Novel Regulation of Calcium Inhibition of the Inositol 1,4,5-trisphosphate Receptor Calcium-release Channel

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ABSTRACT The inositol 1,4,5-trisphosphate (InsP₃) receptor (InsP₃R), a Ca²⁺-release channel localized to the endoplasmic reticulum, plays a critical role in generating complex cytoplasmic Ca²⁺ signals in many cell types. Three InsP₃R isoforms are expressed in different subcellular locations, at variable relative levels with heteromultimer formation in different cell types. A proposed reason for this diversity of InsP₃R expression is that the isoforms are differentially inhibited by high cytoplasmic free Ca²⁺ concentrations ([Ca²⁺]), possibly due to their different interactions with calmodulin. Here, we have investigated the possible roles of calmodulin and bath [Ca²⁺] in mediating high [Ca²⁺] inhibition of InsP₃R gating by studying single endogenous type 1 InsP₃R channels through patch clamp electrophysiology of the outer membrane of isolated Xenopus oocyte nuclei. Neither high concentrations of a calmodulin antagonist nor overexpression of a dominant-negative Ca²⁺-insensitive mutant calmodulin affected inhibition of gating by high [Ca²⁺]. However, a novel, calmodulin-independent regulation of [Ca²⁺] inhibition of gating was revealed: whereas channels recorded from nuclei kept in the regular bathing solution with [Ca²⁺] ~400 nM were inhibited by 290 μM [Ca²⁺], exposure of the isolated nuclei to a bath solution with ultra-low [Ca²⁺] (<5 nM, for ~30 s) before the patch-clamp experiments reversibly relieved Ca²⁺ inhibition, with channel activities observed in [Ca²⁺] up to 1.5 mM. Although InsP₃ activates gating by relieving high [Ca²⁺] inhibition, it was nevertheless still required to activate channels that lacked high [Ca²⁺] inhibition. Our observations suggest that high [Ca²⁺] inhibition of InsP₃R channel gating is not regulated by calmodulin, whereas it can be disrupted by environmental conditions experienced by the channel, raising the possibility that presence or absence of high [Ca²⁺] inhibition may not be an immutable property of different InsP₃R isoforms. Furthermore, these observations support an allosteric model in which Ca²⁺ inhibition of the InsP₃R is mediated by two Ca²⁺ binding sites, only one of which is sensitive to InsP₃.

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

INTRODUCTION

The second messenger, inositol 1,4,5-trisphosphate (InsP₃), is generated in many cell types through the hydrolysis of phosphatidylinositol 4,5-bisphosphate by membrane-bound phospholipase C activated by plasma membrane receptors responding to extracellular stimuli. InsP₃ then diffuses through the cytoplasm to bind to its receptor (InsP₃R) in the ER and activate it as a Ca²⁺ channel to release Ca²⁺ stored in the ER lumen. Modulation of the cytoplasmic free Ca²⁺ concentration ([Ca²⁺]) by InsP₃R-mediated Ca²⁺ release is a ubiquitous intracellular signal transduction mechanism that regulates numerous processes (Berriedge, 1993).

Three isoforms of the InsP₃R, with spliced variants, have been identified (Joseph, 1996). Most mammalian cell types express multiple InsP₃R isoforms in distinct and overlapping intracellular locations with their absolute and relative expression levels regulated by gene transcription, alternative splicing and receptor degra-

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Abbreviations used in this paper: InsP₃, inositol 1,4,5-trisphosphate; InsP₃R, InsP₃ receptor; NCaS, regular [Ca²⁺] bath solution; PCaS, physiological [Ca²⁺] bath solution; r-InsP₃R-3, rat type 3 InsP₃R; CaM, calmodulin; ULCaS, ultra-low [Ca²⁺] bath solution; X-InsP₃R-1, Xenopus type 1 InsP₃R.
for the regulation of intracellular Ca$^{2+}$ signaling (Taylor, 1998), it has been suggested that differential inhibition by high \([\text{Ca}^{2+}],\) of the different InsP$_3$R isoforms may generate distinct Ca$^{2+}$ signals in different cell types with different patterns of InsP$_3$R isoform expression, and that this may be a reason for the diversity of InsP$_3$R expression (Hagar et al., 1998).

It has been suggested that high \([\text{Ca}^{2+}],\) inhibition of the InsP$_3$R is mediated by calmodulin (CaM), a ubiquitous Ca$^{2+}$-binding protein that binds to and regulates the functions of many proteins. CaM was found to bind to the InsP$_3$R-1 in the presence of free Ca$^{2+}$ to a single site in the regulatory domain (Maeda et al., 1991; Yamada et al., 1995; Hirota et al., 1999). Purified InsP$_3$R-1 channels lacking bound CaM were not inhibited by high \([\text{Ca}^{2+}],\) whereas addition of CaM restored inhibition of channel gating by high \([\text{Ca}^{2+}],\) (Hirota et al., 1999; Michikawa et al., 1999). The notion that high Ca$^{2+}$ inhibition of channel gating was mediated by CaM was reinforced by observations that the type 3 InsP$_3$R (InsP$_3$R-3) did not bind CaM (Yamada et al., 1995; Cardy and Taylor, 1998; Lin et al., 2000) and was not inhibited by high \([\text{Ca}^{2+}],\) (Hagar et al., 1998). Nevertheless, other data suggest that the role of CaM in high \([\text{Ca}^{2+}],\) inhibition of InsP$_3$R channel gating is far from unequivocal. Despite the absence of detectable interaction between CaM and a mutant InsP$_3$R-1 in which the putative CaM binding site was eliminated (Yamada et al., 1995), more recent studies have demonstrated that this mutant channel is nevertheless still inhibited by high \([\text{Ca}^{2+}],\) (Mak et al., 2001a) with quantitative features similar to those of inhibition of the InsP$_3$R-1 in the same membrane (Mak et al., 1998).

Here, we investigated the possible effects of CaM on high \([\text{Ca}^{2+}],\) inhibition of the gating of single endogenous InsP$_3$R-1 channels in their native membrane environment using nuclear membrane patch clamp electrophysiology (Mak and Foskett, 1994). Our experiments do not provide evidence supporting any role for CaM in this process. However, we discovered a novel regulation of high \([\text{Ca}^{2+}],\) inhibition of InsP$_3$R-1 channel gating. Inhibition of InsP$_3$R-1 gating by high \([\text{Ca}^{2+}],\) can be reversibly abrogated by exposure of the channel to a bathing solution containing ultra-low \([\text{Ca}^{2+}]\) (<5 nM). Our observations indicate that inhibition of InsP$_3$R-1 channel gating by high \([\text{Ca}^{2+}],\) can be disrupted by environmental conditions experienced by the channel, and therefore may not be an invariant property of a specific InsP$_3$R isoform. Furthermore, these observations support an allosteric model in which Ca$^{2+}$ inhibition of the InsP$_3$R is mediated by two Ca$^{2+}$ binding sites, only one of which is sensitive to InsP$_3$.

**MATERIALS AND METHODS**

**Heterologous Expression of Calmodulin in Xenopus Oocytes**

Maintenance of *Xenopus laevis* and surgical extraction of ovaries were performed as described previously (Mak and Foskett, 1994, 1997, 1998). Oocytes were defolliculated as described (Jiang et al., 1998). cRNA (1 \(\mu\)g/\(\mu\)l) of rat calmodulin (CaM), either wild-type (w.t.) or a quadruple mutant (q.m.) containing a D→A mutation in each of the four EF hands so that Ca$^{2+}$ binding in all EF hands was abolished (Xia et al., 1998; Keen et al., 1999), was synthesized in vitro from cDNA provided as a gift by Dr. John P. Adelman (Vollum Institute, Portland, OR). 23 nl of cRNA (either w.t. or q.m.) was injected into the cytoplasm of oocytes 1 day after defolliculation, as described (Mak et al., 2000). cRNA-injected and uninjected control oocytes were maintained under identical conditions in individual wells in 96-well plates containing 200 \(\mu\)l of ASOS (100 mM NaCl, 2 mM KCl, 1 mM MgCl$_2$, 1.8 mM CaCl$_2$, 5 mM HEPES, pH adjusted to 7.6 with NaOH; with 3 mM Na pyruvate, 100 \(\mu\)g/ml gentamycin, and 100 \(\mu\)M N-acetyl-Leu-Leu-Val-Tyr-AMC (Sigma-Aldrich). 80 \(\mu\)l of ASOS in each well was changed daily. Nuclear patch clamp experiments and immunoprecipitations were performed 2–4 d after cRNA injection when the expression level of exogenous CaM was stable as determined by Western analysis.

**Western Analysis and Immunoprecipitation**

Western analysis was performed on oocyte extracts (cRNA-injected and un.injected), as described in Mak et al. (2000), to ascertain the levels of endogenous and heterologously expressed CaM in the oocytes using a specific antibody (Upstate Biotechnology). Immunoprecipitation of InsP$_3$R (type 1) and CaM was performed using oocyte lysates, as described in (Mak et al., 2000), with a specific type 1 InsP$_3$R antibody (Joseph and Samanta, 1993; Joseph et al., 1995) and protein A agarose (GIBCO BRL), and an antibody to CaM and protein G agarose (GIBCO BRL), respectively.

**Solutions for Patch Clamp Experiments**

All patch clamp experiments were performed with solutions containing 140 mM KCl and 10 mM HEPES with pH adjusted to 7.1 with KOH. The free Ca$^{2+}$ concentration (\([\text{Ca}^{2+}],\)) of the pipette solutions (to which the cytoplasmic side of the InsP$_3$R is exposed in patch-clamp experiments) was tightly controlled by buffering various amounts of added CaCl$_2$ (40–400 \(\mu\)M) with 500 \(\mu\)M of the high-affinity Ca$^{2+}$ chelator, BAPTA (1,2-bis(o-aminophosphonoyl)ethane-N,N,N',N'-tetraacetic acid; Molecular Probes) and 0.5 mM Na$_2$ATP (100 mM < \([\text{Ca}^{2+}],\) < 2.5 \(\mu\)M); or 500 \(\mu\)M of the low-affinity Ca$^{2+}$ chelator, 5.5'-dibromo BAPTA (Molecular Probes) and 0.5 mM Na$_2$ATP (5 \(\mu\)M < \([\text{Ca}^{2+}],\) < 15 \(\mu\)M); or 0.5 mM Na$_2$ATP alone (15 \(\mu\)M < \([\text{Ca}^{2+}],\) < 300 \(\mu\)M). Solutions with \([\text{Ca}^{2+}],\) > 300 \(\mu\)M contained no Ca$^{2+}$ chelator for buffering. The normal Ca$^{2+}$ bath solution (NCaS) contained 500 \(\mu\)M BAPTA and 250 \(\mu\)M CaCl$_2$ (free \([\text{Ca}^{2+}],\) = 400–500 nM), and the physiological Ca$^{2+}$ bath solution (PCaS) contained 500 \(\mu\)M BAPTA and 70 \(\mu\)M CaCl$_2$ (free \([\text{Ca}^{2+}],\) = 48 ± 5 nM). The free \([\text{Ca}^{2+}],\) of these solutions was directly measured using Ca$^{2+}$-selective mini-electrodes (Baudet et al., 1994). The ultra-low Ca$^{2+}$ bath solution...
(ULCaS) contained 1 mM BAPTA and no added CaCl$_2$. The contaminating [Ca$^{2+}$] in the solution was determined by induction-coupled plasma mass spectrometry (Mayo Medical Laboratory) to be $\sim$6–10 µM. Ca$^{2+}$-selective minielectrodes were unable to determine accurately the free [Ca$^{2+}$] in the ULCaS because of the nonlinear response of the electrode in free [Ca$^{2+}$] $<$ 5 nM. Free [Ca$^{2+}$] was calculated using the Maxchelator software (C. Patton, Stanford University, Stanford, CA) to be $\sim$0.9–1.5 nM.

Unless specified otherwise, all pipette solutions contained a saturating concentration (10 µM) of InsP$_3$ (Mak and Foskett, 1994) from Molecular Probes. When specified, the pipette solutions also contained 500 µM W-7 (a CaM binding antagonist; N-(6-aminohexyl)-5-chloro-1-naphthalene-sulfonamide hydrochloride; Calbiochem), or 10 µM purified bovine CaM (Calbiochem). All reagents were used with no further purification.

**Oocyte Nucleus Isolation Protocols**

A stage V or VI oocyte was gently teased open mechanically in the isolation bathing solution, enabling the translucent nucleus to be isolated from the cytoplasmic matrix. The isolated nucleus was either directly transferred to the experimental bathing solution (protocol Nd, Ld, and Pd, Fig. 1), or it was transferred through a series of culture dishes containing 4–5 ml of incubation bath solutions (protocol L, LN, and LNL, in Fig. 1) before it was ultimately transferred to the experimental bath. The nucleus remained in each incubation bath for at least 20 min before the next transfer, to ensure that the solution in the perinuclear lumen between the outer and inner nuclear envelope had attained ionic equilibrium with the bath solution (Mak and Foskett, 1994). Approximately 20 µl of the previous bath solution accompanied the nucleus to the new bath in a transfer. The culture dish containing the nucleus in the experimental bath solution was finally moved onto the stage of the inverted microscope where patch clamp experiments were performed.

**Acquisition and Analysis of Single-Channel Patch-clamp Current Records**

The isolated nucleus was gently immobilized as described previously (Mak and Foskett, 1994) so that membrane patches could be repeatedly obtained from the same region ($\pm$2 µm) of the outer nuclear membrane (Mak and Foskett, 1997). Due to abrupt termination of channel activity (Mak and Foskett, 1994, 1997), patch clamp experiments were performed in “on-nucleus” configuration to maximize the duration of channel activities recorded. To prevent contamination of the pipette solution by the bath solution (especially the Ca$^{2+}$ chelator in the bath solution) by diffusion through the pipette tip during the time when the pipette was immersed in the bath and before giga-Ohm seal formation, a positive pressure ($\sim$10 mmHg) was maintained inside the pipette until the pipette tip was properly positioned on the nuclear membrane. Then suction was applied in the pipette to obtain a giga-Ohm seal. All experiments were performed at room temperature with the pipette electrode at 20 mV relative to the reference bath electrode unless specifically stated otherwise. Each experiment recorded the InsP$_3$R channel activity at a specific [Ca$^{2+}$], and [InsP$_3$], with no change of the pipette or bath solutions during the experiment. Data acquisition was performed as previously described (Mak et al., 1998), with currents recorded with a filtering frequency of 1 kHz and a digitizing frequency of 5 kHz.

The patch clamp current traces were analyzed using MacTac software (Bruxton) to identify channel-opening and -closing events using a 50% threshold. Current traces exhibiting one InsP$_3$R channel, or two InsP$_3$R channels determined to be identical and independently gated (Mak and Foskett, 1997), were used for channel open probability ($P_o$) evaluation. The number of channels in the membrane patch was assumed to be the maximum number of open channel current levels observed throughout the current record. In experimental conditions with $P_o > 0.1$, only current records with longer than 10 s of InsP$_3$R channel activities were used for determination of $P_o$, so there is little uncertainty in the number of channels in the current traces used. In experimental conditions with $P_o < 0.1$, only current records exhibiting one open channel current level with InsP$_3$R channel activities lasting longer than 30 s were used, to ensure that they were truly single-channel records (Mak et al., 2001a). The $P_o$ data shown for each set of experimental conditions are the means of results from at least four separate patch-clamp experiments performed under the same conditions. Error bars indicate the SEM.

**RESULTS**

**Lack of Effect of Calmodulin on Ca$^{2+}$ Inhibition of InsP$_3$R Gating in Endoplasmic Reticulum Membrane**

Previous single-channel patch-clamp studies of the endogenous Xenopus type 1 InsP$_3$R (X-InsP$_3$R-1) in its native ER membrane environment revealed a biphasic regulation by [Ca$^{2+}$], of the single-channel open probability ($P_o$) in its native ER membrane environment revealed a biphasic regulation by [Ca$^{2+}$], of the single-channel open probability ($P_o$) (Mak et al., 1998, 2001b). It has been suggested that calmodulin (CaM) bound to the channel mediates inhibition of InsP$_3$R gating by high [Ca$^{2+}$], (Michikawa et al., 1999). We therefore investigated the possibility that the high [Ca$^{2+}$] inhibition of X-InsP$_3$R-1 channel gating observed in our previous studies was mediated by CaM. Oocyte nuclei were isolated and transferred directly into an experimental bath of NCaS for patch-clamp experiments (protocol Nd in Fig. 1). By repeated patch clamping over the surface of an isolated nucleus, regions on the outer nuclear envelope were identified in which the probability of detecting InsP$_3$R channel activities in membrane patches ($P_o$) was high (Mak and Foskett, 1997). A series of patch-clamp experiments was performed at these regions with pipette solutions (to which the cytoplasmic side of the InsP$_3$R was exposed) alternately containing either

![Figure 1. Schematic diagram showing the various protocols used to isolate oocyte nuclei for nuclear patch clamp experiments.](image)
[Ca$^{2+}$]_i = 755 nM, or very high [Ca$^{2+}$]_i (290 μM) with 500 μM of W-7, a CaM binding antagonist. The former solution is one in which the channel gates with a high $P_o$, thereby ascertaining the presence of functional InsP$_3$R channels in the regions selected during the series of experiments. In contrast, the latter solution has [Ca$^{2+}$]_i sufficiently high to inhibit InsP$_3$R channel gating (Mak et al., 1998). Because CaM is endogenously expressed in Xenopus oocytes (Fig. 2, Lane A and C), we reasoned that if CaM mediated the high [Ca$^{2+}$]_i inhibition of InsP$_3$R channel gating, then inclusion of 500 μM of W-7 in the pipette solution may block high [Ca$^{2+}$]_i inhibition by interfering with CaM binding to the InsP$_3$R channel (Michikawa et al., 1999), making channel gating observable in the 290 μM [Ca$^{2+}$]_i solutions. Nevertheless, no channel activity was detected in any of the five patches with 500 μM W-7 and [Ca$^{2+}$]_i = 290 μM (Fig. 3 B), whereas InsP$_3$R channel activities were readily detected in five out of six patches with [Ca$^{2+}$]_i = 755 nM (Fig. 3 A).

Whereas this result with W-7 is seemingly inconsistent with the hypothesis that CaM mediates Ca$^{2+}$ inhibition of InsP$_3$R gating, CaM-dependent regulation of the small-conductance Ca$^{2+}$-activated K$^+$ (SK) channel gating is insensitive to W-7 and other CaM inhibitors (Xia et al., 1998). However, overexpression of a mutant CaM, in which the Ca$^{2+}$-binding EF hand motifs were disabled, interfered with the Ca$^{2+}$ activation of the SK channel gating by competing with the endogenous CaM for the interaction with the channels (Xia et al., 1998; Keen et al., 1999). The effects of mutant CaM expression on SK channel gating provided evidence that endogenous CaM is tightly and constitutively (even in the absence of Ca$^{2+}$) associated with the SK channel and mediates the effects of Ca$^{2+}$ on SK channel gating. The ability of high Ca$^{2+}$ concentrations to inhibit InsP$_3$R channel gating in our in vitro electrophysiological studies can be observed for long times (up to 2 h) after isolation of the nuclei (Mak et al., 1998; Boehning et al., 2001; Mak et al., 2001b). Thus, if CaM mediates the effect of high [Ca$^{2+}$]_i, it must remain associated with the channel in the isolated nuclei, and therefore must be tightly bound to the InsP$_3$R and not free to diffuse away into the large experimental bath. We therefore explored the possibility that Ca$^{2+}$ inhibition of InsP$_3$R channel gating was mediated by a constitutive tight association of CaM with the channel, by examining the effects of overexpression of the Ca$^{2+}$-insensitive quadruple mutant (q.m.) CaM on the Ca$^{2+}$ regulation of the InsP$_3$R.

The q.m. CaM, which has all EF hands mutated and therefore is Ca$^{2+}$ insensitive, was overexpressed in Xenopus oocytes by cytoplasmic microinjection of cRNA. Western analysis (n = 5) indicated that the exogenous q.m. CaM was expressed to a level that was at least an order of magnitude higher than the endogenous wild-type CaM (Xia et al., 1998; Fig. 2). Patch-clamp experiments using nuclei isolated by protocol Nd (Fig. 1) from q.m. CaM-expressing oocytes revealed that InsP$_3$R channel gating was still inhibited by high [Ca$^{2+}$]; InsP$_3$R channel activities were detected in 11 out of 11 patches with pipette solutions containing [Ca$^{2+}$]_i = 2.1
μM (Fig. 3 C), but no channel activity was detected in any of 9 patches with pipette solutions containing 290 μM [Ca2+]i, (Fig. 3 D). These results therefore also did not support the hypothesis that Ca2+ inhibition of InsP3R channel gating is mediated by CaM.

The lack of effect of overexpression of the q.m. CaM on Ca2+ inhibition of gating may suggest that endogenous CaM is not normally associated with the InsP3R. We examined the biochemical association between the InsP3R and CaM by coimmunoprecipitation. Using lysates prepared from cRNA-injected oocytes overexpressing either w.t. or q.m. CaM (Fig. 2), immunoprecipitation of the endogenous type 1 InsP3R with a specific antibody did not coimmunoprecipitate either w.t. or q.m. CaM (n = 4; unpublished data). In the converse experiments, immunoprecipitation of CaM with an antibody that binds to both w.t. and q.m. forms did not coimmunoprecipitate the InsP3R (n = 4; unpublished data). These results therefore do not provide evidence of an association between CaM and the InsP3R.

In summary, our single-channel patch clamp experiments revealed that neither high concentrations of a CaM antagonist, nor overexpression of a Ca2+-insensitive q.m. CaM had any effect on [Ca2+]i, inhibition of InsP3R channel gating. In addition, coimmunoprecipitation failed to demonstrate an association between CaM and the InsP3R. Thus, our investigations did not provide any evidence supporting the hypothesis that high [Ca2+]i, inhibition of InsP3R gating observed in in vitro patch clamp studies is mediated by CaM. These conclusions are therefore in agreement with those reached in some other studies (Zhang and Joseph, 2001; Nosyreva et al., 2002).

Figure 4. Typical current traces from nuclei in ULCaS bath isolated by protocol L. Arrows indicate closed channel current levels. Pipette solutions contained 10 μM InsP3 and [Ca2+]i, as tabulated. The last current trace was obtained with −20 mV applied transmembrane potential. Other current traces were obtained with +20 mV applied potential.

Figure 5. [Ca2+]i, dependencies of the channel P_o of the InsP3R in oocyte nuclei isolated using various protocols (Nd, L, and LNL) and applied potentials (±20 mV) as tabulated. All pipette solutions used contained 10 μM InsP3. The dashed curve is a simple activating Hill equation fit for the data from nuclei isolated with protocol L (large open circles). For comparison, the biphasic Hill equation fit (continuous curve) for the data points from nuclei isolated directly into NCaS bath (small filled circles) obtained in a previous study (Mak et al., 1998) are also shown. The InsP3R channel P_o was lower in ULCaS than in NCaS at [Ca2+]i = 100 nM. It is possible that this reflects some intrinsic properties of the InsP3R after exposure to the low bath [Ca2+]i. Alternately, this may only be an artifact as a result of the movement of free Ca2+ ions across the open channel. With pipette [Ca2+]i = 100 nM, when the oocyte nucleus was in NCaS ([Ca2+]i = 400–500 nM), the Nernst reversal potential for Ca2+ ions was −35 mV so Ca2+ ions moved across the open InsP3R channel from the luminal side to the cytoplasmic side despite an applied transmembrane voltage of 20 mV. This could cause the effective [Ca2+]i, at the activating Ca2+-binding sites on the cytoplasmic side of the channel to be higher than the free [Ca2+]i in the bulk of the pipette solution if the Ca2+-binding sites are close enough to the ion conducting pore. Conversely, when the nucleus was in ULCaS ([Ca2+]i < 5 nM), Ca2+ ions moved across the open InsP3R channel in the opposite direction, down the electrical and chemical gradients, possibly lowering the effective [Ca2+]i, at the Ca2+-binding sites. In [Ca2+]i < 250 nM, the mean open channel duration (<t_o>) of the InsP3R increases with [Ca2+]i, (Mak and Foskett, 1998). Therefore, if Ca2+ flux across the open InsP3R channel caused the effective [Ca2+]i, at the activating Ca2+-binding sites to deviate from the free [Ca2+]i in the bulk pipette solution, then channels in NCaS bath would have longer <t_o> and higher channel P_o than those in ULCaS bath, as observed. On the other hand, in [Ca2+]i > 300 nM, <t_o> does not exhibit any dependence on [Ca2+]i, although the mean closed channel duration (<t_c>) is still affected by [Ca2+]i, (Mak and Foskett, 1998). Deviation of effective [Ca2+]i at the Ca2+-binding sites from the bulk free [Ca2+]i would dissipate quickly by diffusion once the channel closed and therefore would not affect <t_o>. Thus, there would be no difference between the observed P_o of InsP3R in ULCaS and NCaS bath in [Ca2+]i > 300 nM, as observed.
**Abrogation of Ca\(^{2+}\)-dependent Inhibition of InsP\(_3\)R Channel Gating**

Our experimental results suggested that CaM is not involved in the inhibition of InsP\(_3\)R channel gating by high [Ca\(^{2+}\)]. However, it remained possible that a different molecule may be involved, and that conditions could be identified which would strip such a putative effector from the InsP\(_3\)R in the isolated nucleus, thereby rendering the InsP\(_3\)R insensitive to Ca\(^{2+}\) inhibition. We reasoned that the putative effector, as a sensor of [Ca\(^{2+}\)], might be dependent on normal [Ca\(^{2+}\)], for its association with the InsP\(_3\)R. We therefore incubated the isolated nuclei in an ultra-low Ca\(^{2+}\) bath solution (ULCaS) before using them for nuclear patch clamp experiments to determine the Ca\(^{2+}\) dependence of the InsP\(_3\)R gating.

In the first set of experiments, nuclei were isolated by protocol L (Fig. 1) into a bath of ULCaS ([Ca\(^{2+}\]) < 5 nM). In the presence of 10 \(\mu\)M cytoplasmic (pipette) [InsP\(_3\)] and [Ca\(^{2+}\)] < 20 \(\mu\)M, gating of the InsP\(_3\)R exposed to the ULCaS was very similar to that of InsP\(_3\)R in nuclei isolated directly into NCaS by protocol Nd (Fig. 4; Mak et al., 1998). In both cases, channel \(P_o\) was low (<0.2) in [Ca\(^{2+}\)] < 150 nM, it increased dramatically to 0.8 as [Ca\(^{2+}\)] was increased from 150 nM to 1\(\mu\)M, and then \(P_o\) remained at the maximum level of 0.8 when [Ca\(^{2+}\)] was further increased from 1 to 20 \(\mu\)M (Fig. 5). The InsP\(_3\)R in nuclei isolated by protocol Nd were inhibited by [Ca\(^{2+}\)] > 20 \(\mu\)M (Mak et al., 1998) but, remarkably, InsP\(_3\)R in nuclei isolated into ULCaS by protocol L exhibited robust channel activities in [Ca\(^{2+}\)] as high as 1.5 mM (Fig. 4) with no decrease in channel \(P_o\) (Fig. 5). Thus, a 20-min exposure to the ULCaS containing <5 nM Ca\(^{2+}\) caused the gating of InsP\(_3\)R channel to be no longer inhibited by high [Ca\(^{2+}\)]. All of the InsP\(_3\)R channel activities observed in the ultra-low [Ca\(^{2+}\)] bath solution also terminated abruptly after \(~30\) s, like those previously observed in the regular bath solution (Mak and Foskett, 1994, 1997).

Because of the difference between the free Ca\(^{2+}\) concentration in the high [Ca\(^{2+}\)] pipette solutions and ultra-low [Ca\(^{2+}\)] bath solutions, it is possible that a potential difference may be established across the membrane and affect the high [Ca\(^{2+}\)] inhibition of the InsP\(_3\)R and thus its \(P_o\). We performed patch clamp experiments with \(-20\) mV applied potential, using high [Ca\(^{2+}\)] pipette solution ([Ca\(^{2+}\)] = 221 \(\mu\)M) with nuclei isolated with protocol L. The InsP\(_3\)R channel \(P_d\) (9 out of 20 patches exhibited channel activity), gating kinetics (last current trace in Fig. 4), and \(P_o\) (Fig. 5) were not detectably different from that recorded at \(+20\) mV (\(P_d = 6\) out of 8 patches), indicating that the abrogation of high [Ca\(^{2+}\)] inhibition by exposure to ULCaS is not due to simple electrostatic effects that change the membrane potential.

We previously demonstrated that the Ca\(^{2+}\) dependence of channel \(P_o\) in nuclei isolated by protocol Nd into NCaS was well fitted by a biphasic Hill equation

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

with maximum open probability \(P_{max} = 0.81 \pm 0.02\), half-maximal activating [Ca\(^{2+}\)] \((K_{act}) = 210 \pm 20\) nM, activation Hill coefficient \(H_{act} = 1.9 \pm 0.3\), half-maximal inhibitory [Ca\(^{2+}\)] \((K_{inh}) = 54 \pm 3\) \(\mu\)M, and inhibitory Hill coefficient \(H_{inh} = 3.9 \pm 0.7\) (Mak et al., 1998). Our new data indicated that the InsP\(_3\)R in nuclei isolated by protocol L into ULCaS exhibited no inhibition by high [Ca\(^{2+}\)], so that the Ca\(^{2+}\) dependence of channel \(P_o\) can be fitted by a simple activating Hill equation

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

with maximum open probability \(P_{max} = 0.84 \pm 0.01,\) half-maximal activating [Ca\(^{2+}\)] \((K_{act}) = 280 \pm 30\) nM, and activation Hill coefficient \(H_{act} = 2.7 \pm 0.3\) (Fig. 5).

Nuclei isolated directly into a ULCaS bath by protocol Ld were used to determine the minimum duration of exposure to ULCaS bath required to relieve high [Ca\(^{2+}\)] inhibition of InsP\(_3\)R gating. We found that channel activities could be detected with a pipette solution containing 10 \(\mu\)M InsP\(_3\) and 290 \(\mu\)M [Ca\(^{2+}\)], no earlier than 5 min after the nucleus was isolated into the ULCaS bath. Thus, the process involved in the relief of Ca\(^{2+}\) inhibition of InsP\(_3\)R channel gating by exposure of the isolated nuclei to ULCaS is a slow one, requiring a few minutes.

To determine if normal cytoplasmic [Ca\(^{2+}\)] (~50 nM) is low enough to cause the relief of high [Ca\(^{2+}\)] inhibition of InsP\(_3\)R gating, we isolated oocyte nuclei directly in PCaS bath (protocol Pd, Fig. 1). In a series of experiments performed in areas of the nuclear membrane identified with very high \(P_o\), using pipette solutions with 10 \(\mu\)M InsP\(_3\) and 0.5 mM ATP, containing alternately 630 nM or 221 \(\mu\)M [Ca\(^{2+}\)]. InsP\(_3\)R channels were observed in seven out of seven patches with 630 nM [Ca\(^{2+}\)], but no InsP\(_3\)R channel activity was observed in any of 11 patches with 221 \(\mu\)M [Ca\(^{2+}\)], even when the nucleus was exposed to the PCaS bath for over 160 min. Thus, the normal resting [Ca\(^{2+}\)] of the cytoplasm (~50 nM) is not sufficiently low to induce the relief of Ca\(^{2+}\) inhibition observed in the ultra-low Ca\(^{2+}\) condition.

**InsP\(_3\) Dependence of the InsP\(_3\)R in ULCaS Bath**

Our previous studies (Mak et al., 1998, 2001a) revealed that InsP\(_3\) activates gating by relieving the Ca\(^{2+}\) inhibition of the channel. InsP\(_3\) increases \(K_{inh}\), the inhibitory
half-maximal $[\text{Ca}^{2+}]_i$, with no effect on the values of the channel $\text{Ca}^{2+}$-activation parameters ($K_{\text{act}}, H_{\text{act}}$) or $P_{\text{max}}$ in Eq. 1. It seemed likely that this mode of InsP$_3$ activation cannot operate if the channel is not inhibited by high $[\text{Ca}^{2+}]$, as observed after the channel had been exposed to the ULCaS bath for a few minutes. We therefore examined whether InsP$_3$ was still required to gate the InsP$_3$R under conditions that abrogated Ca$^{2+}$ inhibition of the channel.

A series of experiments was performed using nuclei isolated by protocol L into ULCaS bath, patching in regions of the nuclei identified to exhibit high $P_{\text{act}}$, with pipette solutions alternately containing either 10 $\mu$M InsP$_3$ and $[\text{Ca}^{2+}] = 755$ nM, or no InsP$_3$ and $[\text{Ca}^{2+}]$, between 60 nM and 290 $\mu$M. Again, the former solution was used to ascertain the presence of functional InsP$_3$R channels in the regions of the isolated nuclei selected for our experiments for the entire duration of the series. InsP$_3$R channel activities were observed in 27 out of 30 membrane patches in the presence of InsP$_3$, but no channel activity was detected in any of the 10 patches without InsP$_3$ (Fig. 6 A). Therefore, even though the InsP$_3$R was no longer inhibited by high $[\text{Ca}^{2+}]$, when the nucleus was isolated into ULCaS, InsP$_3$ was nonetheless still necessary for channel gating.

Because it seemed paradoxical that InsP$_3$ activates channel gating by modulating the ability of Ca$^{2+}$ to inhibit the channel, and yet InsP$_3$R channels that exhibit no high $[\text{Ca}^{2+}]$, inhibition still require InsP$_3$ for gating, we examined the effects of subsaturating [InsP$_3$] on channel gating under conditions that abolish high [Ca$^{2+}$], inhibition. It was shown previously that in the presence of a subsaturating concentration of InsP$_3$ (10–33 nM), InsP$_3$R channels isolated directly into NCaS (protocol Nd) were much more sensitive to Ca$^{2+}$ inhibition than those exposed to higher [InsP$_3$] (Mak et al., 1998). In contrast, we observed that channels in nuclei isolated into and incubated in ULCaS (protocol L), and activated by subsaturating concentrations of InsP$_3$ (10–20 nM) exhibited no Ca$^{2+}$ inhibition. Channel activities were observed in 340 $\mu$M [Ca$^{2+}$], a normally inhibiting [Ca$^{2+}$], as well as in 4.2 $\mu$M [Ca$^{2+}$], (Fig. 6 B) with similar channel $P_{\text{act}}$ (Fig. 6 C). Importantly, the maximum $P_{\text{act}}$ observed in subsaturating [InsP$_3$] was lower than that observed in saturating [InsP$_3$] (c.f. Fig. 4, $[\text{Ca}^{2+}] = 5.5$ and 340 $\mu$M, and Fig. 6 B). Within the subsaturating range, i.e., $[\text{InsP}_3] < 100$ nM, increasing [InsP$_3$] affected the channel activity mainly by tuning the value of $P_{\text{max}}$ in the simple activating Hill equation (Eq. 2) (Fig. 6 C), instead of affecting the Ca$^{2+}$ inhibitory parameters ($K_{\text{inh}}$ or $H_{\text{inh}}$), but not $P_{\text{max}}$ in the biphasic Hill equation (Eq. 1), as normally observed in the InsP$_3$R channel exposed to NCaS (Mak et al., 1998). Thus, the effect of InsP$_3$ on the InsP$_3$R channel in ULCaS was dramatically different from that observed in NCaS.

![Figure 6](image)

**Figure 6.** (A and B) Typical current traces from nuclei isolated with protocol L. Arrows indicate closed channel current levels. (A) The pipette solutions contained no InsP$_3$ and 290 $\mu$M $[\text{Ca}^{2+}]$, as tabulated. (B) The pipette solutions contained 10 nM InsP$_3$ and $[\text{Ca}^{2+}]$, as tabulated. (C) $[\text{Ca}^{2+}]$ dependence of the channel $P_{\text{act}}$ of the InsP$_3$R in the presence of various [InsP$_3$] as tabulated. The number of channels used to evaluate each of the data points ($n$) is tabulated next to the corresponding data point. Oocyte nuclei used were isolated using protocol L. The curves are simple activating Hill equation fits (Eq. 2) with the same $K_{\text{act}} = 280$ nM and $H_{\text{act}} = 2.7$. The dashed, dotted, and continuous curves have $P_{\text{max}} = 0.18, 0.35$, and 0.84 for $[\text{InsP}_3] = 10$ nM, 20 nM, and 10 $\mu$M, respectively.

**Reversibility of the Regulation by Bath $[\text{Ca}^{2+}]$ of Ca$^{2+}$ Inhibition of the InsP$_3$R Channel**

It is possible, as we stated before, that the inhibition of InsP$_3$R gating by high $[\text{Ca}^{2+}]$, is mediated by some molecule that is tightly bound to the InsP$_3$R in the NCaS bath, and that dissociates from the channel in the presence of extremely low $[\text{Ca}^{2+}]$ in the ULCaS bath. Dissociation of this putative effector from the InsP$_3$R channel can then render the channel insensitive to inhibition by high $[\text{Ca}^{2+}]$. Accordingly, after dissociation, the putative effector molecule could possibly diffuse away into the essentially infinitely large volume of the bath. If this model is correct, the loss of Ca$^{2+}$ inhibition should be irreversible. To explore the reversibility of the loss of Ca$^{2+}$ inhibition, we performed patch-clamp experiments on nuclei isolated from the same batch of oocytes using different isolation/incubation protocols. As described above, Ca$^{2+}$ inhibition was abrogated when the nuclei were isolated into ULCaS bath by pro-
Inhibition of InsP3R Channel: Is CaM Involved in the Regulation by Bath [Ca2+]?

We explored the possible role of CaM in mediating the novel regulation of Ca2+ inhibition of InsP3R gating by the bath [Ca2+]. The working hypothesis was that addition of CaM would restore normal inhibition of channel gating by high [Ca2+], after it had been relieved by exposure to the low [Ca2+] bath. Patch-clamp experiments were performed on nuclei isolated by protocol L into ULCaS bath, using a pipette solution containing 10 µM purified CaM with 10 µM InsP3 and high [Ca2+], (290 µM). Nevertheless, InsP3R channel gating was observed in the presence of CaM (Fig. 8 A) that was indistinguishable (P > 0.05, Fig. 8 E) from that observed under the same conditions without CaM (compare Fig. 4, [Ca2+]i = 290 µM). Thus, addition of CaM did not reconstitute normal high [Ca2+]i inhibition of channel gating.

We also performed a series of patch-clamp experiments on nuclei isolated from oocytes expressing the Ca2+-insensitive q.m. CaM. When the nuclei were isolated by protocol L into ULCaS bath, InsP3R channel activities were observed in high (290 µM) [Ca2+]i (six of eight patches, Fig. 8 B) as frequently as in the normally "permissive" [Ca2+]i between 500 nM and 5.5 µM (seven of eight patches). Furthermore, the channel P0 was the same as that observed in the channels in nuclei isolated by protocol L from uninjected oocytes (P >

Is CaM Involved in the Regulation by Bath [Ca2+]i of Ca2+ Inhibition of InsP3R Channel?

We explored the possible role of CaM in mediating the novel regulation of Ca2+ inhibition of InsP3R gating by different protocols, all from the same batch of oocytes. Arrows indicate closed channel current levels. All pipette solutions contained 10 µM InsP3. (A) InsP3R channel activity in 290 µM [Ca2+]i, in nuclei isolated by protocol L into ULCaS, n = 5. (B) Absence of InsP3R channel activity in 290 µM [Ca2+]i, in nuclei isolated by protocol LN, n = 11. (C) InsP3R channel gating in 5.5 µM [Ca2+]i, (n = 2) in the same nucleus as used in B. (D) InsP3R channel activity in 290 µM [Ca2+]i, in nuclei isolated by protocol LNL, n = 4.

![Figure 7](image1.png)  
**Figure 7.** Typical current traces obtained from nuclei isolated using different protocols, all from the same batch of oocytes. Arrows indicate closed channel current levels. All pipette solutions contained 10 µM InsP3. (A) InsP3R channel activity in 290 µM [Ca2+]i, in nuclei isolated by protocol L into ULCaS, n = 5. (B) Absence of InsP3R channel activity in 290 µM [Ca2+]i, in nuclei isolated by protocol LN, n = 11. (C) InsP3R channel gating in 5.5 µM [Ca2+]i, (n = 2) in the same nucleus as used in B. (D) InsP3R channel activity in 290 µM [Ca2+]i, in nuclei isolated by protocol LNL, n = 4.

![Figure 8](image2.png)  
**Figure 8.** (A) Typical current traces obtained from nuclei isolated from uninjected oocytes using protocol L with pipette solution containing 10 µM InsP3, 290 µM [Ca2+]i, and 10 µM purified CaM, n = 4. (B–D) Typical current traces obtained from nuclei isolated from oocytes expressing q.m. CaM with pipette solution containing 10 µM InsP3 and 290 µM [Ca2+]i. InsP3R channel activity was observed in 290 µM [Ca2+]i, in nuclei isolated by protocol L (B, n = 6) or protocol LNL (D, n = 8), but not in nuclei isolated by protocol LN (C, n = 9). Arrows indicate closed channel current levels. (E) Histogram of InsP3R channel P0, at 10 µM InsP3 and 290 µM [Ca2+]i, observed in various nuclei under experimental conditions as tabulated.
0.05, Fig. 8 E). In addition, expression of the q.m. CaM had no effect on the reversibility of the low [Ca\(^{2+}\)]\(_{\text{bath}}\) effect. Thus, no InsP\(_3\)R channels were detected in any of the nine patches from nuclei isolated from mutant CaM-expressing oocytes by protocol LN (Fig. 8 C). Moreover, high [Ca\(^{2+}\)]\(_{\text{bath}}\) inhibition of the channel was still completely abrogated in nuclei isolated from mutant CaM-expressing oocytes by protocol LN. Thus, channel activities were observed in 290 \(\mu\text{M} \text{[Ca}^{2+}\text{]}, \) (Fig. 8 D) with \(P_{\text{h}}\) (8 of 13 patches) similar (\(P > 0.05\)) to that in \([\text{Ca}^{2+}]\), between 500 nM and 5.5 \(\mu\text{M}\) (seven of eight patches). InsP\(_3\)R channel \(P_{\text{h}}\) in these nuclei from mutant CaM-expressing oocytes was the same as that observed in nuclei isolated from uninjected oocytes by protocol L or LNL. (\(P\) from \(t\) test was >0.05, Fig. 8 E). Therefore, there were no differences between the Ca\(^{2+}\) inhibition (or lack thereof) of InsP\(_3\)R channel gating observed in nuclei isolated from various protocols from oocytes overexpressing the mutant CaM and that observed in nuclei isolated from uninjected oocytes, under all experimental conditions.

**DISCUSSION**

**Is There a Role of CaM in Inhibition by High [Ca\(^{2+}\)]\(_{\text{bath}}\) of InsP\(_3\)R-1 Gating?**

Numerous investigations have explored the interactions between the InsP\(_3\)R and CaM, but their nature, regulation, and functional effects on intracellular Ca\(^{2+}\) signaling are still far from clear. Although it was reported that CaM binding regulates high [Ca\(^{2+}\)]\(_{\text{bath}}\) inhibition of the InsP\(_3\)R-1 channel (Hirota et al., 1999; Michikawa et al., 1999), subsequent studies using microsomal fluxes or reconstituted channels in lipid bilayers have provided contradictory evidence (Zhang and Joseph, 2001; Nosyreva et al., 2002). In this study, we investigated the possible involvement of CaM in the high [Ca\(^{2+}\)], inhibition of single-channel InsP\(_3\)R-1 gating using the nuclear patch clamp method (Mak and Foskett, 1994). This approach enables single-channel recording of endogenous and recombinant InsP\(_3\)R channels in their native membrane environment. Similar biphasic regulation by [Ca\(^{2+}\)], of both the endogenous *Xenopus* type 1 channel and the recombinant rat type 3 InsP\(_3\)R channel have been observed in previous nuclear patch clamp studies (Mak et al., 1998, 2001a). In this study, we directly explored the role of CaM in high [Ca\(^{2+}\)] inhibition of InsP\(_3\)R channel gating. We have found no evidence to support the hypothesis that inhibition of InsP\(_3\)R-1 channel activities by high [Ca\(^{2+}\)] is mediated by direct interaction between the InsP\(_3\)R channel and CaM. First, in the presence of 500 \(\mu\text{M}\) W-7, a CaM antagonist that was previously reported to alleviate Ca\(^{2+}\) inhibition of InsP\(_3\)R-1 channels reconstituted into bilayers (Michikawa et al., 1999), the X-InsP\(_3\)R-1 was still inhibited by high [Ca\(^{2+}\)], in our nuclear patch-clamping experiments (Fig. 3, A and B). Second, overexpression in oocytes of a dominant-negative, Ca\(^{2+}\)-insensitive q.m. CaM did not interfere with normal Ca\(^{2+}\) inhibition of the X-InsP\(_3\)R-1 in the oocyte nuclear envelope (Fig. 3, C and D). Third, addition of CaM (10 \(\mu\text{M}\)) to the pipette solution did not reconstitute normal Ca\(^{2+}\) inhibition of InsP\(_3\)R channel after it was abrogated by exposure of the channel to ULcAS bath (Fig. 8 A). Fourth, overexpression of q.m. CaM in oocytes did not affect the abrogation of Ca\(^{2+}\) inhibition by exposure of the channel to ULcAS bath, nor did it affect the restoration of Ca\(^{2+}\) inhibition when the channel was placed back in NCaS bath (Fig. 8, B–D). Furthermore, communoprecipitation experiments did not detect any association between CaM and InsP\(_3\)R-1 in the *Xenopus* oocytes. Therefore, whereas CaM may regulate intracellular Ca\(^{2+}\) signaling through other mechanisms, our experimental results, together with other recent publications (Zhang and Joseph, 2001; Nosyreva et al., 2002), indicate that it does not regulate inhibition of InsP\(_3\)R-1 channel gating by high [Ca\(^{2+}\)].

What then could be the mechanism of Ca\(^{2+}\) inhibition? The simplest hypothesis is that the Ca\(^{2+}\)-binding sites responsible for Ca\(^{2+}\) inhibition of channel gating are contained within the structure of the InsP\(_3\)R protein itself. Many regions of the protein have been shown to bind Ca\(^{2+}\) in vitro studies (Sienaert et al., 1996, 1997). One or more of these or as yet unidentified sites may play a role, although there are no data available that address this issue. Alternately, another molecule could perhaps be involved. The InsP\(_3\)R interacts with other proteins (Patel et al., 1999; Yang et al., 2002). Of interest, a calmodulin-like protein, CaBP1, interacts with high affinity with the ligand-binding region of the channel (Yang et al., 2002). Whereas it is highly unlikely that CaBP1 and its isoforms mediate Ca\(^{2+}\) inhibition, since they are likely neurally restricted and have been shown to stimulate channel gating (Yang et al., 2002), the identification of noncalmodulin Ca\(^{2+}\)-binding protein interactions with the receptor lends credence to the notion that a Ca\(^{2+}\)-binding protein could possibly be involved in mediating Ca\(^{2+}\) responses of the channel. Because Ca\(^{2+}\) inhibition of channel activity has been observed in a number of distinct experimental systems from different species, such a putative effector would need to be ubiquitously expressed and tightly associated with the channel.

**A Novel Regulation of [Ca\(^{2+}\)], Inhibition of InsP\(_3\)R-1 Channel Gating**

Our investigations have revealed a novel CaM-independent regulation of the InsP\(_3\)R-1 channel: abrogation of high [Ca\(^{2+}\)], inhibition of InsP\(_3\)R-1 channel gating by
exposure of the channel to ultra-low bath [Ca\(^{2+}\)] (<5 nM). The physical location of the low [Ca\(^{2+}\)]\(_{\text{bath-sensing}}\) mechanism on the InsP\(_3\)R protein is unknown. The abrogation could possibly be caused by low [Ca\(^{2+}\)] in the perinuclear space between the inner and outer nuclear envelope, to which the luminal side of the InsP\(_3\)R-1 channel is exposed. In this case, exposure to the ultra-low bath [Ca\(^{2+}\)] causes the luminal [Ca\(^{2+}\)] to fall to low levels due to uncompensated Ca\(^{2+}\) leak; and the [Ca\(^{2+}\)]\(_{\text{sensing}}\) mechanism responsible for switching high [Ca\(^{2+}\)], inhibition of InsP\(_3\)R-1 channel on and off is located on the luminal side of the InsP\(_3\)R channel. The existence of a Ca\(^{2+}\)-binding site on the luminal side of the InsP\(_3\)R-1 channel has been reported (Sienaert et al., 1996). Our previous studies indicated that the ionic composition of the solution in the perinuclear space between the inner and outer nuclear envelope is likely to be similar to that of the bath solution (Mak and Foskett, 1997). The long lag time (~300 s) between the isolation of the nucleus into the ultra-low [Ca\(^{2+}\)] bath solution and the earliest detection of InsP\(_3\)R channel activities that could not be inhibited by high [Ca\(^{2+}\)], may reflect the time required for the solution in the perinuclear space to become fully equilibrated with the bath solution, or the time taken for Ca\(^{2+}\) bound to the luminal Ca\(^{2+}\)-binding sites of the InsP\(_3\)R channel to dissociate from the sites after the drop in luminal [Ca\(^{2+}\)], or a combination of the two.

Alternately, the [Ca\(^{2+}\)]-sensing mechanism could possibly be located on the cytoplasmic side of the channel. In this case, the long lag time (~300 s) between exposure of the channel to ultra-low [Ca\(^{2+}\)] and the abrogation of high [Ca\(^{2+}\)], inhibition would imply that dissociation of Ca\(^{2+}\) from the sensing mechanism is slow (rate ~0.003 s\(^{-1}\)). Although such a sensing mechanism would be exposed to high [Ca\(^{2+}\)] in the pipette solution as soon as the giga-ohm seal was formed, InsP\(_3\)R channel activities were nevertheless observed for typically >10 s when the channel was exposed to [Ca\(^{2+}\)], ~290 \(\mu\)M before the activities abruptly terminated (Mak and Foskett, 1997; Boehning et al., 2001). Thus, binding of Ca\(^{2+}\) to the sensing mechanism to restore normal high [Ca\(^{2+}\)], inhibition must also be a very slow process (rate <0.1 s\(^{-1}\)). If the [Ca\(^{2+}\)]-sensing mechanism is in equilibrium with the cytoplasmic solution, the forward rate constant \((k_f)\) for Ca\(^{2+}\) dissociation from the [Ca\(^{2+}\)]-sensing mechanism is \(\approx 0.003\) s\(^{-1}\) and the reverse rate constant \((k_r)\) is such that 0.1 s\(^{-1}\) \(\approx k_r \times 290\) \(\mu\)M. If the [Ca\(^{2+}\)]-sensing mechanism is a simple Ca\(^{2+}\)-binding site, then the equilibrium constant \(K_{\text{inh}}\) for Ca\(^{2+}\) dissociation from the site should then be \(\approx 10\) \(\mu\)M. However, abrogation of channel inhibition was not observed in our normal bath solutions that contain 300–500 nM Ca\(^{2+}\) (Mak et al., 1998), or in our physiological Ca\(^{2+}\)-bath solution containing 50 nM free Ca\(^{2+}\). It could only be observed when bath [Ca\(^{2+}\)] was reduced to very low levels. Thus, if the [Ca\(^{2+}\)]\(_{\text{bath-sensing}}\) mechanism is located on the cytoplasmic side of the channel, it is likely to be a set of cooperative Ca\(^{2+}\)-binding sites. Further studies are necessary to distinguish whether cytoplasmic or luminal [Ca\(^{2+}\)] is being sensed in the disruption of high [Ca\(^{2+}\)], inhibition of the InsP\(_3\)R, and to determine the molecular mechanisms involved in that process.

**Mechanism of Regulation of High [Ca\(^{2+}\)], Inhibition of InsP\(_3\)R-1 Channel Gating by Exposure to Low [Ca\(^{2+}\)] Bath**

A novel allosteric model, developed in the accompanying manuscript, can account for the effect of ultra-low [Ca\(^{2+}\)] bath exposure on the abrogation of high [Ca\(^{2+}\)], inhibition as well as the effect of InsP\(_3\) to modulate maximum channel \(P_o\), rather than \(K_{\text{inh}}\) under these conditions. In brief, this model accounts for our results by postulating the existence of two functional inhibitory Ca\(^{2+}\) binding sites associated with each monomer of the tetrameric channel. One site is only inhibitory when the channel is not liganded with InsP\(_3\), because InsP\(_3\) binding relieves the Ca\(^{2+}\) inhibition imposed by this site. In contrast, the properties of the other inhibitory site are not affected by InsP\(_3\) binding. In normal physiological [Ca\(^{2+}\)], conditions, Ca\(^{2+}\) binding to this InsP\(_3\)-insensitive site provides the observed high [Ca\(^{2+}\)], inhibition \(K_{\text{inh}} \approx 50\) \(\mu\)M of the fully InsP\(_3\)-liganded channel. The ability of this InsP\(_3\)-insensitive site to be inhibitory is reversibly lost after exposure of the channel for >5 min to an ultra-low bath [Ca\(^{2+}\)] (<5 nM). Thus, the observed abrogation of high [Ca\(^{2+}\)], inhibition of channel activity in saturating [InsP\(_3\)] can be accounted for by the fact that there is no longer any functional inhibitory Ca\(^{2+}\)-binding site. On the other hand, in the absence of InsP\(_3\), the InsP\(_3\)-sensitive Ca\(^{2+}\) inhibition site is functional and keeps the channel closed. Thus, the channel still requires InsP\(_3\) to gate open even when the InsP\(_3\)-insensitive site has been disrupted by exposure to ultra-low bath [Ca\(^{2+}\)]. A detailed description of this model, which can account for these and many other features of ligand regulation of the channel observed in nuclear patch clamp experiments, is developed in the accompanying manuscript (Mak et al., 2003, in this issue).

**Are Different Sensitivities to Inhibition by High [Ca\(^{2+}\)], a Fundamental Distinguishing Feature among the InsP\(_3\)R Isoforms?**

The three isoforms of InsP\(_3\)R have complicated patterns of expression in various tissues with complex regulation by various mechanisms (Taylor et al., 1999). Because the permeation and conductance properties of the InsP\(_3\)R isoforms are very similar (Mak et al., 2000; Ramos-Franco et al., 2000), differences among the iso-
forms in localization and channel gating and its regulation are likely to be reasons for the existence of InsP$_3$R diversity. A review of published single-channel studies of various InsP$_3$R isoforms suggests that different sensitivities to inhibition by high [Ca$^{2+}$], may be one distinguishing functional feature among the various InsP$_3$R isoforms. Nevertheless, it is not clear whether such differences are intrinsic to the channels, or whether they are perhaps artificially generated by the different experimental protocols used for studying InsP$_3$R channel activity.

In the presence of $\sim$1 mM InsP$_3$, native and recombinant InsP$_3$R-1 channels (including various splice variants) reconstituted into lipid bilayers exhibited similar strong inhibition by [Ca$^{2+}$], with half-maximal inhibitory [Ca$^{2+}$] of 0.1–2 mM (Bezprozvanny et al., 1991; Ramos-Franco et al., 1998a,b; Tu et al., 2002), whereas native X. laevis and recombinant rat InsP$_3$R-1 channels studied in their native membrane environment using nuclear patch clamp techniques exhibited inhibition by high [Ca$^{2+}$], but with a significantly higher half-maximal inhibitory [Ca$^{2+}$] of $\sim$50 mM (Ma et al., 1998; Boehning et al., 2001). When reconstituted into planar bilayers, Ca$^{2+}$-inhibition of InsP$_3$R-1 could be alleviated by very high [InsP$_3$] (180 $\mu$M) (Kaftan et al., 1997; Moraru et al., 1999), whereas Ca$^{2+}$-inhibition of InsP$_3$R-1 studied in the native membrane environment was not further affected by [InsP$_3$] once the channel was saturated with [InsP$_3$] >100 nM (Ma et al., 1998).

InsP$_3$R-2 channels reconstituted in lipid bilayers exhibited variable but low sensitivity to inhibition by high [Ca$^{2+}$], with a half-maximal [Ca$^{2+}$] of $\sim$400 $\mu$M for recombinant InsP$_3$R-2 channels (Ramos-Franco et al., 2000) and >1 mM for native channels (Ramos-Franco et al., 1998b, 2000) in 1 mM InsP$_3$.

Native type 3 InsP$_3$R channels reconstituted into lipid bilayers exhibited no detectable inhibition by high [Ca$^{2+}$], and its $P_o$ remained at its maximum value (0.05) in [Ca$^{2+}$], between 1 and 100 $\mu$M in the presence of 2 $\mu$M InsP$_3$ (Hagar et al., 1998). In marked contrast, recombinant r-InsP$_3$R-3 in the nuclear membrane of oocytes is inhibited by high [Ca$^{2+}$], in an InsP$_3$-dependent manner very similar to that for X-InsP$_3$R-1 under identical experimental conditions (Ma et al., 2001a).

How can we account for such divergent results? Our studies here demonstrate that Ca$^{2+}$ inhibition of the X-InsP$_3$R-1 channel in its native membrane environment can be completely, specifically and reversibly abrogated under certain experimental conditions (after exposure to a nominally Ca$^{2+}$-free bath). Associated with this effect, the InsP$_3$ dependence of the channel $P_o$ was also changed—normally, InsP$_3$ affects the apparent affinity of the inhibitory Ca$^{2+}$-binding sites of the channel (Ma et al., 1998), whereas after UL CaS bath exposure, InsP$_3$ affects the maximum $P_o$ observed (Fig. 6 C). Of note, this InsP$_3$ dependence of maximum $P_o$ is very similar to the observed effect of InsP$_3$ on the $P_o$ of InsP$_3$R-2 channels reconstituted into lipid bilayers (Ramos-Franco et al., 1998b). These observations raise the intriguing possibility that the observed differences in the sensitivities to Ca$^{2+}$ inhibition of various InsP$_3$R isoforms may be a consequence of the different environment and/or isolation conditions to which the channels were exposed, rather than the result of differences in fundamental intrinsic characteristics of the individual isoforms. For example, the InsP$_3$R-1 and InsP$_3$R-3 channel isoforms exhibited very similar inhibition by high [Ca$^{2+}$], when they are studied in a native ER membrane environment (Mak et al., 1998, 2001a), but they behaved differently in reconstitution systems. We suggest that it is worth considering the possibility that procedures employed in the isolation and reconstitution and recording of InsP$_3$R-3 used in (Hagar et al., 1998) disrupted the normal high [Ca$^{2+}$], inhibition of the InsP$_3$R-3, causing the observed lack of Ca$^{2+}$ inhibition, in very much the same way that exposure to a UL CaS bath abrogated the high [Ca$^{2+}$], inhibition of InsP$_3$R-1 observed in this study. Whereas the procedures used in the isolation and reconstitution and recording of InsP$_3$R-1 by themselves did not eliminate high [Ca$^{2+}$], inhibition of the channel (Bezprozvanny et al., 1991; Ramos-Franco et al., 1998a,b; Tu et al., 2002), they may account for the ability, observed only in the reconstituted systems, of extremely high [InsP$_3$] to abrogate high [Ca$^{2+}$], inhibition (Kaftan et al., 1997; Moraru et al., 1999). By the same token, it is possible that the very low sensitivity to high [Ca$^{2+}$], inhibition of the InsP$_3$R-2 channel isoform reconstituted in lipid bilayers (Ramos-Franco et al., 1998b, 2000) was induced by the isolation and reconstitution and recording protocols. Obviously, these issues will need to be resolved in future studies, for example, of the Ca$^{2+}$ responses of type 2 InsP$_3$R channels in the native ER membrane environment, under the same experimental conditions as those used for the types 1 and 3 InsP$_3$R isoforms; and of the sensitivities of Ca$^{2+}$ inhibition of the other InsP$_3$R isoforms to exposure to ultra-low bath [Ca$^{2+}$]. Nevertheless, our identification in this study of conditions that can radically alter the [Ca$^{2+}$], inhibition properties of the channel suggests that careful consideration of the isolation protocols and other conditions to which InsP$_3$R channels are exposed before they are examined will be warranted in future studies.

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