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Regulation of IP₃R Channel Gating by Ca²⁺ and Ca²⁺ Binding Proteins

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I. OVERVIEW

The activity of the inositol 1,4,5-trisphosphate (IP₃) receptor (IP₃R) Ca²⁺ release channel is intricately regulated by a multitude of ligands. The most important ligands regulating IP₃R channel activity are IP₃ and Ca²⁺, its physiological permeant ion. Generally, cytoplasmic Ca²⁺ regulates steady-state IP₃R channel gating with a biphasic concentration dependence—Ca²⁺ at low concentrations activates the channel and increases its open probability P_o , whereas at higher concentrations, Ca²⁺ inhibits the channel. Ca²⁺ modulates channel activity by binding to several apparently distinct sites that regulate Ca²⁺ activation, IP₃-dependent Ca²⁺ inhibition, IP₃-independent Ca²⁺ inhibition, channel inactivation, and channel recruitment. In addition, a separate Ca²⁺-binding site appears to regulate the functionality of the IP₃-independent Ca²⁺ inhibition site. Insights into Ca²⁺ regulation of channel gating have been advanced by recent studies of the kinetic responses of single channels in endoplasmic reticulum membranes to rapid changes in the concentrations of Ca²⁺ and IP₃. Quantitative and qualitative molecular models can account for much of the steady-state and kinetic behaviors of IP₃R channel gating regulation by cytoplasmic Ca²⁺. The identification of modal gating has revealed that Ca²⁺ modulates IP₃R channel activity primary by regulating its propensity to gate in particular modes. Ca²⁺ regulation of the channel is impinged upon by other channel regulators, including IP₃ and ATP, as allosteric modulators. Ca²⁺ can also impinge on the activity of the channel indirectly, by binding to other proteins that interact with it.

II. INTRODUCTION

The activity of the inositol 1,4,5-trisphosphate (IP₃) receptor (IP₃R) Ca²⁺ release channel is regulated by a wide range of ligands. The most important ligands regulating IP₃R channel activity are IP₃ and Ca²⁺, its physiological permeant ion. Generally, Ca²⁺ regulates steady-

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state IP₃R channel gating with a biphasic concentration dependence—Ca²⁺ at low concentrations activates the channel and increases its open probability P_o , whereas at higher concentrations, Ca²⁺ inhibits the channel. IP₃ and Ca²⁺ are essential for IP₃R channel activation, but other physiological ligands, such as ATP, are not. ATP-free acid potentiates IP₃ channel activity allosterically by enhancing the sensitivity of the channel to Ca²⁺ activation. Regulation of channel activity by IP₃ is also by mainly modifying the sensitivity of the channels to Ca²⁺ regulation. The most systematic studies have revealed that IP₃, as well as adenophostin A and its analogs, regulates the IP₃R channel by allosterically modulating the sensitivity of the channels to Ca²⁺ inhibition, with little effect on the Ca²⁺ activation properties.

Besides steady-state channel gating kinetics, other aspects of IP₃R channel activity are also regulated by Ca²⁺ and IP₃. In the constant presence of IP₃, IP₃R channel activity inevitably terminates. This reversible, IP₃-induced inactivation of the channel progresses faster in high Ca²⁺ concentrations and in subsaturating concentrations of IP₃. In addition, channel recruitment is a distinct process in which suboptimal Ca²⁺ concentrations (too low or too high) and subsaturating IP₃ concentrations activate fewer IP₃R channels than optimal concentrations of either ligand.

Thus, ligand regulation of the IP₃R channel is complex, with Ca²⁺ as the major determinant of the channel properties. Ca²⁺ modulates channel activity by binding to several apparently distinct binding sites that regulate Ca²⁺ activation, IP₃-dependent Ca²⁺ inhibition, IP₃-independent Ca²⁺ inhibition, channel inactivation, and channel recruitment. In addition, a separate Ca²⁺-binding site appears to regulate the properties of the IP₃-independent Ca²⁺ inhibition site. Ca²⁺ regulation of the channel is impinged on by other channel regulators, including IP₃ and ATP, as allosteric modulators. Furthermore, Ca²⁺ can impinge on the activity of the channel indirectly, by binding to other proteins that interact with the channel (Foskett, White, Cheung, & Mak, 2007; and Section III).

III. CYTOPLASMIC Ca²⁺ REGULATION OF IP₃R CHANNEL GATING

A. Insights from Steady-State Measurements

1. Biphasic Regulation of IP₃R Channel Gating by Ca²⁺—The most widely studied aspect of IP₃R channel activity regulation is that by Ca²⁺. Patch-clamp experiments on outer membranes of isolated nuclei or whole cell recordings of plasma membrane-localized IP₃R isoforms from different species (*Xenopus* type 1, rat types 1 and 3, and IP₃R from insect *Spodoptera*; Boehning, Joseph, Mak, & Foskett, 2001; Gin, Falcke, Wagner, Yule, & Sneyd, 2009; Ionescu et al., 2006; Mak, McBride, & Foskett, 1998, 2001b; Wagner, Joseph, & Yule, 2008) have revealed that in the presence of saturating concentrations of IP₃, IP₃R channels have very low activity in resting cytoplasmic Ca²⁺ concentrations ($[Ca^{2+}]_i \sim 50$ nM), but that as Ca²⁺ is raised through the sub-micromolar range ($< 1 \mu M$), IP₃R channel activity increases to a maximum level with $P_o \sim 0.8$ (Fig. 1). In nuclear patch-clamp recordings, P_o remains at the maximal level over a wide range of $[Ca^{2+}]_i$ before further increases begin to inhibit it, at $[Ca^{2+}]_i > 10 \mu M$. Thus, the Ca²⁺ dependence of channel activity for these IP₃R channels is biphasic, with a broad, plateau-shaped P_o versus $[Ca^{2+}]_i$ curve (Fig. 2).

Although the general shapes of the channel P_o versus $[Ca^{2+}]_i$ curves in nuclear patch-clamp studies are similar, the Ca^{2+} dependencies have minor differences among different IP₃R isoforms, which may be physiologically important. Ca^{2+} activation of type 1 IP₃R channels is positively cooperative, enabling P_o to increase sharply and reach the maximum value within a narrow range of $[Ca^{2+}]_i$. Such a behavior is ideal for Ca^{2+} -induced Ca^{2+} release (CICR). In contrast, P_o of the type 3 IP₃R channel increases over a broader range of $[Ca^{2+}]_i$ and with a higher Ca^{2+} affinity. Such a behavior is ideal as a trigger that responds to low IP₃ concentrations. Ca^{2+} inhibition of the vertebrate IP₃R channels in these studies is highly cooperative, so channel P_o decreases rapidly over a narrow range of $[Ca^{2+}]_i$, whereas Ca^{2+} inhibition of the insect Sf9 cell channel exhibits no cooperativity, and P_o decreases gently over a broader range of $[Ca^{2+}]_i$. The physiological relevance of these differences is not clear.

In some other single-channel studies, different $[Ca^{2+}]_i$ dependencies have been observed. In some (Marchenko, Yarotsky, Kovalenko, Kostyuk, & Thomas, 2005; Ramos-Franco, Caenepeel, Fill, & Mignery, 1998; Ramos-Franco, Fill, & Mignery, 1998; Striggow & Ehrlich, 1996; Tu et al., 2002, 2003; Tu, Wang, & Bezprozvanny, 2005), IP₃R channels in saturating IP₃ were very sensitive to Ca^{2+} inhibition, such that their biphasic P_o versus $[Ca^{2+}]_i$ curves are narrow and bell-shaped. In others, high Ca^{2+} inhibition was significantly reduced or completely absent (Hagar, Burgstahler, Nathanson, & Ehrlich, 1998; Mak, McBride, Petrenko, & Foskett, 2003; Ramos-Franco et al., 2000; Ramos-Franco, Fill, et al., 1998), so that the channel remained active in physiologically attainable supra-micromolar $[Ca^{2+}]_i$ ($> 500 \mu M$).

2. Use of Biphasic Hill Equation to Describe Ca^{2+} Regulation of IP₃R Channel Activity—A useful way to quantify Ca^{2+} regulation of IP₃R channel activity is to fit the P_o versus $[Ca^{2+}]_i$ data with an empirical, model-independent Hill equation. The biphasic equation

$$P_o = P_{Hill} \left\{ 1 + \left(\frac{K_{act}}{[Ca^{2+}]_i} \right)^{H_{act}} \right\}^{-1} \left\{ 1 + \left(\frac{[Ca^{2+}]_i}{K_{inh}} \right)^{H_{inh}} \right\}^{-1} \quad (1)$$

has been used to describe the P_o of channels that are both activated and inhibited by Ca^{2+} (Foskett et al., 2007). The Ca^{2+} dependencies of the channel can be characterized in terms of five Hill equation parameters: P_{Hill} , K_{act} , H_{act} , K_{inh} , H_{inh} . For IP₃R channels that have very different sensitivities to Ca^{2+} activation and inhibition, with a plateau-shaped P_o versus $[Ca^{2+}]_i$ curve in saturating IP₃ (Ionescu et al., 2006; Mak et al., 1998, 2001b), the data can be fitted by the Hill equation with a unique set of parameters, with each parameter describing one aspect of the Ca^{2+} dependence. P_{Hill} is the maximum P_o that the channel could attain if there was no Ca^{2+} inhibition. Because of the low sensitivity of the channel to Ca^{2+} inhibition in saturating IP₃, the channel is fully activated before Ca^{2+} starts to inhibit it. Thus, the observed maximum open probability P_{max} under optimal ligand conditions is equal to P_{Hill} . The parameters K_{act} and K_{inh} are then EC_{50} and IC_{50} for Ca^{2+} , that is, the $[Ca^{2+}]_i$ at which $P_o = 0.5P_{Hill}$. They are inversely related to the functional sensitivity of the

channel to Ca^{2+} activation and inhibition, respectively. H_{act} and H_{inh} describe the level of cooperativity of Ca^{2+} activation and inhibition, respectively.

IP_3R channels that have similar sensitivities to Ca^{2+} activation and inhibition display narrow bell-shaped P_o versus $[\text{Ca}^{2+}]_i$ curves, because the channel is not yet fully activated by Ca^{2+} when Ca^{2+} inhibition starts to reduce P_o . In such cases, the set of Hill equation parameters that provide a good fit to the data is not unique. Accordingly, no physical significance can be assigned to one particular set of Hill equation parameters, and conclusions drawn from them are questionable (Foskett et al., 2007).

Since IP_3 regulates IP_3R channels by modifying their sensitivity to Ca^{2+} inhibition (see later discussion), channels with broad, plateau-shaped P_o versus $[\text{Ca}^{2+}]_i$ curves at saturating $[\text{IP}_3]$ exhibit narrow bell-shaped P_o versus $[\text{Ca}^{2+}]_i$ curves at subsaturating $[\text{IP}_3]$ (see Fig. 2A with 10 or 20 nM IP_3), with P_{max} significantly less than observed in saturating $[\text{IP}_3]$. However, P_o at subsaturating $[\text{IP}_3]$ can still be well fitted by the same biphasic Hill equation (1) assuming that only K_{inh} was affected by $[\text{IP}_3]$ (Mak et al., 1998, 2001b).

Hill equations are empirical, and not based on any specific model for ligand regulation of channel gating. The equations enable the gating behaviors of the IP_3R to be characterized with a small number of parameters, provided that the Ca^{2+} dependence curve has a plateau shape with a well-defined P_{Hill} value. However, the empirical fit does not provide insights into the molecular mechanism(s) responsible for ligand regulation. For instance, the parameters H_{act} and H_{inh} do not have a direct model-independent relation with the number of activating or inhibitory Ca^{2+} -binding sites in the channel.

3. Ca^{2+} Activation of IP_3R Channels— Ca^{2+} activation of channel activity has been observed with comparable functional sensitivity to Ca^{2+} in most electrophysiological studies of the IP_3R , suggesting that the characteristics of Ca^{2+} activation are likely determined by intrinsic features of the IP_3R molecule that are conserved throughout evolution and among isoforms. This conservation probably exists because activation of IP_3R by a rise of Ca^{2+} from resting levels (~ 50 nM) to hundreds of nanomolar plays a key role in intracellular Ca^{2+} signaling. In the presence of activating levels of IP_3 , elevations of $[\text{Ca}^{2+}]_i$ due to Ca^{2+} released by one IP_3R channel can in turn activate IP_3R channels nearby to release more Ca^{2+} in a positive feedback. CICR mediated by IP_3R channels acts as a critical communication mechanism between channels to coordinate their activities, generating large-scale Ca^{2+} signals (puffs and waves) from elementary Ca^{2+} release events emanating from individual IP_3R channels (Berridge, 1997).

Ca^{2+} activation of IP_3R channels is mostly positively cooperative (Fig. 2), enabling them to be activated sharply by Ca^{2+} within a narrow range. This property likely provides fine-tuning of the channel activity. Nevertheless, noncooperative Ca^{2+} activation has also been observed (Hagar et al., 1998; Mak et al., 2001b; Ramos-Franco, Fill, et al., 1998), suggesting that cooperativity is not an essential feature, but may rather provide diversity in Ca^{2+} signals generated by different isoforms and under different conditions. For example, ATP regulation of the Ca^{2+} activation properties of the type 3 channel was associated with changes in the degree of cooperativity (Mak et al., 2001b).

IP₃R isoforms with higher sensitivity to Ca²⁺ activation (especially type 3 IP₃R; Fig. 2B) have higher P_o at resting [Ca²⁺]_i when IP₃ is present, than do channels formed by other isoforms. Thus, when an extracellular stimulus elicits production of IP₃ in a resting cell, such channels will have a higher probability of opening to release Ca²⁺ from the ER. They can therefore act as triggers to initiate IP₃-induced Ca²⁺ release (IICR; Mak et al., 2001b). Ca²⁺ released by these triggers may then raise local [Ca²⁺]_i sufficiently to cause nearby IP₃R channels to release Ca²⁺ through CICR, thus propagating the Ca²⁺ signals.

4. Ca²⁺ Inhibition of IP₃R Channels—Whereas Ca²⁺ activation of IP₃R is consistently observed in all single-channel experiments with comparable functional sensitivity to Ca²⁺, the reported sensitivities of IP₃R channels to Ca²⁺ inhibition are highly variable even in saturating IP₃, so that a wide range of different shapes of channel P_o versus [Ca²⁺]_i curves has been reported (Foskett et al., 2007).

An understanding of the Ca²⁺ inhibition properties of the channels is important. Ca²⁺ inhibition can possibly serve as a negative feedback mechanism to either terminate or prevent Ca²⁺ release as the local [Ca²⁺]_i is raised by IP₃R-mediated Ca²⁺ release, even in the continuous presence of IP₃. This process may play a significant role in the generation of Ca²⁺ spikes and oscillations, as well as the generation of highly localized Ca²⁺ signals, by preventing runaway Ca²⁺ release due to the positive feedback of CICR. Furthermore, the range of [Ca²⁺]_i over which inhibition is exerted is also important, because it defines the spatial domain over which the channels may experience such regulation. For example, if the apparent affinity of the inhibitory Ca²⁺-binding site is 20 μM, only channels quite close to an open Ca²⁺ channel will experience feedback inhibition. On the other hand, if the apparent affinity of the inhibitory Ca²⁺-binding site is in the sub-micromolar range, then channels throughout the cytoplasm, even those that are quite far from individual release sites, will experience inhibitory [Ca²⁺]_i.

The diversity of properties of Ca²⁺ inhibition of IP₃R channels observed in different studies may reflect a range of physiologically relevant modifications of Ca²⁺ inhibition by environmental factors that provide additional regulation of IP₃R-mediated Ca²⁺ signals. Alternately, such diversity may reflect a lack of control of important experimental variables during preparation or recording of the channels. There is no clear correlation between the sensitivity of an IP₃R channel to Ca²⁺ inhibition and the molecular structure of the IP₃R. Nuclear patch-clamp studies revealed very similar Ca²⁺ inhibition characteristics (low Ca²⁺ sensitivity and high level of cooperativity) for homotetrameric channels formed by types 1 or 3 IP₃R isoforms (Mak et al., 1998, 2001b; Fig 2A and B). On the other hand, whereas similar Ca²⁺ inhibition characteristics for different splice variants of the same isoform of IP₃R (Ramos-Franco, Caenepeel, et al., 1998; Tu et al., 2002) or different isoforms (Tu, Wang, & Bezprozvanny, 2005) have been observed in some reconstitution studies, the same isoforms displayed very different Ca²⁺ inhibition properties in others (see Fig. 2 and cf. Mak et al., 1998; Tu, Wang, & Bezprozvanny, 2005). Ca²⁺ inhibition was absent for both type 2 (Ramos-Franco et al., 2000) and type 3 (Hagar et al., 1998) IP₃R channels in some planar bilayer reconstitution studies, but not in others (Tu, Wang, & Bezprozvanny, 2005). Furthermore, the Ca²⁺ inhibition properties are largely, but not completely, distinct between the reconstitution and nuclear patch-clamp studies. For example, similar high sensitivity to

Ca^{2+} inhibition was observed for IP_3R channels formed by the same isoform studied using nuclear patching (Marchenko et al., 2005) or bilayer reconstitutions (Tu et al., 2002).

It is likely that various forms of regulation, both physiological and non-physiological, impinge on the Ca^{2+} inhibition properties of the channel. The sensitivity of recombinant rat type 3 (IP_3R) to Ca^{2+} inhibition was altered by cytoplasmic ATP (Tu, Wang, & Bezprozvanny, 2005). Importantly, because Ca^{2+} inhibition is regulated by IP_3 , as discussed later, different IP_3 sensitivities of channels in different studies may contribute to different Ca^{2+} inhibition properties. Different IP_3 sensitivities could in turn be generated by extrinsic factors such as membrane lipid composition (Cannon et al., 2003), covalent modifications of the channel (e.g., phosphorylation, redox), presence or absence of interacting proteins (Foskett et al., 2007), or to intrinsic differences in channel properties between species and/or isoforms. Even a simple non-physiological maneuver can radically alter the channel's Ca^{2+} inhibition properties. Exposure of IP_3R channels to a very low $[\text{Ca}^{2+}]_i$ (<10 nM) for a few minutes before they are exposed to IP_3 completely and reversibly relieves high $[\text{Ca}^{2+}]_i$ inhibition of types 1 and 3 IP_3R channels (Mak, McBride, Petrenko, et al., 2003; Fig. 2D). Thus, the Ca^{2+} inhibition properties of the channel are regulated by a distinct Ca^{2+} -binding site(s), which could be a locus for either regulation or disruption of Ca^{2+} inhibition. It is possible that this site was disrupted during protocols involved in channel reconstitution in those studies of the types 2 (Ramos-Franco et al., 2000) and 3 (Hagar et al., 1998) channel isoforms that failed to observe high- $[\text{Ca}^{2+}]_i$ inhibition.

One possible mechanism of regulating Ca^{2+} inhibition of IP_3R channels that has been repeatedly invoked is through interaction of the channel with calmodulin. However, single-channel studies of IP_3R are consistent with the absence of any regulatory role for calmodulin in Ca^{2+} inhibition of IP_3R . A fuller discussion of the calmodulin interaction with IP_3R is provided in Section III.A.

5. IP_3 Regulates IP_3R Channel Activity Through Modulation of its Sensitivity to Ca^{2+} Inhibition—Spontaneous IP_3R activity with very low P_o (approximately a few percentage) can be observed in the absence of IP_3 (Mak, McBride, & Foskett, 2003; Ramos-Franco, Fill, et al., 1998). However, both Ca^{2+} (in appropriate concentrations) and IP_3 must be present on the cytoplasmic side of the channel to activate it appreciably. IP_3 and Ca^{2+} are not simply equivalent coagonists of the channel, since the two ligands activate the IP_3R in very different manners. Systematic studies of the *Xenopus* type 1 ($X\text{-IP}_3\text{R-1}$), r- $\text{IP}_3\text{R-3}$, and Sf9 IP_3R channels under a broad range of $[\text{IP}_3]$ and $[\text{Ca}^{2+}]_i$ in steady-state conditions revealed that the channel becomes more sensitive to inhibition by high $[\text{Ca}^{2+}]_i$ in the presence of subsaturating $[\text{IP}_3]$. That is, at lower $[\text{IP}_3]$, the channel is inhibited by lower $[\text{Ca}^{2+}]_i$ (Fig. 2A–C). In contrast, the sensitivity to Ca^{2+} activation, and the cooperativity of Ca^{2+} activation and inhibition are not significantly affected by IP_3 . At very low $[\text{IP}_3]$, the maximum channel P_o observed and the range of $[\text{Ca}^{2+}]_i$ over which the channel is active are both reduced (Fig. 2A–C). Both effects can be fully accounted for by an increase in the sensitivity of the channel to Ca^{2+} inhibition. At very low $[\text{IP}_3]$, the channel is so sensitive to Ca^{2+} inhibition that it begins to be inhibited before it is fully activated. This pattern of IP_3 regulation of channel activity by solely modulating the sensitivity of the channel to inhibition by Ca^{2+} is consistently observed for IP_3R channels of different isoforms (types 1

and 3) from very different species (*Xenopus*, rat, and insect; Ionescu et al., 2006; Mak et al., 1998, 2001b), suggesting that it is a common mechanism underlying ligand regulation of all IP₃R (although see Wagner et al., 2008).

Because IP₃ affects only the sensitivity to Ca²⁺ inhibition, the IP₃ sensitivity of the channel can be defined by the changes it causes in this parameter. A half-maximal effect was observed at ~50 nM IP₃ for both *X*-IP₃R-1 and r-IP₃R-3 (Fig. 2A and B). The response of these channels is fully saturated by 100 nM IP₃ (Mak et al., 1998, 2001b). In general, this functional sensitivity to IP₃ is in reasonable agreement with dissociation constants K_d derived from IP₃ binding assays, and EC₅₀s for IP₃ stimulation of Ca²⁺ release (Kaznatcheyeva, Lupu, & Bezprozvanny, 1998; Mauger, Liévremon, Piétri-Rouxel, Hilly, & Coquil, 1994; Meyer, Holowka, & Stryer, 1988; Miyakawa et al., 2001; Ramos-Franco et al., 2000; Taylor & Richardson, 1991). In contrast, the Sf9 IP₃R has lower sensitivity to IP₃, with half-maximal effect at about 400 nM, and its response is not saturated until IP₃ ≈ 1 μM (Fig. 2C; Ionescu et al., 2006). Canine cerebellar IP₃R-1 apparently has a very low sensitivity to IP₃ such that the P_o versus [Ca²⁺]_i curve of the channel was still substantially changed when IP₃ was increased from 2 to 180 μM (Kaftan, Ehrlich, & Watras, 1997). This significantly lower IP₃ sensitivity agrees with the low IP₃ sensitivity of IP₃R-mediated Ca²⁺ release observed in cerebellar neurons (Khodakhah & Ogden, 1993, 1995), and may be due to the differences in IP₃R splice variants: IP₃R examined in Mak et al. (1998) was peripheral SII— form, whereas in Kaftan et al. (1997), the cerebellar SII+ form was examined, or due to the interference of IP₃R channel gating by phosphatidylinositol 4,5-bisphosphate bound to the cerebellar IP₃R (Lupu, Kaznatcheyeva, Krishna, Falck, & Bezprozvanny, 1998).

a. Physiological Significance of IP₃ Modulation of Ca²⁺ Inhibition of IP₃R

Channel.: The modulation of IP₃R channel K_{inh} by IP₃ provides a possible mechanism intrinsic to the channel to generate graded Ca²⁺ release in response to different levels of extracellular stimulus intensity, instead of an all-or-nothing signal expected for the process of CICR (Berridge, 1997; Bootman & Berridge, 1995). Because IP₃R exposed to higher [IP₃] has lower susceptibility to Ca²⁺ inhibition, [Ca²⁺]_i that can inhibit channel activity at low [IP₃] will be insufficient to inhibit it when [IP₃] is increased. Higher [Ca²⁺]_i at the vicinity of an open channel can therefore be achieved before reaching levels that will inhibit other closed channels in the same channel cluster before they can open (Berridge, 1997). This enables coordinated release of channels in the same local cluster, whereas in lower [IP₃], Ca²⁺ released by the stochastic opening of one channel suppresses gating of the rest of the channels in the cluster. By enabling higher [Ca²⁺]_i to be achieved at the local level by coordinated Ca²⁺ release, higher [IP₃] associated with more intense stimuli would promote greater diffusive spread of the local Ca²⁺ signal to other IP₃R channel clusters, thereby transforming highly localized signals at low levels of stimulation to more global coordinated Ca²⁺ release signals as the intensity of the stimulus is increased.

In addition, modulation by IP₃ of Ca²⁺ inhibition enables channel activity to be exquisitely sensitive to small changes in [IP₃] within the subsaturating range. For *X*-IP₃R-1 in 10 nM IP₃, the channel is appreciably active only within a very narrow range of [Ca²⁺]_i (50–300 nM) with very low P_o ($P_{max} \sim 0.1$), whereas with IP₃ raised only to 100 nM, the channel is

active over a very wide range of $[Ca^{2+}]_i$ (50 nM to 20 μ M; Fig. 2A). Thus, the IP_3 -mediated Ca^{2+} signal can be finely controlled by small differences in the $[IP_3]$.

6. IP_3 and Ca^{2+} Regulate IP_3 R Channel Activities Through Multiple Ca^{2+} Sensors

—The most comprehensive studies of ligand regulation of IP_3 R channel activity indicate that IP_3 regulates gating mainly by modulating its sensitivity to Ca^{2+} inhibition (Ionescu et al., 2006; Kaftan et al., 1997; Mak et al., 1998, 2001b). However, IP_3 R channels that lack inhibition by Ca^{2+} (Ramos-Franco, Fill, et al., 1998), or with Ca^{2+} inhibition abolished by preexposure to very low levels of Ca^{2+} (Mak, McBride, & Petrenko et al., 2003), nevertheless still require IP_3 for activity (Fig. 2D). In these channels, increases in $[IP_3]$ increase the observed P_{max} , with little effect on the properties of Ca^{2+} activation. This suggests that there are at least two distinct kinds of Ca^{2+} sensors responsible for ligand regulation of IP_3 R channel P_0 . One kind of Ca^{2+} sensor is responsible for Ca^{2+} inhibition in saturating $[IP_3]$. This sensor has an apparent Ca^{2+} affinity of ~20–50 μ M in most nuclear patch-clamp studies (Boehning et al., 2001; Ionescu et al., 2006; Mak et al., 1998, 2001b) and accounts for the descending phase of the P_0 versus $[Ca^{2+}]_i$ curve in saturating $[IP_3]$. This Ca^{2+} -binding site is nonfunctional in the IP_3 R channels that are not inhibited by high Ca^{2+} . The second kind of Ca^{2+} sensor is IP_3 -dependent. It is responsible for the IP_3 sensitivity of the channel regardless of the functionality of the other inhibitory Ca^{2+} sensor. In the absence of IP_3 , this site has very high Ca^{2+} affinity and inhibits channel opening. Thus, channels that lack high Ca^{2+} inhibition still require IP_3 for activation. In addition, comparisons of the sensitivities and levels of cooperativity for Ca^{2+} and IP_3 activation of different IP_3 R isoform channels argues for the existence of a third Ca^{2+} sensor that is activating yet IP_3 -independent. The following describes the properties of each of these sensors separately.

a. The IP_3 -Independent Inhibitory Ca^{2+} Sensor. As noted earlier, single-channel studies of IP_3 R have revealed diverse properties for Ca^{2+} inhibition of the channel, with very different sensitivities observed even for the same IP_3 R isoform and splice variant in different studies. As a result, the P_0 versus $[Ca^{2+}]_i$ curves observed in some studies are narrow and bell-shaped, whereas in others, they are broad and plateau-shaped. Ca^{2+} inhibition appeared to be totally absent in some studies. It has also been demonstrated that this inhibitory Ca^{2+} sensor can be reversibly rendered completely nonfunctional, thus abolishing high Ca^{2+} inhibition of the channel, by treating the channel with nanomolar $[Ca^{2+}]_i$ for a few minutes before exposure of the channel to IP_3 (Mak, McBride, & Petrenko et al., 2003). Channels that are not sensitive to Ca^{2+} inhibition are nevertheless fully IP_3 -dependent (Ramos-Franco et al., 2000; Mak, McBride, & Petrenko et al., 2003; Fig. 2D). Together, these observations indicate that this inhibitory Ca^{2+} sensor is not responsible for IP_3 dependence of the channel and is therefore likely IP_3 -independent. Furthermore, the functional affinity for Ca^{2+} for this IP_3 -independent inhibitory Ca^{2+} sensor is malleable, suggesting that it could possibly be under physiological regulation. Besides establishing the malleability of the inhibitory Ca^{2+} sensor, abolition of Ca^{2+} inhibition by preexposure of the channel to nanomolar $[Ca^{2+}]_i$ further indicates that its functionality is controlled by another, different type of Ca^{2+} -binding site(s) (Mak, McBride, & Petrenko et al., 2003).

b. The IP₃-Dependent Ca²⁺ Sensor.: Another type of Ca²⁺ sensor is regulated by IP₃ binding to the channel. This IP₃-dependent Ca²⁺ sensor is extremely sensitive to small changes in [IP₃] within the subsaturating range. For example, the sensitivity of X-IP₃R-1 channels to Ca²⁺ inhibition was reduced dramatically as [IP₃] was raised from 10 to 100 nM. The channel was significantly inhibited by 160 nM Ca²⁺ in 10 nM IP₃, but it was not inhibited until cytoplasmic [Ca²⁺]_i was > 30 μM in 100 nM IP₃ (Fig. 2A). To reconcile this exquisite sensitivity of the channel to subsaturating levels of IP₃ with the tetrameric structure of the channel consisting of four IP₃R molecules each with a single IP₃-binding site, an allosteric model was proposed in which the IP₃-dependent Ca²⁺ sensors in the channel (one per IP₃R molecule, total of four in each channel) act as inhibitory Ca²⁺-binding sites to inhibit channel gating when bound to Ca²⁺ in the absence of IP₃ (Mak, McBride, & Foskett, 2003). However, as IP₃ concentration is raised and IP₃ binds to the channel, Ca²⁺ binding to the IP₃-dependent Ca²⁺ sensors starts to favor opening of the channel. In effect, this Ca²⁺ sensor becomes an activating Ca²⁺-binding site. Thus, IP₃ regulates IP₃ R channel activity with very high effectiveness by modifying not only the functional affinity of the IP₃-dependent Ca²⁺ sensors, but also their functional nature, changing them from inhibitory to activating sites.

The interplay of the two different types of Ca²⁺ sensors, one IP₃-sensitive, the other IP₃-insensitive, enables the response of IP₃R channel to IP₃ to saturate very abruptly despite its high sensitivity to subsaturating [IP₃]. Once IP₃ exceeds the saturating level of 100 nM, the *P*_o versus [Ca²⁺]_i curve of the X-IP₃R-1 exhibits no change even as IP₃ is further increased by over 3 orders of magnitude (Fig. 2A). This behavior results from the influence of the inhibitory Ca²⁺ sensor(s) that is IP₃-independent. Thus, at IP₃ > 100 nM, Ca²⁺ inhibition of the channel is caused by the IP₃-independent, purely inhibitory Ca²⁺ sensor. The abruptness in the saturation of the response of the channel to changes in IP₃ concentration is due to the insensitivity to IP₃ of these inhibitory Ca²⁺ sensors.

c. The IP₃-Independent Activating Ca²⁺ Sensor.: In nuclear patch-clamp experiments, X-IP₃R-1 and r-IP₃R-3 channels exhibited similar sensitivities to activation by IP₃, even though the sensitivity and degree of cooperativity for Ca²⁺ activation of the two types of channels were very different (Fig. 2A and B; Mak et al., 1998, 2001b). On the other hand, the sensitivity and level of cooperativity for IP₃ activation of X-IP₃R-1 and Sf9 IP₃R channels are very different, even though the two types of channels have similar sensitivities and levels of cooperativity for Ca²⁺ activation. To quantitatively account for these different characteristics of Ca²⁺ and IP₃ activation, a third type of Ca²⁺ sensor must also play a role. This Ca²⁺ site is IP₃-independent and responsible for the consistent sensitivity to Ca²⁺ activation observed in various IP₃R channels despite their differences in IP₃ sensitivity or the presence or absence of Ca²⁺ inhibition.

7. A Model to Account for Multiple Ca²⁺ Sensor Regulation of IP₃R Steady-State Channel Gating—Numerical calculations (Mak, McBride, & Foskett, 2003) indicate that an allosteric model postulating the three types of Ca²⁺ sensors described above can account for all single-channel behaviors of various IP₃R channels studied by nuclear patch-clamp experiments (Foskett & Mak, 2004; Ionescu et al., 2006; Mak, McBride, &

Foskett, 2003). In the simplest allosteric model that fits all observations in *Xenopus* oocyte nuclear patch-clamp studies of ligand regulation of steady-state channel gating behavior of *Xenopus* type 1 and rat type 3 IP₃R isoforms, the tetrameric channel can adopt six different conformations, the equilibria among which are controlled by one IP₃-binding site and three different functional Ca²⁺-binding sites in each IP₃R monomer that directly affect the equilibria among active and closed conformations of the channel in a manner similar to the Monod-Wyman-Changeux (MWC) model (Fig. 3). One of the Ca²⁺-binding sites is activating (so-called “F” site), whereas another is inhibitory (so-called “H” site), but both are independent of IP₃. In contrast, the third Ca²⁺-binding site (so-called “G” site) is affected by IP₃, being inhibitory in the absence of IP₃ but becoming activating as [IP₃] increases. Thus, two different Ca²⁺-binding sites in each IP₃R monomer, the G and H sites, contribute to Ca²⁺ inhibition. Ca²⁺ binding to the IP₃-dependent G site inhibits IP₃R activity in the absence of IP₃, which is responsible for the lack of channel activity in the absence of IP₃ in normal physiological [Ca²⁺]_i. IP₃ binding to the channel changes the effective affinities of the G site, and in so doing transforms it into an activating site. This IP₃-mediated transformation of the nature of this Ca²⁺-binding site is responsible for all the IP₃ dependence of the channel, accounting for the extremely high sensitivity of channel gating to small changes in [IP₃]. The other Ca²⁺-binding H site is strictly inhibitory with a lower Ca²⁺ affinity (10–30 μM) that is not modulated by IP₃ binding. The IP₃-independence of this site is responsible for the lack of further effect of IP₃ on types 1 and 3 channels in *Xenopus* oocytes once [IP₃] > 1 μM, accounting for the observation that the effects of IP₃ abruptly saturate around this concentration. Furthermore, Ca²⁺ binding to the H site is also partly responsible for the observed inhibition of the channels even at lower [Ca²⁺]_i (< 10–30 μM), when [IP₃] is less than 1 μM. Whereas the properties of the H sites are insensitive to IP₃ 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 IP₃ because the other Ca²⁺-binding sites, specifically the IP₃-dependent G site, are not affected by the low bath [Ca²⁺]. Ca²⁺ binding to the G site inhibits IP₃R activity in the absence of IP₃.

8. Ligand-Dependent, IP₃-Induced IP₃R Channel Inactivation—An unusual aspect of IP₃R-mediated intracellular signaling is the phenomenon of “quantal release,” defined (Meyer & Stryer, 1990; Muallem, Pandol, & Beeker, 1989) as the ability of cells to have graded release of Ca²⁺ from intracellular stores in response to incremental levels of extracellular agonist or IP₃ (reviewed in Bootman, 1994; Missiaen, Parys, De Smedt, Oike, & Casteels, 1994; Parys, Missiaen, Smedt, Sienaert, & Casteels, 1996; Taylor, 1998). This entails two processes: (i) an initial Ca²⁺ release whose rate is proportional to IP₃ concentration followed by (ii) a substantial reduction in rate or termination of Ca²⁺ release despite the presence of constant IP₃. Consequently, sustained exposure to submaximal levels of agonists, even over extensive periods, only mobilizes a fraction of total releasable Ca²⁺ in a cell. This is surprising because, with the steady-state ligand regulation of IP₃ R channel *P*₀ discussed so far, it might have been expected that all IP₃R channels should become activated in response to sufficient agonist stimulation, releasing all of the IP₃-sensitive Ca²⁺ stores, albeit at different rates depending on the agonist concentration.

In nuclear patch-clamp studies, abrupt termination of IP₃R channel activities despite the constant presence of agonist has been observed for all IP₃R investigated (Boehning et al., 2001; Ionescu et al., 2006; Mak & Foskett, 1994, 1997; Mak, McBride, & Foskett, 2001a; Mak et al., 2000). The mean duration of channel activity observed from its initial activation by IP₃ until the termination of activity in the presence of constant IP₃ (T_a) for vertebrate IP₃R is typically ~30s (Mak & Foskett, 1997; Mak et al., 2000). A $[Ca^{2+}]_i$ dependence of T_a was qualitatively described for recombinant r-IP₃R-1 expressed in COS cell outer nuclear membrane (Boehning et al., 2001), but it was impossible, due to technical difficulties, to rule out the possibility that such abrupt termination of channel activity was a nonphysiological artifact associated with patching, for example, collisions of the channels with the walls of the glass pipette. In a nuclear patch-clamp study of the Sf9 IP₃R channel, it was demonstrated that T_a was dependent on the concentrations of both ligands (Ionescu et al., 2006). In optimal ligand conditions, T_a was ~120 s, substantially longer than the vertebrate channels. In optimal [IP₃], T_a was reduced in $[Ca^{2+}]_i > 1 \mu M$, with reduction by over 10-fold at 89 μM Ca²⁺. In subsaturating IP₃, T_a already began to decrease in $[Ca^{2+}]_i \sim 300$ nM, substantially lower than that observed in saturating IP₃. The IP₃-induced termination of IP₃R channel activity was fully reversible upon ligand removal (Ionescu et al., 2006). These results suggest that the observed inevitable termination of channel activity is not an experimental artifact, and may be due to the entry of IP₃-liganded channels into a true inactivated state, driven by binding of Ca²⁺ to the channel at a relatively slow rate (Ionescu et al., 2006).

The inactivation kinetics observed in nuclear patch-clamp experiments: $T_a \sim 10$ – 100 s in Sf9 IP₃R (Ionescu et al., 2006) and ~20–30 s in vertebrate IP₃R (Boehning et al., 2001; Mak & Foskett, 1997; Mak et al., 2000) are comparable to those estimated by ER permeability measurements in permeabilized hepatocytes (Hajnóczky & Thomas, 1994), as well as to the kinetics of IP₃-induced increases in IP₃ affinity of an apparently desensitized IP₃R in cerebellar microsomes (Coquil, Mauger, & Claret, 1996; half-life of channel activity ~15–45 s) and the kinetics of the transient fast phase of Ca²⁺ release in response to initial exposure to IP₃ in permeabilized and intact cells (see Meyer & Stryer, 1990; Muallem et al., 1989; Taylor & Potter, 1990). Of note, IP₃R inactivation observed in permeabilized hepatocytes (Hajnóczky & Thomas, 1994), which had kinetics similar to those observed in the single-channel studies, was shown to account for release termination associated with $[Ca^{2+}]_i$ oscillations (Hajnóczky & Thomas, 1997). These results suggest that the kinetics of inactivation observed in single-channel studies are of physiological relevance for $[Ca^{2+}]_i$ signaling in cells. However, it remains unclear whether distinct inactivation kinetics observed in different studies reflects methodological differences, distinct types of inactivation or inhibition, or a physiologically relevant range of kinetics of a common inactivation mechanism.

9. Ligand-Dependent IP₃R Channel Recruitment—In addition to termination of Ca²⁺ release in the presence of constant IP₃, quantal Ca²⁺ release requires that the initial rate of Ca²⁺ release from intracellular stores be proportional to IP₃ concentration. One mechanism to achieve this is by IP₃-tuning of Ca²⁺ inhibition of channel activity, as

discussed earlier. Additional mechanisms were discovered to also play a role in recruiting channels into activity as a function of ligand stimulation.

A consistent rate of detection of IP₃R channel activity in nuclear patch-clamp experiments using isolated Sf9 nuclei enabled quantification of the average number of IP₃R channels detected in a nuclear patch-clamp experiment (N_A) under various ligand concentrations (Ionescu et al., 2006). In saturating IP₃, the number of active IP₃R in each patch was a biphasic function of $[Ca^{2+}]_i$. The observation that N_A was a function of stimulus strength is unexpected since it was anticipated that the entire channel population in a membrane patch would always become activated, albeit to different levels of activity (P_o) depending on the strength of ligand activation. Instead, these results indicate that suboptimal ligand concentrations are insufficient to activate all available IP₃R channels in a membrane patch that can be activated by optimal ligand concentrations. To account for these observations, a model was proposed in which Ca^{2+} binding at a very fast rate to a “sequestration” site, before these channel could actively gate open and be observed, sequestered some of the available IP₃R channels into a nonactive state when ligand conditions were suboptimal (Ionescu et al., 2006).

Thus, two mechanisms revealed by single-channel patch-clamp electro-physiology exist to grade Ca^{2+} release through a population of IP₃R channels: regulation of channel activity level (P_o) as well as recruitment of additional channels. Both mechanisms coexist and can occur even in the absence of crosstalk among channels by released Ca^{2+} (CICR). The importance of this channel recruitment mechanism can be appreciated by considering that the rate of Ca^{2+} flux through a population of IP₃R channels (J) is given by

$$J = \gamma NP_o, \quad (2)$$

where γ is the single-channel conductance, N is the number of activated channels, and P_o is the average single-channel open probability. Single-channel studies determined that the IP₃R channel conductance γ is largely IP₃-independent. Since both channel P_o and N_A are regulated by Ca^{2+} and IP₃, the ligand dependence of Ca^{2+} flux released through IP₃R channels is more accurately estimated in terms of N_AP_o . In saturating [IP₃], the dependence of N_AP_o on $[Ca^{2+}]_i$ is biphasic: N_AP_o increases by over 10-fold as Ca^{2+} is increased from 50 to 500 nM and then gradually decreases as $[Ca^{2+}]_i$ is further increased. N_AP_o is also strongly dependent on IP₃ concentration. With IP₃ reduced to 33 nM, the dependence of N_AP_o on $[Ca^{2+}]_i$ remains biphasic with peak N_AP_o observed in $Ca^{2+} \approx 0.5\text{--}1 \mu\text{M}$, but maximum N_AP_o is an order of magnitude lower than that observed in saturating IP₃ (Ionescu et al., 2006).

B. Insights from Kinetic Responses to Rapid Changes in Ligand Concentrations

Recently, it became possible, with the development of the cytoplasmic side-out configuration in nuclear patch-clamp studies, to obtain kinetic information concerning single IP₃R channel responses to ligand concentration changes with millisecond resolution (Mak et al., 2007).

1. Ca^{2+} Activation and Deactivation—The kinetics of Ca^{2+} regulation were first studied by applying rapid $[\text{Ca}^{2+}]_i$ jumps in the presence of constant, saturating ($10\ \mu\text{M}$) IP_3 . Channel activation by a jump from low ($< 10\ \mu\text{M}$) to optimal ($2\ \mu\text{M}$) $[\text{Ca}^{2+}]_i$ had mean latency of $\sim 40\ \text{ms}$ (Fig. 4C), an order of magnitude faster than the response to IP_3 activation ($350\text{--}500\ \text{ms}$; Fig. 4A). The latency for a $[\text{Ca}^{2+}]_i$ jump to $300\ \mu\text{M}$ was shorter but detectable ($\sim 24\ \text{ms}$; Fig. 4I), indicating that Ca^{2+} binding and subsequent conformation changes both contribute to the Ca^{2+} activation latency. Channel deactivation when Ca^{2+} was returned to $< 10\ \text{nM}$ had a latency of $\sim 160\ \text{ms}$ (Fig. 4D), also significantly faster compared with channel deactivation due to removal of IP_3 ($\sim 690\ \text{ms}$; Fig. 4B). These results demonstrate that IP_3 -bound channels respond rapidly to changes in $[\text{Ca}^{2+}]_i$, a property ideally suited for CICR to couple IP_3R channels in Ca^{2+} signaling.

Of note, the activation latency in response to simultaneous jumps of $[\text{IP}_3]$ (from 0 to $10\ \mu\text{M}$) and $[\text{Ca}^{2+}]_i$ (from $< 10\ \text{nM}$ to $2\ \mu\text{M}$) was $\sim 60\ \text{ms}$ (Fig. 4E), much shorter than that for IP_3 activation in constant optimal $[\text{Ca}^{2+}]_i$ and comparable to the latency for Ca^{2+} activation in constant saturating $[\text{IP}_3]$. If IP_3 binding was required before Ca^{2+} can bind, then the activation latency for a simultaneous jump of the two ligands should be comparable to that for IP_3 in optimal $[\text{Ca}^{2+}]_i$. Thus, there is no requirement for strict sequential IP_3 and Ca^{2+} binding to activate the channel, and IP_3 and Ca^{2+} binding is not independent, since independence predicts that the activation latency for simultaneous ligand jumps should be longer than both activation latencies for $[\text{IP}_3]$ or $[\text{Ca}^{2+}]_i$ jumps individually. Instead, the kinetic data are consistent with a *negative* cooperative interference in which high-affinity Ca^{2+} binding to a channel not bound to IP_3 retards subsequent activation of the channel by IP_3 . The deactivation latency for simultaneous removal of IP_3 (from $10\ \mu\text{M}$ to 0) and Ca^{2+} (from $2\ \mu\text{M}$ to $< 10\ \text{nM}$) of $\sim 70\ \text{ms}$ (Fig. 4F) was significantly shorter than deactivation latencies for removal of either IP_3 or Ca^{2+} alone. This implies that unbinding of IP_3 and Ca^{2+} from the IP_3R is also neither sequential nor independent. These results rule out many models for ligand regulation of IP_3R activity (Fraiman & Ponce Dawson, 2004; Hirose, Kadowaki, & Iino, 1998; Marchant & Taylor, 1997; Sneyd & Falcke, 2005; Swillens, Combettes, & Champeil, 1994; Swillens, Champeil, Combettes, & Dupont, 1998; Tang, Stephenson, & Othmer, 1996).

2. A Qualitative Model to Account for Rapid Ca^{2+} Regulation of Channel Activity—The observed response latencies point to a seemingly paradoxical interaction between IP_3 and Ca^{2+} in activation of the IP_3R . Longer activation latencies in response to jumps in $[\text{IP}_3]$ in constant optimal $[\text{Ca}^{2+}]_i$ compared to the activation latencies in response to simultaneous jumps in $[\text{IP}_3]$ and $[\text{Ca}^{2+}]_i$ indicate that Ca^{2+} can bind with high affinity to the channel in the absence of IP_3 . However, unlike the high-affinity Ca^{2+} binding that activates the channel in concert with IP_3 binding established from steady-state studies (above), this Ca^{2+} binding interferes with IP_3 activation by predisposing the channel to activate through slower conformation transition pathways. On the other hand, the much more rapid deactivation of the channel in response to simultaneous drops in $[\text{IP}_3]$ and $[\text{Ca}^{2+}]_i$ compared to channel deactivation caused by drops in either ligand alone indicates that the IP_3 and the high-affinity Ca^{2+} sites have *positive* cooperative coupling in an activated channel, such that

occupation of the IP₃ or Ca²⁺ sites causes the channel to deactivate through slower conformation transition pathways, thereby stabilizing the channel in active conformation(s).

A simplified version of the quantitative model proposed to account for steady-state ligand regulation of channel activity (above; Fig. 3) (Mak, McBride, & Foskett, 2003), which assumes only basic allosteric interactions between IP₃ and Ca²⁺ that arise solely from preferential binding of the two ligands to various channel conformations, is also able to account for these kinetic responses qualitatively. In its simplest form (Fig. 5), the model hypothesizes that the channel can assume four different conformations. In the absence of IP₃, it is energetically favorable for the channel to assume two conformations, one active (A) and one inactive (B), with Ca²⁺ binding to the high-affinity sites stabilizing the inactive B conformation. Thus, in the absence of IP₃, the high-affinity Ca²⁺ sites (G sites) are actually *inhibitory*. IP₃ binding to the channel energetically stabilizes the other two conformations, also one active (C) and one inactive (D). Ca²⁺ binding to the high-affinity sites stabilizes the active C conformation relative to the inactive D conformation. Consequently, the high-affinity G sites are *activating* when the channel is IP₃-bound. Thus, because of the different relative affinities of the high-affinity Ca²⁺ sites in the four channel conformations, IP₃ binding to the channel changes the nature of the high-affinity G Ca²⁺ sites from inhibitory to activating. This transformative effect of IP₃ on the nature of the high-affinity Ca²⁺ binding G sites can account for the seemingly paradoxical channel response latencies measured here. Because Ca²⁺ binding to the high-affinity G sites in the absence of IP₃ stabilizes the inactive B conformation which has low affinity for IP₃, preexposure of the channel to 2 μ M Ca_i²⁺ slows binding of IP₃ to the channel and consequent activation kinetics compared with activation by simultaneous [Ca²⁺]_i and [IP₃] jumps. Conversely, occupation of the IP₃ and high-affinity Ca²⁺ G sites stabilizes an active channel in the active C conformation with high affinity for IP₃ and Ca²⁺. This accounts for the shorter mean deactivation latency by simultaneous removal of IP₃ and Ca²⁺_i compared with that due to removal of either ligand alone. The model hypothesizes that the channel can assume the different conformations in any ligand-binding state, with IP₃ and Ca²⁺ binding regulating channel activity by affecting the probability of the channel being in various conformations. Thus, active and inactive conformations are connected by multiple branched pathways through various ligand binding and conformation changes.

3. Channel Inhibition and Recovery Kinetics—Switching [Ca²⁺]_i from 2 μ M (optimal) to 300 μ M (inhibitory) in the presence of 10 μ M IP₃ inhibited channel gating (Fig. 4G), demonstrating that IP₃ binding does not render the low-affinity inhibitory (H) Ca²⁺ sites inaccessible for Ca²⁺ binding, disproving previous claims (Adkins & Taylor, 1999). The mean latency for Ca²⁺ inhibition (~300 ms) was substantially longer than that for Ca²⁺ activation. The mean latency for channel recovery from Ca²⁺ inhibition was ~2.4 s (Fig. 4H). This remarkably long latency defines a refractory period between successive Ca²⁺ release events mediated by the same IP₃R channel or channel cluster. A refractory period has been invoked to account for mutual annihilation of colliding intracellular Ca²⁺ waves (Lechleiter, Girard, Peralta, & Clapham, 1991) and the periodicity of Ca²⁺ oscillations (Parker & Ivorra, 1990), but its molecular basis remained unknown. The observed latency is in quantitative agreement with the mean refractory periods (~2.5 s) between two successive

spontaneous IP₃R-mediated elementary Ca²⁺ release events (puffs) observed in the presence of saturating [IP₃] (Fraiman, Pando, Dargan, Parker, & Ponce Dawson, 2006). This suggests that the latency for channel recovery from Ca²⁺ inhibition, rather than other mechanisms such as lingering high [Ca²⁺]_i in the vicinity of the release sites after a Ca²⁺ release event, is responsible for the refractory behavior of Ca²⁺ release sites observed *in vivo* (Ilyin & Parker, 1994).

4. Kinetics of a Single-Channel Ca²⁺ Release Burst—In cells, Ca²⁺ released by an individual channel raises [Ca²⁺]_i to inhibitory (~60 μM) levels in the microdomain surrounding it. The kinetic results predict that this Ca²⁺ will feed back to inhibit it and terminate release after the burst of activity. [Ca²⁺]_i jumps from low subactivating (<10 nM) directly to inhibitory (300 μM) levels in the presence of 10 μM IP₃ produced just such a burst of channel activity (Fig. 4I) as Ca²⁺ first bound to the high-affinity (F and G) to activate the channel before the low-affinity inhibitory (H) site(s) became occupied, with mean activation and inhibition latencies of channel activity bursts = ~24 and ~470 ms, respectively. The mean single-channel burst duration was ~470 ms. The time course of these channel activity bursts represents the kinetics of the fundamental Ca²⁺ release event—the basic building block from which local concerted Ca²⁺ release by clusters of IP₃R channels (puffs) and global propagating IP₃R-mediated Ca²⁺ signals (waves) are generated through CICR.

A considerable fraction (~10%) of [Ca²⁺]_i jumps from < 10 nM to 300 μM failed to generate a burst. In comparison, < 1% of [Ca²⁺]_i jumps from < 10 nM to 2 μM failed to activate gating. Thus, Ca²⁺ binding to the high-affinity activating G sites and low-affinity inhibitory H sites are not sequential, and the failure of [Ca²⁺]_i jumps (<10 nM to 300 μM) to generate an activity burst simply reflects the nonzero probability of Ca²⁺ binding to an inhibitory H site before the activating site(s) becomes occupied. Because the mean latency for recovery from Ca²⁺ inhibition is over an order of magnitude longer than the mean latency of deactivation by Ca²⁺_i removal, during most [Ca²⁺]_i drops (from 300 mM to < 10 nM), no channel activity was observed (Fig. 4J) as the channels were deactivated due to Ca²⁺ dissociation from the high-affinity activating F and G sites before they recovered from Ca²⁺ inhibition. However, in ~6% of [Ca²⁺]_i drops, the channel recovered from Ca²⁺ inhibition first, generating a brief burst of activity before it was deactivated by Ca²⁺ unbinding (Fig. 4K). This indicates that Ca²⁺ dissociation from the inhibitory and activating sites are also not sequential.

C. Insights from Modal Gating Analyses

Examination of long current recordings of single Sf9 IP₃R channels in the presence of constant [IP₃] and [Ca²⁺]_i 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 (Ionescu et al., 2007). Three distinct gating modes of the IP₃R were identified: a mode in which the channel is mostly bursting, another with fast channel gating kinetics, and one with long quiescent periods, with channel *P*_o of 0.85, 0.24, and 0.007, respectively. 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. In the mode with Intermediate P_o (I mode), the IP₃R gates frequently with mostly short openings and closings. In the Low P_o (L mode), the channel has long closed periods interrupted with brief, infrequent openings (Fig. 6).

Strikingly, the P_o within each mode (P_o^M , with M standing for H, I, or L) remains relatively consistent over all $[Ca^{2+}]_i$ (Ionescu et al., 2007). Accordingly, $[Ca^{2+}]_i$ regulates P_o predominantly by regulating the fraction of time the channel spends in the different gating modes (relative prevalence π^M ; Fig. 7). The π^I exhibited relatively little $[Ca^{2+}]_i$ dependence, whereas both π^H and π^L showed strong ligand dependencies. In the presence of saturating IP₃ and optimal $[Ca^{2+}]_i$, the channel is found predominantly in the H mode and in the L mode for relatively little time, resulting in high overall P_o . The high π^H is due to the channel entering the H mode more frequently and staying there longer. In nonoptimal $[Ca^{2+}]_i$ (0.1 and 89 μM), the channel has low overall activity mainly because it enters the L mode more frequently.

Although the IP₃R channel P_o^M stayed within a narrow range over all $[Ca^{2+}]_i$ examined, the gating kinetics within a mode are not completely $[Ca^{2+}]_i$ -independent. The mean open and closed channel durations and mean durations of channel activity bursts and gaps between those bursts in the three modes all exhibited some variations over the $[Ca^{2+}]_i$ range examined. Nevertheless, mode switching is the most relevant mechanism of ligand regulation of IP₃R gating.

The important role that modal gating plays in ligand regulation of IP₃R activity indicates that besides the time scales of channel openings and closings (approximately milliseconds), other, longer time scales in IP₃R gating kinetics are likely to be relevant for the kinetics of IP₃-mediated $[Ca^{2+}]_i$ signaling *in vivo*. One such time scale is associated with the channel burst and burst-terminating (interburst) gap durations. Whereas most of the short closings are the result of ligand-independent channel gating (Foskett & Mak, 2004; Mak, McBride, & Foskett, 2003), 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 IP₃R conformational changes. The studies of the kinetic responses of single IP₃R channels to rapid ligand concentration changes (above) observed that in the constant presence of saturating 10 μM IP₃, the mean lag times to termination of IP₃R channel activity from abrupt changes in $[Ca^{2+}]_i$ from optimal (2 μM) to subactivating (<10 nM) or inhibitory (300 μM) levels were 160 and 290 ms, respectively. In constant 2 μM Ca^{2+}_i ; the mean lag time to channel activity termination from an abrupt drop in [IP₃] from 10 μM to 0 was 700 ms. In those experiments, the channels were most likely in the H mode before the activity-terminating ligand concentration change, with burst duration of 200–600 ms. The similarity between the mean channel lag times observed in rapid perfusion experiments and the mean burst duration 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 IP₃R Ca^{2+} -release event.

The weak dependence of channel burst duration on $[Ca^{2+}]_i$ may possibly be a mechanism by which an active IP₃R channel can avoid being prematurely inhibited by the Ca^{2+} that it releases, because an increase of $[Ca^{2+}]_i$ 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. Moreover, the stabilization by activating $[Ca^{2+}]_i$ of the H mode, which has significantly longer burst durations, may possibly play an important role in CICR. Thus, in the presence of sufficiently high $[IP_3]$, an increase in $[Ca^{2+}]_i$ above the resting level can encourage an IP₃R channel to enter the H mode from the L mode. As a result, the burst duration of the channel increases from that for a L mode (~10 ms), which may not release enough Ca^{2+} to recruit nearby channels to propagate a Ca^{2+} signal (individual blips or puffs), to that of a H mode (~200 ms), which enables the IP₃R channel to continue releasing Ca^{2+} even when the local $[Ca^{2+}]_i$ is raised to a high level. Such long channel bursts can release sufficient Ca^{2+} to recruit neighboring IP₃Rs or IP₃R clusters for a regenerative Ca^{2+} signal.

D. Molecular Bases for Ca^{2+} Regulation of IP₃R Channel Activity

Single-channel studies of ligand regulation of IP₃R channel activity have revealed that Ca^{2+} is intimately involved in regulating many aspects of IP₃R channel activity, including activation (increasing steady-state channel P_o), inhibition (decreasing steady-state channel P_o), inactivation (termination of channel activity in the presence of constant ligand concentrations), and recruitment (changing the fraction of channels in a population that is activated). Moreover, other ligands exert their effect on IP₃R channel activity by altering the functional Ca^{2+} affinities of the channel: the main effect of IP₃ is modulating sensitivity of the channel to Ca^{2+} inhibition, and ATP potentiates channel activity by increasing its sensitivity to Ca^{2+} activation. However, despite the identification of multiple putative Ca^{2+} -binding sites in the IP₃R primary sequences (Sienaert et al., 1996, 1997), relatively little progress has been made in determining the amino acid sequences that constitute the functional Ca^{2+} sensors in the IP₃R.

1. Residues in the IP₃R Sequence—Eight GST-fused denatured peptide fragments of the IP₃R located throughout the linear sequence were found to bind Ca^{2+} in gel-overlays (Sienaert et al., 1996, 1997), and although the biochemical detection of several sites is consistent with the presence of multiple Ca^{2+} -binding sites inferred from studies of single-channel gating (above), the physiological implications of such data are unclear. Mutagenesis of a conserved glutamic acid residue in IP₃R (Glu2100) affected $[Ca^{2+}]_i$ signals (Miyakawa et al., 2001) and shifted the apparent Ca^{2+} dependence of reconstituted channel P_o by approximately fivefold, from 0.2 to 1 μM (Tu et al., 2003). A peptide spanning residues Glu1932-Arg2270 bound Ca^{2+} with an apparent affinity of 160 nM as measured by tryptophan fluorescence, which was decreased to approximately 1 μM when Glu2100 was mutated (Tu et al., 2003). Although it has been concluded that this residue and region are important for Ca^{2+} regulation (Miyakawa et al., 2001; Tu et al., 2003), it was not determined whether the observed effects on channel function were due to modification of one of the Ca^{2+} -binding sites discussed above, or whether they were secondary effects caused by long-range allosteric mechanisms. Second, metal-binding sites in proteins generally comprise several interacting residues that help to coordinate and stabilize the ion in a binding pocket. Ca^{2+} -binding sites usually consist of 6 or 7 coordinating oxygen atoms provided by side-

chain carboxyls, main chain carbonyls, and water (Yang, Lee, Hellinga, & Yang, 2002). However, additional residues that might interact with Glu2100 to coordinate and bind Ca^{2+} have not been identified. With these caveats in mind, this region of the channel may play a role in Ca^{2+} sensing, but further experimentation is necessary to determine whether it is a Ca^{2+} -binding site, and which of the functional sites it represents. Another region of the receptor that has been considered to be involved in Ca^{2+} regulation of channel gating is the ligand-binding domain. Two surface acidic clusters were observed in the crystal structure of the IP_3 -bound 225–604 fragment (Bosanac et al., 2002). Site 1 was contained completely within the β -trefoil domain, whereas site 2 was located across the two domains. Both sites had been shown previously to bind Ca^{2+} in gel overlay experiments (Sienart et al., 1997). The residues that contribute to the acidic patches are highly conserved among isoforms. Nevertheless, mutagenesis studies failed to provide evidence for a role for residues in either site in Ca^{2+} activation since none of the mutations affected the ability of the channel to function at low cytoplasmic $[\text{Ca}^{2+}]_i$ in Ca^{2+} release assays (Joseph, Brownell, & Khan, 2005). However, only single point mutations were examined, and the relatively low resolution of Ca^{2+} release assays for measuring detailed channel properties may require multiple residues in a Ca^{2+} binding motif to be mutated to observe functional consequences. Single-channel studies of these mutant channels may reveal more subtle effects on the Ca^{2+} dependence of gating.

2. Summary of Functional Ca^{2+} Regulatory Sites—Detailed single-channel studies have revealed that Ca^{2+} regulates different aspects of IP_3R channel activity with very different functional affinities, binding kinetics, and ligand dependencies. Therefore, multiple different distinct Ca^{2+} sensors are likely to be involved. There is one Ca^{2+} sensor responsible for IP_3 modulation of Ca^{2+} inhibition of the channel. It changes from an inhibitory Ca^{2+} -binding site to an activating binding site depending on the concentration of IP_3 . Another IP_3 -independent Ca^{2+} sensor is responsible for the Ca^{2+} activation with consistent Ca^{2+} sensitivity observed in a wide variety of IP_3R channels. Yet another Ca^{2+} sensor is IP_3 -independent but inhibitory, with variable functional Ca^{2+} affinity that can be changed by factors extrinsic to the IP_3R channel like membrane lipid composition and exposure to low $[\text{Ca}^{2+}]_i$. Elimination of the functionality of this purely inhibitory Ca^{2+} -binding site by exposure to very low bath Ca^{2+} concentrations implies that the channel possesses a fourth Ca^{2+} -binding site with very high Ca^{2+} affinity that must be occupied by Ca^{2+} for the IP_3 -independent Ca^{2+} inhibitory site to be functional. There is a fifth Ca^{2+} sensor that binds Ca^{2+} at a low rate ($\sim 0.01 \text{ s}^{-1}$), but only when the channel is liganded with IP_3 . This sensor is responsible for IP_3 -induced inactivation of IP_3R channels. Ca^{2+} binding to this site causes the channel to enter into an inactive state from which it cannot emerge in the continuous presence of IP_3 . A sixth Ca^{2+} sensor is responsible for regulation of IP_3R channel recruitment.

Thus, the evidence points to a complex picture with multiple functional Ca^{2+} sensors in the channel, and the molecular identities of these sensors remain to be properly elucidated. Regulation of IP_3R channel activity by these Ca^{2+} sensors is probably complicated, with various degrees of cooperativity and hierarchical organization so that Ca^{2+} binding to one sensor can regulate functionality of another Ca^{2+} sensor. Structurally, these Ca^{2+} sensors are

most likely to be intrinsic to the channel, although some could reside in a protein(s) associated with the IP₃R channel complex.

IV. Ca²⁺ BINDING PROTEIN REGULATION OF IP₃R CHANNEL GATING

A. Calmodulin

The ubiquitous and conserved Ca²⁺-binding protein calmodulin (CaM) confers Ca²⁺-dependent regulation on many proteins, including ion channels (Saimi & Kung, 2002). Binding of Ca²⁺ to CaM (Ca²⁺-CaM) triggers conformational changes that promote or modulate its interaction with binding partners. Ca²⁺-free CaM (apoCaM) can also interact with target proteins, enabling CaM to function, in essence, as a Ca²⁺-regulated subunit of the protein. In this way, CaM mediates Ca²⁺-dependent and -independent regulation of many ion channels, including the ryanodine receptor (Balshaw, Yamaguchi, & Meissner, 2002). Although a substantial literature suggests that CaM interacts with the IP₃R, the *in vivo* association between the two proteins as well as the functional implications are far from unequivocal (recently reviewed in Foskett et al., 2007; Nadif Kasri et al., 2002; Rossi & Taylor, 2004; Taylor & Laude, 2002).

CaM was reported to bind within residues 1564–1585 (Fig. 2B) of the coupling domain of the mouse type 1 channel (Maeda et al., 1991). The interaction was Ca²⁺-dependent in CaM-sepharose binding assays (Maeda et al., 1991; Yamada et al., 1995). In an early report, purified IP₃R-1 channels reconstituted into lipid bilayers lacked inhibition by high [Ca²⁺]_i, whereas channels reconstituted from cerebellar microsomes displayed high [Ca²⁺]_i inhibition (Michikawa et al., 1999). This result was interpreted as indicating that a component of Ca²⁺-dependent inhibition was lost during purification. The authors inferred that Ca²⁺ inhibition of IP₃R gating was therefore not mediated by Ca²⁺ binding to the IP₃R itself (Michikawa et al., 1999). Addition of CaM to the bath restored Ca²⁺ inhibition of purified receptors, suggesting that it mediated high [Ca²⁺]_i inhibition of channel gating (Michikawa et al., 1999). Addition of the CaM antagonists W7 or the CaM binding domain of CaM kinase II activated reconstituted cerebellar IP₃R, which was interpreted as indicating that microsomal IP₃R is constitutively associated with and inhibited by CaM. The conclusion that high [Ca²⁺]_i inhibition of IP₃R gating is mediated by CaM was reinforced by observations that IP₃R-3 did not bind CaM (Cardy & Taylor, 1998; Lin, Widjaja, & Joseph, 2000; Yamada et al., 1995) and its gating was not inhibited by high [Ca²⁺]_i (Hagar et al., 1998).

Michikawa et al. (1999) has been repeatedly cited as evidence that Ca²⁺ inhibition of IP₃R channel gating is mediated by CaM. However, many experimental results argue against this conclusion. The *K*_d for Ca²⁺-CaM binding to the channel was reported to be 27 μM (Hirota, Michikawa, Natsume, Furuichi, & Mikoshiba, 1999), suggesting a low-affinity interaction, inconsistent with constitutive association with reconstituted channels. Indeed, high concentrations (10 μM) of CaM were required to inhibit Ca²⁺ release from purified IP₃R-1 (Hirota et al., 1999). Second, IP₃R-3 is not immune to Ca²⁺ inhibition as originally claimed. Biphasic [Ca²⁺]_i regulation of type 3 channels has been demonstrated in electrophysiological recordings by nuclear membrane patching (Mak et al., 2001b) and in planar bilayer reconstitutions (Tu, Wang, Nosyreva, De Smedt, & Bezprozvanny, 2005). Because the type 3

channel does not bind CaM, Ca^{2+} inhibition of channel gating can therefore occur in the absence of CaM binding (Cardy & Taylor, 1998). Third, in nuclear patch-clamp recordings of *X-IP₃R-1*, inhibition of channel activity by high $[\text{Ca}^{2+}]_i$ was observed in the presence of W7 and despite the overexpression of a dominant-negative, Ca^{2+} -insensitive CaM (Mak, McBride, Petrenko, et al., 2003). Fourth, mutation of the CaM-binding site eliminated CaM interaction with the IP₃R (Yamada et al., 1995), but it did not affect Ca^{2+} inhibition of channel activity (Nosyreva et al., 2002; Zhang & Joseph, 2001). The published data taken together provide little support for the proposition that high $[\text{Ca}^{2+}]_i$ inhibition of IP₃R gating is mediated by CaM.

In addition to a putative role in Ca^{2+} inhibition of channel gating, CaM has also been invoked to regulate other properties of the IP₃R. These studies have been reviewed elsewhere (Foskett et al., 2007). There are few biochemical data to suggest that CaM and IP₃R interact *in vivo*. The evidence of interactions relies almost exclusively on the various *in vitro* binding and pharmacological studies, while successful coimmunoprecipitation of the two proteins has not been demonstrated. Therefore, despite considerable effort to place CaM in the IP₃R signaling complex, there is little consensus on its functional role.

B. CaBP and CIB1

The IP₃R interacts with a family of CaM-related Ca^{2+} -binding proteins termed CaBPs, a subset of the neuronal Ca^{2+} sensor (NCS) family of EF-hand-containing proteins (Yang et al., 2002). Eight CaBP genes have been identified (CaBP1–8) with alternate splicing generating long and short forms of CaBP1 and CaBP2 (Haeseleer, Imanishi, Sokal, Filipek, & Palczewski, 2002; Haeseleer et al., 2000). CaBP1 binds with high affinity (apparent $K_d < 50$ nM) in a Ca^{2+} -dependent manner (apparent affinity $\sim 1 \mu\text{M}$) to the amino-terminal 600 residues of all three rat IP₃R isoforms (Yang et al., 2002), a region that encompasses the IP₃-binding domain. The Ca^{2+} dependence of the interaction was mediated by the EF hands of CaBP (White, Yang, Monteiro, & Foskett, 2006; Yang et al., 2002). Truncation mutagenesis of the IP₃R ligand-binding domain localized CaBP1 binding to the amino-terminal 225 residues (Kasri et al., 2004), although the binding observed was Ca^{2+} -independent, whereas the high-affinity interaction of CaBP1 with the IP₃R is strongly Ca^{2+} -dependent (White et al., 2006; Yang et al., 2002). Thus, the significance of CaBP1 binding to this region is unclear.

In nuclear membrane patch-clamp recording of *X-IP₃R-1*, channel activity with high P_o and gating kinetics similar to those elicited by IP₃ was observed in the absence of IP₃ when recombinant CaBP1 was included in the pipette solution at optimal $[\text{Ca}^{2+}]_i$. In contrast, the triple-EF-hand mutant CaBP1 that lacked Ca^{2+} binding failed to activate the channel (Yang et al., 2002). Purified CaBP2 and CaBP5 each had similar effects (Yang et al., 2002). These results identified the CaBP Ca^{2+} sensors as a family of protein ligands of the IP₃R channel. It was proposed that this regulation might enable the channel to become activated in the absence of phosphoinositide metabolism, and that it may modulate the sensitivity of the channel to IP₃, which may play a role in spatially restricting Ca^{2+} release (Yang et al., 2002). Because IP₃R-mediated Ca^{2+} signals are shaped by messenger diffusion, degradation and removal, processes that will have distinct kinetics for IP₃ compared with CaBPs, the

identification of novel ligands for the IP₃R provided new insights into the dimensions and versatility of this ubiquitous signaling pathway.

Members of the CaBP family are exclusively expressed in brain and retina (Haeseleer et al., 2000), but this mode of IP₃R regulation likely extends more widely to nonneuronal cell types as well. The ubiquitously expressed protein CIB1 (calcium- and integrin-binding protein; also called calmyrin or KIP) was subsequently shown to interact with the IP₃R (White et al., 2006). CIB1 belongs to a distinct subfamily of NCS proteins (Gentry et al., 2005). Nevertheless, like CaBP1, CIB1 binds to the first 600-residue ligand-binding region of all isoforms of the IP₃R in a Ca²⁺-sensitive fashion, with an affinity in the μ M range, and has a similar dependence on functional EF-hands. Like CaBPs, CIB1 activated *Xenopus* and Sf9 IP₃R-1 channel gating in the absence of IP₃, establishing it as a novel protein agonist. CIB1 behaves as a partial agonist of the channel, since it was less efficacious than optimal [IP₃] in activating channel gating (White et al., 2006).

Although acute exposure of the IP₃R to purified recombinant CaBP/CIB1 proteins *activates* channel gating, overexpression of either CaBP or CIB1 was found to *inhibit* agonist-induced IP₃R-dependent Ca²⁺ release in intact cells (Haynes, Tepikin, & Burgoyne, 2004; Kasri et al., 2004; White et al., 2006). This apparent conflict was resolved by the demonstration that CaBP/CIB1 initially activates IP₃R channel activity, but then subsequently drives the channel into an inactivated state that cannot be activated by IP₃ (White et al., 2006). In CaBP1/CIB1 overexpression studies, much of the IP₃R channel population would be in the inactivated state, accounting for the muted IP₃-mediated responses observed in CaBP1 or CIB1-expressing cells (Haynes et al., 2004; Kasri et al., 2004; White et al., 2006). These results suggest that prolonged exposure to CIB1 can effectively remove functional channels from the total IP₃R population by a process of ligand-induced channel inactivation.

Phosphorylation or other covalent modifications, and other protein interactions may regulate the affinities of the interactions of CaBP1 and CIB1 with the IP₃R. Mutation of a conserved phosphorylation site in CaBP1 resulted in even greater [Ca²⁺]_i signaling inhibition (Kasri et al., 2004), although this site is not conserved in CIB1. Regulated unbinding of the protein ligands could enable the channel to escape from inactivation, allowing it to again be activated by protein ligand rebinding, in a mechanism of repeated channel activation that is independent of IP₃.

C. Chromogranins

Chromogranins are high-capacity, low-affinity Ca²⁺ binding proteins that are enriched in secretory granules but are also found in ER and nuclear compartments (Aunis & Metz-Boutigue, 2000). Both chromogranins A and B (CGA/CGB) interact within the third luminal loop of IP₃R-1 within the stretch of residues that link the luminal end of the pore selectivity filter to the beginning of TM6 (Yoo & Lewis, 1994, 2000; Yoo et al., 2000). The *P*_o of reconstituted IP₃R-1 incorporated into bilayers was dramatically increased by both CGA and CGB, from ~5%, the typical maximum *P*_o observed for the reconstituted channel, to 20–80% in different studies (Choe, Harrison, Grant, & Ehrlich, 2004; Thrower et al., 2003; Thrower, Park, So, Yoo, & Ehrlich, 2002). This remarkable level of stimulation was associated with the appearance of a new, long open state (Thrower et al., 2003, 2002).

Remarkably, the chromogranin-activated channel lacked all cytoplasmic Ca^{2+} sensitivity, being equally active 10 nM, 1 μM , and 100 μM Ca^{2+} (Thrower et al., 2002). However, it is not known whether the interaction is modulated by physiological changes in luminal $[\text{Ca}^{2+}]$.

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FIGURE 1.

Typical single-channel current traces of *Xenopus* type 1 IP₃R in various cytoplasmic Ca²⁺ concentrations and saturating 10 μM IP₃. Current traces were recorded during nuclear patch-clamp experiments at cytoplasmic Ca²⁺ concentrations as tabulated in 0.5 mM free ATP (modified from Mak et al., 1998). All current traces in this and other graphs were recorded at 20 mV. Arrows indicate closed-channel current level in all current traces. Channel P_o evaluated for the single-channel patch-clamp experiments yielding the current traces shown in A, B, C, and D are 0.008, 0.50, 0.89, and 0.002, respectively. From Foskett et al. (2007).

FIGURE 2.

[Ca²⁺]_i and [IP₃] regulation of IP₃R channel activity. (A) [Ca²⁺]_i dependence of mean P_o of endogenous X-IP₃R-1 channels (filled symbols) in various [IP₃] as tabulated (modified from Mak et al., 1998). Each data point in this and subsequent P_o versus [Ca²⁺]_i plots is the average of channel P_o from at least four experiments using the same ligand concentrations. The curves are least-square fit of the data points using the biphasic Ca²⁺ regulation Hill equation (Eq. (1)) with parameters as tabulated. The large open circles represent P_o for recombinant rat IP₃R-1 channels in various [Ca²⁺]_i in saturating 10 μ M IP₃ (modified from Boehning et al., 2001). Inset: Plot of K_{inh} derived from the biphasic Hill equation fit of P_o data versus [IP₃] used. The curve is the least-square fit of the K_{inh} values using the activation Hill equation $\log(K_{inh}) = \log(K_{inh}^{\infty}) - IP_3^{IP_3} H \log\left(1 + IP_3^{IP_3} K/[IP_3 R]\right)$ with parameters as tabulated. (B) [Ca²⁺]_i dependence of mean P_o of recombinant rat type 3 IP₃R channels in

various $[IP_3]$ as tabulated (modified from Mak et al., 2001b). Data points and fitted curves are obtained as described for A. (C) $[Ca^{2+}]_i$ dependence of mean P_o of endogenous IP_3R channels from Sf9 cells in various $[IP_3]$ as tabulated (modified from Ionescu et al., 2006). Data points, fitted curves, and inset graph are obtained as described for (A). (D) $[Ca^{2+}]_i$ dependence of mean P_o of $X-IP_3R-1$ IP_3R channels that have been exposed to bath solution with very low $[Ca^{2+}]_i$ (<5 nM) for a few minutes before the patch-clamp experiments, in various $[IP_3]$ as tabulated. The curves are least-square fits to the data using activation Hill equation $P_o = P_{Hill} \left\{ 1 + \left(K_{act} / [Ca^{2+}]_i \right)^{H_{act}} \right\}^{-1}$ with parameters as tabulated. Modified from Mak, McBride, Petrenko, et al. (2003).

FIGURE 3.

The MWC-based four + two-conformation model for IP₃R channel gating. Only conformation transitions are represented in the schemes. Reactions involving binding of IP₃ and Ca²⁺ to the IP₃R channel and the state of occupation of the various ligand-binding sites of the channel are omitted from the schemes for clarity. The shaded rectangles represent the grouping of the open A* and closed A' conformations into the active A conformation, and the grouping of the C* and C' conformations into the active C conformation. Modified from Mak, McBride, & Foskett (2003).

FIGURE 4.

Nuclear patch-clamp studies of single IP₃R channel responses to rapid ligand concentration changes. (A-K) Single-channel current traces from cyto-out patches showing responses of Sf9 IP₃R channels to abrupt changes in ligand concentrations indicated by color bars above the traces. Ligand concentrations were changed by switching the perfusing bath solutions, which also caused a change in the closed channel baseline current level because of the different [KCl] in the perfusing solutions. This allowed the time course of the perfusing solution switch to be monitored. Response latencies are denoted by gray bars below the traces. Modified from Mak et al. (2007).

FIGURE 5.

Kinetic scheme accounting for the observed IP₃R channel response latencies. A qualitative allosteric model stipulating one kind of high-affinity Ca²⁺ site in an IP₃R channel. Occupation of the IP₃-binding sites causes the channel to adopt the C and D conformations instead of the A and B conformations, so that Ca²⁺ binding to the high affinity Ca²⁺ site becomes activating instead of inhibitory. Only conformation changes are shown in this kinetic scheme. IP₃ and Ca²⁺ binding to the channel, and the ligand-binding state of the channel conformations, are omitted for clarity. Reproduced from Mak et al. (2007).

FIGURE 6.

IP₃R channel modal gating. Single IP₃R channel gating records observed in saturating 10 μM IP₃ and suboptimal (100 nM), optimal (1 μM) and inhibiting (89 μM) [Ca²⁺]_i as tabulated. Each section consists of a set of four graphs derived from one 50-s long continuous single-channel record. From the top: raw single-channel current trace (in black with arrow indicating the closed-channel current level), idealized current trace (in blue), idealized current trace after burst analysis (in purple), and the gating mode of the channel with green, yellow, and red representing H, I, and L modes. The current traces were selected to show modal transitions among all three gating modes in one continuous recording under various [Ca²⁺]_i.

FIGURE 7.

IP₃R channel modal gating parameters. Channel gating parameters observed in various $[Ca^{2+}]_i$ and $[IP_3]$ as tabulated (in μM). (A) Channel open probability P_o . (B) Relative prevalence π^M (amount of time the channel spent in a mode/total duration of all analyzed channel current records) for the three gating M (M stands for H, I, or L, depending on the mode involved). The H, I, and L modes are plotted in bar graphs in green, yellow, and red, respectively, as indicated by the key. Modified from Ionescu et al. (2007).