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<td>Author(s)</td>
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Mode Switching Is the Major Mechanism of Ligand Regulation of InsP₃ Receptor Calcium Release Channels

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The inositol 1,4,5-trisphosphate (InsP₃) receptor (InsP₃,R) plays a critical role in generation of complex Ca²⁺ signals in many cell types. In patch clamp recordings of isolated nuclei from insect Sf9 cells, InsP₃,R channels were consistently detected with regulation by cytoplasmic InsP₃ and free Ca²⁺ concentrations ([Ca²⁺]ᵢ) very similar to that observed for vertebrate InsP₃,R. Long channel activity durations of the Sf9-InsP₃,R have now enabled identification of a novel aspect of InsP₃,R gating: modal gating. Using a novel algorithm to analyze channel modal gating kinetics, InsP₃,R gating can be separated into three distinct modes: a low activity mode, a fast kinetic mode, and a burst mode with channel open probability (Pₒ) within each mode of 0.007 ± 0.002, 0.24 ± 0.03, and 0.85 ± 0.02, respectively. Channels reside in each mode for long periods (tens of opening and closing events), and transitions between modes can be discerned with high resolution (within two channel opening and closing events). Remarkably, regulation of channel gating by [Ca²⁺]ᵢ and [InsP₃] does not substantially alter channel Pₒ within a mode. Instead, [Ca²⁺]ᵢ and [InsP₃] affect overall channel Pₒ, primarily by changing the relative probability of the channel being in each mode, especially the high and low Pₒ modes. This novel observation therefore reveals modal switching as the major mechanism of physiological regulation of InsP₃,R channel activity, with implications for the kinetics of Ca²⁺ release events in cells.

INTRODUCTION

Inositol 1,4,5-trisphosphate (InsP₃) is a second messenger generated together with diacylglycerol by phospholipase C activated by G-protein-coupled and tyrosine kinase receptors in response to extracellular stimuli (Berridge and Irvine, 1989; Berridge, 1993). The free InsP₃ binds to its receptor (InsP₃,R), a ubiquitous, ER-localized Ca²⁺ channel, activating it to release Ca²⁺ from the ER lumen to increase cytoplasmic free Ca²⁺ concentration ([Ca²⁺]ᵢ). InsP₃,R-mediated [Ca²⁺]ᵢ changes play critical roles in multiple signaling pathways and are involved in generation and regulation of multiple biological processes, including synaptic transmission, gene expression, and apoptosis. Analyses of InsP₃,R-mediated [Ca²⁺]ᵢ signals in nonexcitable cells have revealed complex spatial and temporal features, providing highly regulated global as well as localized control of Ca²⁺-dependent processes (Woods et al., 1986; Petersen et al., 1991; Tregear et al., 1991; Thorn et al., 1993; Bootman et al., 2002).

Characterization of InsP₃,R channel activity and its regulation is essential for molecular insights into these intricate intracellular Ca²⁺ signaling pathways. Application of the patch clamp technique to isolated nuclei (Mak and Foskett, 1994) has provided the most direct approach to study the detailed permeation and gating properties of single InsP₃,R ion channels in their native ER membrane environment. The endogenous InsP₃,R of cultured insect Spodoptera frugiperda (Sf9) cells shares many basic properties with Xenopus and rat InsP₃,R channels studied previously, including a biphasic dependence of its activity on [Ca²⁺]ᵢ, that is critical for generation of spatially and temporally complex [Ca²⁺]ᵢ signals in cells (Ionescu et al., 2006; Foskett et al., 2007). The InsP₃,R channel activities observed in Sf9 nuclear patches last longer than the channels in the other systems before they inevitably inactivate (Boehning et al., 2001; Mak et al., 2005; Ionescu et al., 2006). The longer activity durations of Sf9 InsP₃,R channels provide more event transitions for gating analyses, enabling observations of novel gating behaviors over longer time scales.

Examination of extensive current records of single Sf9 InsP₃,R channels in the presence of well-controlled and constant levels of [InsP₃] and [Ca²⁺], revealed that the channels did not display steady-state gating behavior, but instead exhibited spontaneous changes in gating kinetics through distinct patterns of behavior, or modes. We developed a new algorithm to analyze modal gating kinetics of the channel and identified three distinct

Abbreviations used in this paper: InsP₃, inositol 1,4,5-trisphosphate; InsP₃,R, InsP₃ receptor.
gating modes of the InsP$_3$R: a mode in which the channel is mostly bursting, another with fast channel gating kinetics, and one with long quiescent periods, with channel open probability $P_o$ of 0.85, 0.24, and 0.007, respectively. Unexpectedly, the channel $P_o$ within each mode remain relatively consistent over a wide range of $[\text{Ca}^{2+}]$, and $[\text{InsP}_3]$. Remarkably, our analysis therefore indicates that the observed biphasic $[\text{Ca}^{2+}]$ dependence and $[\text{InsP}_3]$ regulation of InsP$_3$R channel activity are generated primarily by ligand regulation of the relative prevalence of the three gating modes.

**MATERIALS AND METHODS**

**Sf9 Cell Culture and Nuclear Isolation**

*S. frugiperda* (Sf9) cells (Invitrogen) were grown and maintained in SF-900II serum-free media (GIBCO BRL) in suspension culture according to the manufacturer’s protocols. Each batch of cells was subcultured three to four times before being used for electrophysiology and then propagated and used for nuclear isolation for up to 7–8 wk in culture before a new lot was thawed and expanded. Nuclei were prepared for patch clamping as previously described (Mak et al., 2005; Ionescu et al., 2006). Isolated nuclei (5–10 μm in diameter) were distinguished from intact cells based on their unique morphology and selected for electrophysiology (Mak et al., 2005; Ionescu et al., 2006).

**Data Acquisition**

Nuclear patch clamping was performed as previously described (Mak et al., 1998). To maximize the duration of the observed channel activity, current recording was started as soon as seal resistance exceeded 150 MΩ. The standard pipette solution contained (in mM) 140 KCl, 10 HEPES (pH 7.3 by KOH), 0.5 Na$_2$ATP, 0.5 Ca$^{2+}$ chelator, and various $[\text{Ca}^{2+}]$ and $[\text{InsP}_3]$, as indicated. The bath solution contained (in mM) 140 KCl, 10 HEPES (pH 7.3 by KOH), 0.5 BAPTA (1,2-bis(o-aminophenoxy) ethane-N,N,N′,N′-tetraacetic acid; Molecular Probes), and 0.225 CaCl$_2$ (free $[\text{Ca}^{2+}] = 300$ nM). All solutions were carefully buffered to desired free $[\text{Ca}^{2+}]$ and $[\text{InsP}_3]$, as confirmed by fluorometry. All current traces used for analysis were recorded under 20 mV in room temperature. Data were acquired using an Axopatch 200B amplifier (Axon Instruments), filtered at 1 kHz, and digitized at 5 kHz with an ITC-16 interface (Instrutech) and Pulse software (HEKA Electronik).

**Data Analysis**

Segments of current records exhibiting current levels for a single InsP$_3$R channel under various ligand conditions (Table I) were idealized using QuB software (University of Buffalo) with SKM algorithm (Qi et al., 2000a,b). Channel gating kinetics and modal gating behaviors were characterized using our custom algorithm (Appendix) written using Igor Pro software (WaveMetrics). Statistical analyses were performed and figures were generated using Igor Pro software.

**RESULTS**

Application of the nuclear patch clamp technique to nuclei isolated from cultured insect Sf9 cells provided long, uninterrupted single-channel current records of the endogenous InsP$_3$R in its native ER membrane environment over a wide range of concentrations of cytoplasmic ligands ($\text{InsP}_3$ and $\text{Ca}^{2+}$) (Ionescu et al., 2006). With rigorous control of constant ligand and ionic conditions in the pipette and bath solutions during our experiments (Ionescu et al., 2006), we found that in all ligand conditions used, the InsP$_3$R exhibited apparent modal gating behaviors: it gated with steady kinetics for extensive periods (significantly longer than the mean open and closed channel durations) before gating abruptly changed into a discernibly different pattern. In experiments yielding single InsP$_3$R channel current records, channels were regularly observed to exhibit many such transitions through several modes of gating kinetics (Fig. 1).

**Characterization of Modal Gating**

To characterize this modal gating of the Sf9 InsP$_3$R channel and its regulation by $[\text{Ca}^{2+}]$, and $[\text{InsP}_3]$, we examined thousands of seconds of single-channel current records that were obtained in many experiments in the presence of saturating (10 μM) and subsaturating (33 nM) $[\text{InsP}_3]$, and subactivating (0.1 μM), optimal (1 μM), and inhibitory (89 μM) $[\text{Ca}^{2+}]$ (Table I). One characteristic of InsP$_3$R channel gating is the wide range of closed channel durations observed. The channels sometimes remained closed for extensive periods (seconds to tens of seconds) that were orders of magnitudes longer than other closing durations (∼10 ms). In addition, the transitions of InsP$_3$R channel gating from one mode to another occurred abruptly (Fig. 1). To avoid inherent limitations of conventional modal gating analysis algorithms, we developed a novel algorithm that is able to (a) determine when modal transitions occurred with high temporal resolution, and (b) identify the gating modes of InsP$_3$R channels in current records of arbitrary durations without the requirement to average channel kinetic parameters like channel $P_o$ or open or closed channel durations (Appendix).

Our modal gating analysis algorithm uses insights into InsP$_3$R channel gating derived from an allosteric model previously developed to quantitatively account for single InsP$_3$R channel gating behaviors under a wide range of $[\text{InsP}_3]$ and $[\text{Ca}^{2+}]$: that open InsP$_3$R channels close either via brief ligand-independent closings or via closings with durations regulated by $[\text{InsP}_3]$ and $[\text{Ca}^{2+}]$. Therefore $[\text{InsP}_3]$ and $[\text{Ca}^{2+}]$: that open InsP$_3$R channels close either via brief ligand-independent closings or via closings with durations regulated by $[\text{InsP}_3]$ and $[\text{Ca}^{2+}]$. Because the ligand regulation of InsP$_3$R channel gating is of primary interest, burst analysis was used (Magleby and Pallotta, 1983a) in the channel gating analysis to remove the majority of the brief ligand-independent channel closings so that kinetics of the ligand-dependent channel gating can be more easily discerned (Appendix and Table II therein). After burst analysis, the remaining channel openings and closings should be predominantly due to ligand-regulated channel gating. For clarity, those channel openings are referred to as...
bursts with durations $t_b$ separated by burst-terminating gaps with durations $t_g$.

Based on the values of $t_b$ and $t_g$ of adjacent burst-gap pairs, our algorithm consistently identified three distinct gating patterns (modes) in all the InSP$_3$R channel current traces obtained in all $[\text{Ca}^{2+}]_i$ and $[\text{InsP}_3]$ examined (Appendix). In the gating mode with high $P_o$ (H mode), the channel exhibits mainly bursting behavior with only brief gaps interrupting the long bursts of channel activity (Fig. 2, A and D). In the gating mode with intermediate $P_o$ (I mode), the InSP$_3$R channel gates frequently with mostly short openings and closings (Fig. 2, B and E). In the low-$P_o$ (L) mode, the channel has long closed periods interrupted with brief, infrequent openings, so channel $P_o$ is very low (Fig. 2, C and F).

**[Ca$^{2+}$], Regulation of Modal Gating**

Single Sf9 InSP$_3$R channel current records obtained in saturating 10 μM InSP$_3$ under three different $[\text{Ca}^{2+}]_i$ (0.1, 1, and 89 μM) were examined for modal gating behaviors. As observed during the basic characterization of the channel gating properties (Ionescu et al., 2006), the three $[\text{Ca}^{2+}]_i$ covered the full range of biphasic InSP$_3$R channel responses under saturating [InSP$_3$]: in $[\text{Ca}^{2+}]_i = 0.1$ μM, the InSP$_3$R channel is suboptimally activated by Ca$^{2+}$ so channel $P_o$ is low (~0.1); in $[\text{Ca}^{2+}]_i = 1$ μM, the channel is optimally activated by Ca$^{2+}$ with high channel $P_o$ (~0.6–0.8); and in $[\text{Ca}^{2+}]_i = 89$ μM, the channel is inhibited by high [Ca$^{2+}$], so that channel $P_o$ is again low (~0.1) (Fig. 3 A). In agreement with the previous characterization, the $[\text{Ca}^{2+}]_i$ dependence of channel $P_o$ is mainly reflected in changes in mean closed

![Figure 1](image)

**Figure 1.** Single InSP$_3$R channel current traces showing long-term modal gating behavior. Current records were obtained with $[\text{InSP}_3] = 10$ μM (saturating), and $[\text{Ca}^{2+}]_i$ = (A) 100 nM, (B) 1 μM, and (C) 89 μM. A continuous current record is shown for each condition. The arrows on the left indicate the closed-channel background current levels for this and all subsequent current traces. In B and C, arrowheads indicate some transitions when the InSP$_3$R channel gating behavior changed discernibly.

**TABLE I**

Statistics of Data Analyzed

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<tr>
<th>$[\text{Ca}^{2+}]_i$</th>
<th>$[\text{InSP}_3]$</th>
<th>Number of membrane patches</th>
<th>Total record duration</th>
<th>Mean duration per patch$^a$</th>
<th>Total opening events</th>
<th>Mean opening events per patch$^a$</th>
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<tr>
<td>μM</td>
<td>μM</td>
<td></td>
<td>s</td>
<td>s</td>
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<tr>
<td>0.1</td>
<td>10</td>
<td>16</td>
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<td>14,775</td>
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<tr>
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<td>10</td>
<td>26</td>
<td>3394.2</td>
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<tr>
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<td>15</td>
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<td>42 ± 7</td>
<td>11,850</td>
<td>790 ± 260</td>
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$^a$Mean ± SEM are tabulated.
channel duration ($t_o$) over more than an order of magnitude as \([Ca^{2+}]\) was changed, while mean open channel duration ($t_c$) remained relatively constant in all \([Ca^{2+}]\), (Fig. 3 E).

Examination of the gating properties of the InsP$_3$R channel during the periods in which the channel remained in a particular gating mode revealed that the channel $P_o$ within each gating mode ($P_o^M$, with M standing for H, I, or L) has a distinct value 0.85 ± 0.02, 0.24 ± 0.03, and 0.007 ± 0.002 for the H, I, and L modes, respectively, that remains surprisingly consistent over all \([Ca^{2+}]\) examined (Fig. 3 B). Because the modal gating analysis algorithm assigns modes only by the values of $t_o$ and $t_c$ after burst analysis, without directly taking into account the channel $P_o$ (Appendix), the consistency of the channel $P_o^M$ over the various ligand concentrations strongly suggests that the gating modes detected are true representations of the gating kinetics of the channel and not an artifact of the analysis protocol.

Because the InsP$_3$R channel $P_o$ within a mode ($P_o^M$) exhibited relatively small \([Ca^{2+}]\) dependencies, \([Ca^{2+}]\), must regulate the overall channel $P_o$ predominantly by regulating the fraction of time the channel spends in the different gating modes (relative prevalence $\pi^M$) (Fig. 3 C). Examination of modal kinetics revealed that $\pi^H$ is comparatively \([Ca^{2+}]\) independent (Fig. 3 C); the frequency of the channel entering the I mode (relative modal frequency $f^I$) (Fig. 3 D) as well as the mean modal dwell time $\langle \tau^I \rangle$ of the channel in the I mode (Fig. 3 J) exhibited very little \([Ca^{2+}]\) dependence. In contrast, both $\pi^H$ and $\pi^I$ showed profound ligand dependencies. In the presence of saturating 10 M InsP$_3$ and optimal 1 M \([Ca^{2+}]\), the InsP$_3$R channel is found most of the time in the H mode with high $P_o^H$, and in the L mode for relatively little time (Fig. 3 C), resulting in the observed high overall channel activity (Fig. 3 A). The high $\pi^H$ observed is due to the channel entering the H mode more frequently (higher $f^H$, Fig. 3 D) and staying in that mode longer (higher $\langle \tau^H \rangle$, Fig. 3 J). In nonoptimal \([Ca^{2+}]\) (0.1 and 89 M), the channel has lower overall activity (Fig. 3 A) mainly because it enters the L mode more frequently (higher $f^L$, Fig. 3 D). Unlike $\langle \tau^H \rangle$, which exhibits a strong \([Ca^{2+}]\) dependence, $\langle \tau^I \rangle$ remains within a relatively narrow range in all \([Ca^{2+}]\), (Fig. 3 J).

Although InsP$_3$R channel $P_o^M$ stayed within a narrow range over all \([Ca^{2+}]\) examined, the gating kinetics within a mode are not \([Ca^{2+}]\) independent. The channel open, closed, burst, and gap duration distributions in the three modes ($\langle t_o^M \rangle$, $\langle t_c^M \rangle$, $\langle t_b^M \rangle$, and $\langle t_g^M \rangle$, respectively) all exhibited statistically significant variations over the \([Ca^{2+}]\) range examined (Fig. 3, F–I).

**InsP$_3$ Regulation of Modal Gating**

InsP$_3$R channel gating is regulated by InsP$_3$ as well as by \([Ca^{2+}]\). It has been established that InsP$_3$ regulates InsP$_3$R channel gating by tuning the sensitivity of the channel to inhibition by high \([Ca^{2+}]\), (Mak et al., 1998; 2001; Ionescu et al., 2006). Thus, in the presence of subsaturating [InsP$_3$] (33 nM), the Sf9 InsP$_3$R channel is inhibited at a relatively low \([Ca^{2+}]\), so that $P_o$ at [InsP$_3$] = 33 nM and \([Ca^{2+}]\) = 1 M is lower than that at saturating [InsP$_3$] = 10 M and \([Ca^{2+}]\) = 1 M (Fig. 3 A). This reduction in channel $P_o$ is mainly due to increases in $t_o$ whereas $t_c$ shows little dependence on [InsP$_3$] (Fig. 3 E). Modal analysis of InsP$_3$R channel current records obtained in [InsP$_3$] = 33 nM and \([Ca^{2+}]\) = 1 M revealed that the channels in subsaturating (33 nM) [InsP$_3$] exhibit the same three gating modes (H, I, and L modes) with very similar $P_o^M$ in each of the modes as
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compared with channels in saturating (10 μM) [InsP3] (Fig. 3 B). Regulation of the overall channel $P_o$ by [InsP3] is therefore mediated by the effects of [InsP3] on $\pi^d$, which agrees with the observation that in subsaturating [InsP3], the channel is inhibited by lower $[Ca^{2+}]_i$ (Fig. 3 C). In 33 nM [InsP3] and 1 μM $[Ca^{2+}]_i$, $\pi^l$ is not very different from that for the channel in saturating (10 μM) [InsP3]. The ratio of $\pi^l$: $\pi^d$ in $[Ca^{2+}]_i = 1$ μM and [InsP3] = 33 nM is smaller than that for a channel in the same $[Ca^{2+}]_i$, but saturating 10 μM [InsP3], as inhibition by $Ca^{2+}$ stabilizes the L mode relative to the H mode. Since the gating kinetic properties ($\langle t_o^M \rangle$, $\langle t_c^M \rangle$, $\langle t_b^M \rangle$, and $\langle t_g^M \rangle$) of the channel in each mode are significantly regulated by $[Ca^{2+}]_i$ and InsP3 regulates InsP3R channel activity by altering the sensitivity of the channel to $Ca^{2+}$ regulation, these gating kinetic properties of the channel in each mode display dependence on [InsP3] as expected (Fig. 3, F-I).

**DISCUSSION**

We have here obtained long single-channel current records with robust InsP3R channel activities over a wide range of $[Ca^{2+}]_i$ and [InsP3] by patch clamp electrophysiology of isolated nuclei from Sf9 cells. These records have provided new insights into the gating behavior of the InsP3R channel. InsP3R gating is modal, with three distinct patterns of activity identified. Importantly, ligand regulation of channel activity impinges directly upon this modal gating behavior, with $Ca^{2+}$ and InsP3 regulating the propensity of the channel to be in each of the three stereotypic, relatively ligand-independent modes.
The InsP₃R Channel Gates in Three Modes

Based on an allosteric model that quantitatively accounted for single InsP₃R channel gating behaviors under a wide range of [InsP₃] and [Ca²⁺], Mak et al., 2003; Fraiman and Dawson, 2004) have the potential to account for the observed modal gating behaviors. However, since all these models were developed to account for steady-state channel gating behavior under constant [InsP₃] and [Ca²⁺], they all need to be substantially expanded to describe the modal gating behaviors of the InsP₃R channel in which spontaneous transitions from one gating mode to another occurred regularly even in the presence of constant [InsP₃] and [Ca²⁺]. As a starting point, we present in the Appendix (and Fig. 8 therein) the simplest model that quantitatively accounts for the InsP₃R channel modal gating behaviors observed.

Ligand Regulation of InsP₃R Channel Activity Is Mainly Mediated through Mode Switching

A surprising result of our modal analysis is that ligand regulation of InsP₃R channel activity (Pₒ), a critical aspect of regulation of InsP₃-mediated intracellular Ca²⁺ signaling, is mediated mainly by ligand regulation of the relative prevalence of the H mode vs. the L mode (Fig. 3 C). Within the range of [Ca²⁺], and [InsP₃] examined, all the kinetics of the L mode (relative prevalence π₁, relative frequency f¹, and mean dwell time (〈τ¹〉) exhibit little [Ca²⁺] or [InsP₃] dependencies (Fig. 3, C, D, and J). Mode switching is not the only mechanism for ligand regulation of InsP₃R channel kinetics, because detailed gating kinetics of the channel in individual modes (〈τₖ>ₗₗ and 〈τₖ>ₗₖₖ) are also significantly regulated by [InsP₃] and [Ca²⁺]. We suggest that mode switching is nevertheless the most relevant mechanism of ligand regulation of InsP₃R-mediated Ca²⁺ release. InsP₃R channels are spatially localized in the ER in clusters (Mak and Foskett, 1994; Ionescu et al., 2006) with more than one active channel involved in the generation of various Ca²⁺ signaling events ranging from blips and puffs to propagating saltatory waves (Yao et al., 2007). Because the opening and closing kinetics of individual channels are averaged out over the multiple active channels involved, it is the Pₒ of the active InsP₃R channels that directly govern the amount of Ca²⁺ released and therefore the characteristics of the Ca²⁺ signal generated. Thus, ligand-dependent mode switching, which directly impinges on the channel Pₒ, is the major mechanism for ligand regulation of InsP₃R-mediated Ca²⁺ signals. Deeper understanding of the kinetic mechanisms responsible for modal gating behaviors of the channel will therefore provide further insights into ligand regulation of InsP₃R-mediated Ca²⁺ signaling.

Physiological Significance of InsP₃R Modal Gating

Modal gating kinetics have been observed in many different ion channels, including Cl⁻ channels (Blatz and Magleby, 1986; Catacuzzeno et al., 1999), “maxi” BK (Magleby and Pallotta, 1983a,b; McManus and Magleby,
Significance of modal gating is in most cases not clear. Although different channel gating modes have been associated with distinct long-term channel kinetic features, including inactivation (Imredy and Yue, 1994), desensitization (Naranjo and Brehm, 1993), ligand inhibition (Delcour et al., 1995), P/Q- (Luvisetto et al., 2004), and L-type (Imredy and Yue, 1994) Ca$^{2+}$ channels, to name a few. Although the physiological significance of “adaptation” is also not clear, and the InsP$_3$R does not display similar “adaptation” behaviors in response to rapid changes in InsP$_3$ or Ca$^{2+}$ concentrations (Mak et al., 2007). The results here, by demonstrating that important ligand regulation of InsP$_3$R channel activity impinges primarily on modal gating, provide a clear demonstration of the physiological relevance of channel modal gating.

The modal gating analysis presented here was performed on single-channel current traces from insect S9 InsP$_3$R mainly because these channels remain active for long extensive periods during nuclear patch clamp experiments (mean channel activity duration $\sim$120 s; Ionescu et al., 2006). In retrospect, it appears that modal gating behavior was previously observed in nuclear patch-clamp records of diverse endogenous or recombinant channels from different InsP$_3$R isoforms (type 1 and 3) and splice variants (SII+/−) of different species (Xenopus laevis frogs and rat). Bursts of high channel activities (H mode) separated by long quiescent periods (L mode) were observed in endogenous Xenopus type 1 InsP$_3$R channels (Mak and Foskett, 1997; Mak et al., 1998, 2001). Single-channel current records of recombinant rat type 3 InsP$_3$R expressed in Xenopus oocytes presented by Mak et al. (2000, 2001) are reminiscent of the S9 InsP$_3$R channel current records exhibiting modal gating behaviors presented here (Fig. 2). Current records of recombinant rat type 1 InsP$_3$R channels expressed in mammalian COS-7 cells presented by Boehning et al. (2001) clearly exhibited the three gating modes. Furthermore, biphasic regulation of the relative prevalence of the H mode by Ca$^{2+}$ paralleling the biphasic Ca$^{2+}$ regulation of InsP$_3$R channel activity that is reported here was also clearly observable in the current records obtained in various [Ca$^{2+}$], for different InsP$_3$R isoform channels from different species (Mak et al., 1998, 2001; Boehning et al., 2001). Thus, although a comprehensive study of modal gating behavior (like the one performed here for the S9 InsP$_3$R channels) was not feasible for the other InsP$_3$R channels because of their short channel activity durations due to channel rundown or inactivation (Mak and Foskett, 1997; Mak et al., 2000; Boehning et al., 2001), modal gating appears to have been widely observed in many different types of InsP$_3$R and probably plays a major role in ligand regulation of many if not all InsP$_3$R channels.

The important role modal gating plays in ligand regulation of InsP$_3$R activity indicates that besides the time scales of channel openings and closings ($t_c$ and $t_o$ $\sim$ ms, Fig. 3 E), other, longer time scales in InsP$_3$R channel gating kinetics are likely to be relevant for the kinetics of InsP$_3$-mediated intracellular Ca$^{2+}$ signaling in vivo. One such time scale is associated with the channel burst ($t_{b}$) and burst-terminating (interburst) gap ($t_{g}$) durations. Whereas most of the short channel closings are the result of ligand-independent channel gating (Mak et al., 2003; Foskett and Mak, 2004), the time scales of the bursts and gaps probably reflect the kinetics of ligand unbinding from and binding to, respectively, the channel and the associated InsP$_3$R conformational changes. This assumption is supported by the results of the modal gating analysis here (Appendix and Table II therein). Thus, the durations of the bursts and gaps, rather than the durations of channel opening and closing, probably provide a better measure of the kinetics of the response of the InsP$_3$R channel to ligand concentration changes.

A recent study of the kinetic responses of single InsP$_3$R channels to rapid ligand concentration changes observed that in the constant presence of saturating 10 $\mu$M InsP$_3$, the mean lag times to termination of InsP$_3$R channel activity from abrupt changes in [Ca$^{2+}$]$_{\text{i}}$ from optimal ($2 \mu$M) to subactivating (<10 nM) and/or inhibitory (300 $\mu$M) levels were 160 and 290 ms, respectively. In constant 2 $\mu$M Ca$^{2+}$, the mean lag time to channel activity termination from an abrupt drop in [InsP$_3$] from 10 $\mu$M to 0 was 700 ms (Mak et al., 2007). In those experiments, the channels were most likely in the H mode before the activity-terminating ligand concentration change, with mean burst duration $\langle t_{b} \rangle$ of 200–600 ms (Fig. 3 H). The similarity between the mean channel lag times observed in rapid perfusion experiments and the mean burst duration determined here suggests that the kinetics of channel responses to changes in ligand concentrations are likely determined by how fast the channel can exit from a burst when the ligand concentration change occurs. If that is the case, then instead of a channel opening, a channel burst probably constitutes a stereotypical single-channel InsP$_3$R Ca$^{2+}$-release event.

The weak dependence of channel burst duration on [Ca$^{2+}$], may possibly be a mechanism by which an active
InsP₃R channel can avoid being prematurely inhibited by the Ca²⁺ that it releases, because an increase of [Ca²⁺], from 1 μM (optimal) to 89 μM (inhibitory) only reduces the burst duration from ~500 to ~300 ms when the channel is in H mode (Fig. 3 H). Moreover, the stabilization by activating [Ca²⁺], of the H mode (Fig. 3 J), which has significantly longer burst durations (Fig. 3 H), may possibly play an important role in Ca²⁺-induced Ca²⁺ release. Thus, in the presence of sufficiently high [InsP₃], an increase in [Ca²⁺], above the resting level can encourage an InsP₃R channel to enter the H mode from the L mode. As a result, the burst duration of the channel increases from that for an L mode (~10 ms), which may not release enough Ca²⁺ to recruit nearby channels to propagate a Ca²⁺ signal (individual blips or puffs) to that of an H mode (~200 ms), which enables the InsP₃R channel to continue releasing Ca²⁺ even when the local [Ca²⁺], is raised to a high level. Such long channel bursts can release sufficient Ca²⁺ to recruit neighboring InsP₃Rs or InsP₃R clusters for a regenerative Ca²⁺ signal (Berridge, 1997; Ionescu et al., 2006).

In summary, we have demonstrated that the InsP₃R gates with stereotypic behaviors in three distinct modes, and that mode switching accounts for most of the ligand regulation of InsP₃R Ca²⁺ release channel. Modal switching is therefore a novel major mechanism of physiological regulation of InsP₃R channel activity, with implications for the kinetics of Ca²⁺ release events in cells.

**APPENDIX**

**New Algorithm Needed to Analyze Modal Gating Kinetics of InsP₃R Channels**

Conventional modal gating analysis algorithms assign modes according to the value of a channel kinetic parameter, e.g., \( P_o \) or channel opening or closing durations, averaged over current record segments either with a fixed duration (Delcour and Tsien, 1993; Delcour et al., 1993; Satenku et al., 2001; Popescu and Auerbach, 2003; Popescu et al., 2004) or containing a fixed number of channel openings and closings (Blatz and Magleby, 1986; McManus and Magleby, 1988; Catacuzzeno et al., 1999). The InsP₃R channel exhibited closed channel durations that span several orders of magnitude (a few milliseconds to tens of seconds) in all ligand conditions examined (Fig. 1). Furthermore, the transitions between different gating patterns of the InsP₃R channel were abrupt (Fig. 1). Consequently, when short averaging segments were used in conventional algorithms, the value of the averaged parameter fluctuated wildly from segment to segment due to the small number of gating events present in each segment available for averaging, rendering separation of gating modes impossible. Conversely, using long averaging segments in conventional algorithms resulted in loss of temporal resolution and failure to capture the abrupt nature of the modal transitions. Thus, we developed a novel modal gating algorithm to determine the gating mode of the InsP₃R channel from its current record with high accuracy and high temporal resolution.

**Burst Analysis of Idealized Single-Channel InsP₃R Channel Current Traces**

Previous analyses of the gating kinetics of various InsP₃R channels have revealed that the regulation of channel \( P_o \) by [Ca²⁺] and [InsP₃] is predominantly accounted for by ligand regulation of the mean channel closed duration (\( \langle t_c \rangle \) (Mak et al., 1998, 2001; Ionescu et al., 2006). In addition, the maximum channel \( P_o \) is ~0.8, significantly <1, even under optimal ligand conditions, when the channel gates mainly with short openings (channel open duration \( t_o \) ~10 ms) separated by very brief closings (channel closed duration \( t_c \) ~1 ms). It was previously suggested that open InsP₃R channels can close either via ligand-independent or -dependent conformational transitions (Mak et al., 2003), with closures under optimal ligand conditions mediated predominantly via ligand-independent transitions. By visual inspection of current records of the Sf9 InsP₃R channel, we surmised that such ligand-independent transitions accounted for the majority of channel closings in one of the channel gating modes. To properly identify such a gating mode, we performed a burst analysis (Magleby and Pallotta, 1983a) to remove brief channel closings that probably originated from these ligand-independent transitions. Because the ligand-dependent channel closings occur more frequently under ligand conditions that engender low channel \( P_o \), channel closed duration distributions under such conditions are more likely to reveal a clear segregation of the two populations of channel closings originating from ligand-dependent and -independent conformational transitions. The InsP₃R channel \( t_c \) distribution (Fig. 4) for all current records obtained in [Ca²⁺] = 0.1 μM, when \( P_o \) was low, suggested that 10 ms is a reasonable value for the minimum duration of a burst-terminating gap \( T_{\text{min}} \) (Magleby and Pallotta, 1983a). Therefore, all channel closings with \( t_c \leq T_{\text{min}} = 10 \) ms were considered to be caused by ligand-independent channel conformation transitions, and were removed in our burst analysis as if they never occurred. Consequently, all channel closings have durations >10 ms after the burst analysis. Those channel openings that remained after burst analysis, presumably resulting from ligand-dependent transitions, are referred to as bursts with duration \( t_o \), separated by burst-terminating gaps with duration \( t_c > 10 \) ms. Burst analysis with \( T_{\text{min}} = 10 \) ms was applied to all idealized single InsP₃R channel current records obtained in [Ca²⁺] = 0.1, 1, and 89 μM.

It should be noted that even when channel exhibited low \( P_o \) in 10 μM InsP₃ and 0.1 μM Ca²⁺, the duration
distribution of the short channel closing events assumed to be caused by ligand-independent conformation transitions and that of the longer channel closing events supposed to be caused by ligand-dependent transitions overlap substantially (see Fig. 4). With no information about the conformation transitions other than their durations, we applied an abrupt cut-off criterion to separate the channel closing events into two populations, one (with \( t_c \leq T_{\text{gmin}} = 10 \text{ ms} \)) to be filtered out in our burst filtering protocol and one (with \( t_c > T_{\text{gmin}} \)) retained. Under the circumstances, there is no “ideal” choice of the value of \( T_{\text{gmin}} \). Any choice of \( T_{\text{gmin}} \) will inevitably leave a population of closings with \( t_c \) just \(< T_{\text{gmin}} \),

which, upon visual inspection, seem long enough to be retained, and a population of closings with \( t_c \) just \(> T_{\text{gmin}} \), which seem to be short enough to be filtered out. Our choice of \( T_{\text{gmin}} \) as indicated in Fig. 4 minimized the number of such “ambiguous” closings for channels in 10 \( \mu \text{M} \) InsP\(_3\) and 0.1 \( \mu \text{M} \) Ca\(^{2+}\).

The incomplete segregation of the short ligand-independent channel closings and the longer, ligand-dependent ones even in 0.1 \( \mu \text{M} \) Ca\(^{2+}\) and 10 \( \mu \text{M} \) InsP\(_3\) when channel \( P_o \) is low (Fig. 4) also means that because of the stochastic nature of channel gating, a fraction of the ligand-dependent channel closings have short durations. Consequently, they are indistinguishable from the ligand-independent ones and are removed by the burst analysis. To gauge how well the abrupt cut-off criterion \((t_c < 10 \text{ ms})\) worked to remove only short channel closings that were due to ligand-independent transitions, the frequencies of short closing removal were evaluated under different ligand conditions. Assuming that separate independent mechanisms are responsible for generating ligand-independent and ligand-dependent channel closings, ligand-independent channel closings are only observable when the channel is not already closed by ligand-dependent mechanism(s). Ideally, if all ligand-independent closings can be identified, the frequency of ligand-independent closings (the number of such channel closings observed)/(total duration of all bursts after all such closings have been removed) should remain the same under all ligand concentrations. The frequencies of short closings removed by burst analysis of current records obtained under various ligand conditions are tabulated in Table II. Although the frequencies of removed short channel closings are not completely ligand independent, they are not very different from one another, considering the experiment-to-experiment variability. This suggests that the burst analysis using an abrupt cut-off criterion, while obviously imperfect, did remove a substantial fraction of the ligand-independent short channel closings to reveal the underlying modal channel gating kinetics with considerably longer time scales (Figs. 2 and 3) without filtering out too many ligand-dependent closings.

### Three Modes of InsP\(_3\)R Channel Gating

After burst analysis, three distinct patterns (modes) of InsP\(_3\)R channel gating were revealed in all [Ca\(^{2+}\)]. The channel gating kinetics in each mode were not significantly affected by the burst analysis. In the first mode, the channel has long bursts interrupted with gaps that are relatively brief (though \(>10 \text{ ms} \)) so channel \( P_o \) is high (Fig. 2, A and D). In the second mode, the InsP\(_3\)R channel gates frequently with mostly short openings and closings, so channel \( P_o \) is moderate (Fig. 2, B and E). In the third mode, the channel has long closed periods interrupted with brief, infrequent openings, so channel \( P_o \) is very low. Burst analysis does not significantly affect the kinetics of this mode (Fig. 2, C and F). Based on their kinetic characteristics, we refer to the three InsP\(_3\)R channel gating patterns as the high- (H), intermediate- (I), and low- (L) activity modes, respectively.

### Detection of Modal Transitions and Gating Mode Assignment

To identify with high temporal resolution changes among the gating modes of the InsP\(_3\)R channel (modal transitions), durations of channel bursts and burst-terminating gaps \((t_b \) and \( t_g \)) in idealized, burst-analyzed single channel records were monitored. Either \( t_b \) or \( t_g \) crossing over some predefined abrupt thresholds \( T_g \)
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and $T_b$, respectively, from above or below, could signify a modal transition. Visual examination of idealized current traces (both before and after burst analysis) together with plots of $t_g$ and $t_b$ for all single channel current records indicated that setting $T_b = 100 \text{ ms}$ and $T_g = 200 \text{ ms}$ allowed objective detection of channel modal transitions that correlated closely with observed changes in the patterns of channel gating kinetics (Fig. 5). Because a majority of burst-terminating gaps had $t_g \leq 200 \text{ ms}$ in all ligand conditions examined, a hysteresis requirement was implemented in the modal transition detection protocol to avoid overfragmenting the channel gating modes. Thus, a modal transition was recognized only when two or more consecutive burst-terminating gaps had $t_g \leq 200 \text{ ms}$ following one or several consecutive gaps with $t_g > 200 \text{ ms}$ (long purple arrow in Fig. 5 B). However, a modal transition was recognized when a single burst-terminating gap with $t_g > 200 \text{ ms}$ followed one or several consecutive gaps with $t_g \leq 200 \text{ ms}$ (blue arrowheads in Fig. 5 B). Similarly, a majority of channel bursts had $t_b \leq 100 \text{ ms}$ in all ligand conditions examined. Therefore, whereas a modal transition was registered when a single channel burst had $t_b > 100 \text{ ms}$ following a series of bursts with $t_b \leq 100 \text{ ms}$, a modal transition was only registered when two consecutive channel bursts had $t_b \leq 100 \text{ ms}$ following a series of bursts with $t_b > 100 \text{ ms}$.

After the modal transitions were identified, the channel was then classified as being in the I mode if $t_g \leq T_g$ and $t_b \leq T_b$; in the H mode if $t_g \leq T_g$ and $t_b > T_b$; and in the L mode if $t_g > T_g$ and $t_b \leq T_b$ (Fig. 6). Cases in which a burst-terminating gap with $t_g > T_g$ occurred adjacent to a burst with $t_b > T_b$ were rare and considered to be modal transitions between L and H modes occurring between the burst and the gap (Fig. 6).

Channel mode assignments in this algorithm are based on the durations of two consecutive pairs of channel bursts and gaps rather than on the values of channel parameters ($t_o$, $t_c$, or $P_o$) averaged over some window.
activity of the InsP₃R channel before inactivation or rundown. To provide proper weighing for experiments of different durations, the kinetic parameters for InsP₃R channel gating were evaluated using event-based statistics. This means that each opening (or closing) event from any experiment performed with the same set of ligand conditions was considered equivalent. Thus the mean open (or closed) duration ⟨t⟩ is given by

\[
⟨t⟩ = \left\{ \frac{1}{\sum_{j=1}^{M} \sum_{i=1}^{N_j} t_{ij}} \right\}^{-1},
\]

where \( t_{ij} \) is the duration of the \( j^{th} \) of \( N_j \) opening (or closing) events in the \( j^{th} \) of \( M \) experiments performed in the set of ligand conditions in question (Fig. 3 E). Channel \( P_0 \) (Fig. 3 A) was evaluated as the ratio of the sum of all open durations to the sum of all durations (open or closed). Similarly, the mean open (closed, burst, or gap) duration for each gating mode was evaluated by averaging with equal weight the durations of all the openings (closings, bursts, or gaps) in all the periods when the channel was determined to be in that mode by the modal analysis algorithm, regardless of which experiment the event was recorded in as long as the experiment was performed under the same set of ligand conditions (Fig. 3, F–I). Channel \( P_0^M \) was evaluated as the ratio of the sum of all open durations to the sum of all durations (open or closed) in a particular mode (Fig. 3 B). The mean modal dwell times \( ⟨τ^M⟩ \) (Fig. 3 J) were evaluated using Eq. A1 with all dwell times of the mode from any experiment (using the same ligand conditions) weighted equally.

The duration distributions for channel openings and closings in general, and the distributions within each gating mode for channel openings, closings, bursts, and gaps, were all determined to be non-Gaussian by the Jarque-Bera (Jarque and Bera, 1987) and Kolmogorov-Smirnov (Khamis, 2000) tests. Thus, nonparametric statistical analyses were used to characterize and compare the distributions. The ranges of the distributions were described in terms of the standard deviations evaluated by the random bootstrap resampling method (Efron and Tibshirani, 1993; Mooney and Duval, 1993), and statistical significance of the differences between the durations was evaluated using the two-tailed nonparametric Wilcoxon-Mann-Whitney rank-sum test (Cheung and Klotz, 1997).

Because of the large numbers of events available from extensive experimental current records for each set of ligand conditions, the standard deviations of the durations \( t_0, t_o, t_b, t^M, t_c, t^M_c, t_g, t^M_g \), and \( t^M \) are too small to be plotted in Fig. 3 (the error bars are all smaller than the symbols in the graphs). The errors in the values of channel \( P_0 \) and \( P_0^M \), derived from the durations, are also too small to be plotted. According to the nonparametric Wilcoxon-Mann-Whitney rank-sum test, most kinetic parameters were consistent among various ligand concentration conditions (Table I). Segments from each of the many experiments performed under different conditions (Table I) were evaluated as the ratio of the sum of all open durations to the sum of all durations (open or closed). Similarly, the mean open (closed, burst, or gap) duration for each gating mode was evaluated by averaging with equal weight the durations of all the openings (closings, bursts, or gaps) in all the periods when the channel was determined to be in that mode by the modal analysis algorithm, regardless of which experiment the event was recorded in as long as the experiment was performed under the same set of ligand conditions (Fig. 3, F–I).
quantities obtained under optimal (1 μM) Ca\(^{2+}\) and saturating (10 μM) InsP\(_3\) were significantly different when compared with corresponding quantities obtained under other ligand conditions, low (100 nM) Ca\(^{2+}\) and saturating InsP\(_3\), inhibitory (89 μM) Ca\(^{2+}\) and saturating InsP\(_3\), optimal Ca\(^{2+}\) and subsaturating (33 nM) InsP\(_3\).

Whereas event-based statistics may provide more accurate estimation of the channel gating parameters by taking into consideration the very different lengths of channel activity recorded in various experiments, the calculation gives no estimate of the experiment-to-experiment variability of the kinetic parameters. To gauge the reproducibility of the experiments, the kinetic parameters (\(\langle t_c \rangle\), \(\langle t_o \rangle\), \(\langle t_{oM} \rangle\), \(\langle t_{cM} \rangle\), \(\langle t_{bM} \rangle\), \(\langle t_{gM} \rangle\), \(\langle t_{gM} \rangle\)) were also evaluated by experiment-based statistics, so that

\[
\langle t \rangle = \frac{1}{M} \left\{ \sum_{j=1}^{M} \left( \frac{1}{N_j} \sum_{i=1}^{N_j} t_{ij} \right) \right\} = \frac{1}{M} \left\{ \sum_{j=1}^{M} \langle t \rangle_j \right\},
\]

where symbols are similar to those used in Eq. A1. As shown in the second half of Eq. A2, the average durations are evaluated as the mean of the mean durations from individual experiments. The channel open probabilities (\(P_o\) and \(P_{oM}\)) derived by experiment-based statistics are similarly evaluated as the mean of channel open probabilities from individual experiments. More importantly, the standard deviations of these quantities derived by random bootstrap resampling provide some measure of the reproducibility of the experiments. The kinetic parameters and their standard deviations derived through experiment-based statistics are plotted in Fig. 7.

Despite the larger standard deviations of the kinetic quantities evaluated by experiment-based statistics, a comparison, using Wilcoxon-Mann-Whitney rank-sum test, of kinetic quantities obtained under optimal [Ca\(^{2+}\)] (1 μM) and saturated [InsP\(_3\)] (10 μM), and saturating InsP\(_3\) with those obtained in other ligand conditions revealed statistically significantly difference in a majority of the quantities.

**Validation of Modal Gating Analysis Algorithm**

The algorithm developed to analyze channel modal gating kinetics used three parameters: the minimum

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Figure 7. InsP\(_{3}\)R channel gating parameters in various [Ca\(^{2+}\)] and [InsP\(_3\)] evaluated using experiment-based statistics (see Appendix). The same channel gating parameters are plotted in the same panels with the same symbols and color code as in Fig. 3: (A) channel open probability \(P_o\); (B) channel open probability in each of the three gating modes \(P_{oM}\); (C) relative prevalence \(\pi^{M}\) for the three gating modes; (D) relative frequency \(f^{M}\) of the gating modes; (E) mean overall channel opening \(\langle t_o \rangle\) and closing \(\langle t_c \rangle\) duration; (F–J) mean durations of channel opening \(\langle t_{oM} \rangle\), closing \(\langle t_{cM} \rangle\), bursts \(\langle t_{bM} \rangle\), and burst-terminating gaps \(\langle t_{gM} \rangle\) respectively, for the gating modes; (J) mean duration \(\langle t_{gM} \rangle\) of the gating modes. Error bars in (A, B, and E–J) indicate standard errors of the mean evaluated using experiment-based statistics (see Appendix). Parameters marked with asterisks are those observed in suboptimal ligand concentrations that are statistically significantly different from those observed in optimal [Ca\(^{2+}\)] (1 μM) and saturated [InsP\(_3\)] (10 μM).
Figure 8. Schematic diagram showing the simplest completely-interconnected Markov kinetic model for three distinct gating modes. Each of the three modes (L, H, and I modes) consists of one closed (C\textsubscript{o}) and one open (O\textsubscript{a}) kinetic states, with state transition rates for channel opening (k\textsubscript{oM}) and closing (k\textsubscript{cM}) as tabulated. The three modes are completely interconnected through the closed kinetic states (C\textsubscript{H}, C\textsubscript{L}, and C\textsubscript{I}) with six mode transition rates (k\textsubscript{HL}, k\textsubscript{IH}, k\textsubscript{IL}, k\textsubscript{LI}, k\textsubscript{HI}, and k\textsubscript{IL}). Through stochastic simulation, these kinetic rates were used to generate virtual channel current records, which in turn were used to check the accuracy of the modal analysis algorithm.

burst-terminating gap duration $T_{\text{gmin}}$, the limit of channel burst duration $T_B$, and the limit of gap duration $T_G$. The values of $T_{\text{gmin}} = 10$ ms, $T_B = 100$ ms, and $T_G = 200$ ms were selected by visual inspection of single InsP\textsubscript{3}R channel current records and dwell time histograms. To check if the conclusions of the modal analysis are dependent on the choice of these parameters, we systematically examined the effects of using different sets of parameters on the results of our modal analysis. We found that for $7\text{ ms} \leq T_{\text{gmin}} \leq 15\text{ ms}$, $50\text{ ms} \leq T_B \leq 200\text{ ms}$, and $100\text{ ms} \leq T_G \leq 300\text{ ms}$, the modal analysis algorithm still yielded three gating modes each with a distinct value of $P_o$ ($\sim 0.8$, $0.3$, and $0.01$ for H, I, and L modes, respectively) that were largely independent of ligand concentrations.

Because the gating activities and modal transitions of the channel are stochastic in nature, any modal analysis algorithm based on the kinetic properties of the channel ($t_o$ and $t_c$) during a current record is inherently inaccurate to some extent. For example, although most channel closings are brief when the channel is in H mode, long closings will inevitably occur even when the channel is in H mode, causing the modal analysis algorithm to misidentify the channel as being in the I or L mode. Moreover, even though the kinetics of modal transitions are significantly slower than that of channel gating: $\langle M \rangle >\langle t_o \rangle$, $\langle t_c \rangle$, $\langle t^M_o \rangle$, or $\langle t^M_c \rangle$, our algorithm will not detect some brief residences of the channel in a mode, particularly because of the hysteresis requirement used. The complexity of the modal analysis algorithm precludes an analytical approach to evaluate the error rate of the algorithm, i.e., the fraction of time when the algorithm misidentifies the mode of the channel. Furthermore, there is no detectable difference among the values of channel conductance for the InsP\textsubscript{3}R channel in the three modes (Fig. 1) so that there is no assured means to derive the mode that a channel is in from the experimental patch-clamp current record. To estimate the error rate of the modal analysis algorithm, virtual channel current records were generated by stochastic simulation (Shuai et al., 2007) from the kinetic state of an InsP\textsubscript{3}R channel in the simplest three-mode Markov model (Fig. 8). The simple model does not fully capture all the details of the observed InsP\textsubscript{3}R channel modal behavior, but is simple enough that all the necessary state transition rates can be directly calculated from the experimentally observed modal properties of the channel ($\langle t^M_o \rangle$, $\langle t^M_c \rangle$, $\langle t^M \rangle$, $\pi^M$, and $P^M_o$). The virtual current records were analyzed using our modal analysis protocol. The error rate of our algorithm was estimated by comparing the results of the analysis with the known kinetic states of the channel from the simulation. The error rate of the algorithm was $\sim 3.5\%$ for all $[\text{Ca}^{2+}]_i$ examined (0.1, 1, and 89 $\mu$M). The error rate approximately doubled for every threefold increase in the modal transition rates (H$\leftrightarrow$I, I$\leftrightarrow$L, and L$\leftrightarrow$H rates) used in the simulation. Thus, although there must be some inherent inaccuracies in modal transition rates used in the simulation because they were based on the modal properties derived by the modal analysis algorithm, the effects of such inaccuracies on the error rates are not significant. Finally, only 3% of a virtual current record generated for a channel in the H mode only (H$\rightarrow$I and H$\rightarrow$L rates = 0) and $<1\%$ of virtual current records generated for channels only in the I or L modes were misidentified. Thus, our modal analysis protocol identifies the kinetic modes of an InsP\textsubscript{3}R channel from its current record with high accuracy and high temporal resolution.

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