM</td>
</tr>
<tr>
<td>( K^B )</td>
<td>650 nM</td>
<td>70 nM</td>
</tr>
<tr>
<td>( K_{GA} )</td>
<td>200 nM</td>
<td>200 nM</td>
</tr>
<tr>
<td>( K_{GB} )</td>
<td>200 nM</td>
<td>200 nM</td>
</tr>
<tr>
<td>( K_{1A} )</td>
<td>310 nM</td>
<td>310 nM</td>
</tr>
<tr>
<td>( K_{1B} )</td>
<td>25 nM</td>
<td>31 nM</td>
</tr>
<tr>
<td>( K_{2A} )</td>
<td>800 nM</td>
<td>800 nM</td>
</tr>
<tr>
<td>( K_{2B} )</td>
<td>&gt;10 mM</td>
<td>&gt;10 mM</td>
</tr>
<tr>
<td>( R^\text{Ga} )</td>
<td>20 μM</td>
<td>20 μM</td>
</tr>
<tr>
<td>( R^\text{Gb} )</td>
<td>85 nM</td>
<td>85 nM</td>
</tr>
<tr>
<td>( R^\text{Qa} )</td>
<td>6 nM</td>
<td>6 nM</td>
</tr>
<tr>
<td>( R^\text{Qb} )</td>
<td>1.8 nM</td>
<td>1.8 nM</td>
</tr>
<tr>
<td>( R )</td>
<td>5.74</td>
<td>5.74</td>
</tr>
</tbody>
</table>

Despite the good agreement between the calculated \( P_o \) derived from these sets of parameters and the experimental data, these sets of parameters may not be unique. In fact, many of these parameters, like \( K^A, K^B, K^\text{Ga}, K^\text{Gb}, K_{1A}, \) and \( K_{1B} \), have little impact on the calculated \( P_o \).

*Channel \( P_o \) of \( X\)-InsP₃R-1 exposed to regular and ultra-low bath [Ca²⁺] were fitted using the same set of parameters except that \( K_{H1} = K_{H2} \) for channel exposed to ultra-low bath [Ca²⁺].

**a**These parameters can only be determined to be greater than the tabulated values because the calculated \( P_o \) are not very sensitive to these parameters.
parameters as the modified type II non-MWC model, which can describe the observed experimental channel \( P_o \) well. Therefore, we will not consider these models without extra experimental evidence indicating that further relaxing of the constraints of the MWC-based model is warranted.

**Conclusions**

Examination of various molecular models reveals some that can account for the regulation of channel \( P_o \) of both types 1 and 3 InsP\(_3\)R isoforms in various conditions (various \([\text{Ca}^{2+}]_i\), \([\text{InsP}_3]\), regular and ultra-low bath \([\text{Ca}^{2+}]\)). Among them, the four-plus-two-conformation MWC-based model with three \(\text{Ca}^{2+}\)-binding sites per InsP\(_3\)R monomer requires only 14 free parameters to fit the observed channel \( P_o \) for the \(X\)-InsP\(_3\)R-1 exposed to both regular and ultra-low bath \([\text{Ca}^{2+}]\).

In comparison, the three-conformation MWC-based model with two \(\text{Ca}^{2+}\)-binding sites per monomer requires 22 free parameters—2 sets of 11 parameters, one each for channels exposed to regular and ultra-low bath \([\text{Ca}^{2+}]\) (Table S1); and the modified non-MWC model with three \(\text{Ca}^{2+}\) binding sites per monomer requires 20 parameters (Table S2). Thus, the four plus two conformation MWC-based model is the simplest molecular model (involves the fewest free parameters) that can adequately account for our observed channel regulation by \([\text{Ca}^{2+}]_i\) and \([\text{InsP}_3]\).

**References**


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