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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 ($InsP_3$) receptor ($InsP_3R$), a Ca^{2+}-release channel localized to the endoplasmic reticulum, plays a critical role in generating complex cytoplasmic Ca^{2+} signals in many cell types. Three $InsP_3R$ 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 $InsP_3R$ expression is that the isoforms are differentially inhibited by high cytoplasmic free Ca^{2+} concentrations ($[Ca^{2+}]_i$), possibly due to their different interactions with calmodulin. Here, we have investigated the possible roles of calmodulin and bath $[Ca^{2+}]$ in mediating high $[Ca^{2+}]_i$ inhibition of $InsP_3R$ gating by studying single endogenous type 1 $InsP_3R$ 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 Ca^{2+}-insensitive mutant calmodulin affected inhibition of gating by high $[Ca^{2+}]_i$. However, a novel, calmodulin-independent regulation of $[Ca^{2+}]_i$ inhibition of gating was revealed: whereas channels recorded from nuclei kept in the regular bathing solution with $[Ca^{2+}]$ ~400 nM were inhibited by 290 μM $[Ca^{2+}]_i$, exposure of the isolated nuclei to a bath solution with ultra-low $[Ca^{2+}]$ (<5 nM, for ~300 s) before the patch-clamp experiments reversibly relieved Ca^{2+} inhibition, with channel activities observed in $[Ca^{2+}]_i$ up to 1.5 mM. Although $InsP_3$ activates gating by relieving high $[Ca^{2+}]_i$ inhibition, it was nevertheless still required to activate channels that lacked high $[Ca^{2+}]_i$ inhibition. Our observations suggest that high $[Ca^{2+}]_i$ inhibition of $InsP_3R$ 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 $[Ca^{2+}]_i$ inhibition may not be an immutable property of different $InsP_3R$ isoforms. Furthermore, these observations support an allosteric model in which Ca^{2+} inhibition of the $InsP_3R$ is mediated by two Ca^{2+} binding sites, only one of which is sensitive to $InsP_3$.

key words: single-channel electrophysiology • patch clamp • calcium • *Xenopus* oocyte • nucleus

INTRODUCTION

The second messenger, inositol 1,4,5-trisphosphate ($InsP_3$), 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. $InsP_3$ then diffuses through the cytoplasm to bind to its receptor ($InsP_3R$) in the ER and activate it as a Ca^{2+} channel to release Ca^{2+} stored in the ER lumen. Modulation of the cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) by $InsP_3R$-mediated Ca^{2+} release is a ubiquitous intracellular signal transduction mechanism that regulates numerous processes (Berridge, 1993).

Three isoforms of the $InsP_3R$, with spliced variants, have been identified (Joseph, 1996). Most mammalian cell types express multiple $InsP_3R$ isoforms in distinct and overlapping intracellular locations with their absolute and relative expression levels regulated by gene transcription, alternative splicing and receptor degradation that differ during different stages of cell development and in response to extracellular stimuli (Taylor et al., 1999). Furthermore, formation of hetero-tetrameric channels is possible in cell types expressing more than one $InsP_3R$ isoform (Joseph et al., 1995; Monkawa et al., 1995; Wojcikiewicz, 1995; Nucifora et al., 1996). Although this diversity of $InsP_3R$ expression is impressive, its functional correlates and physiological implications remain unclear. Studies of the single-channel properties of the various $InsP_3R$ isoforms have revealed that whereas their permeation and conductance properties are very similar (Mak et al., 2000; Ramos-Franco et al., 2000), their gating may be differentially inhibited by high $[Ca^{2+}]_i$ (Bezprozvanny et al., 1991; Hagar et al., 1998; Mak et al., 1998; Ramos-Franco et al., 1998a,b, 2000; Boehning et al., 2001; Mak et al., 2001a). Because high $[Ca^{2+}]_i$ inhibition of $InsP_3R$ channel gating may be a pivotal feedback mechanism

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Abbreviations used in this paper: $InsP_3$, inositol 1,4,5-trisphosphate; $InsP_3R$, $InsP_3$ receptor; NCaS, regular $[Ca^{2+}]$ bath solution; PCaS, physiological $[Ca^{2+}]$ bath solution; r-$InsP_3R$-3, rat type 3 $InsP_3R$; CaM, calmodulin; ULCaS, ultra-low $[Ca^{2+}]$ bath solution; *X*-$InsP_3R$-1, *Xenopus* type 1 $InsP_3R$.

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for the regulation of intracellular Ca^{2+} signaling (Taylor, 1998), it has been suggested that differential inhibition by high $[Ca^{2+}]_i$ of the different $InsP_3R$ isoforms may generate distinct Ca^{2+} signals in different cell types with different patterns of $InsP_3R$ isoform expression, and that this may be a reason for the diversity of $InsP_3R$ expression (Hagar et al., 1998).

It has been suggested that high $[Ca^{2+}]_i$ inhibition of the $InsP_3R$ 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 $InsP_3R$-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 $InsP_3R$-1 channels lacking bound CaM were not inhibited by high $[Ca^{2+}]_i$, whereas addition of CaM restored inhibition of channel gating by high $[Ca^{2+}]_i$ (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 $InsP_3R$ ($InsP_3R$-3) did not bind CaM (Yamada et al., 1995; Cardy and Taylor, 1998; Lin et al., 2000) and was not inhibited by high $[Ca^{2+}]_i$ (Hagar et al., 1998). Nevertheless, other data suggest that the role of CaM in high $[Ca^{2+}]_i$ inhibition of $InsP_3R$ channel gating is far from unequivocal. Despite the absence of detectable interaction between CaM and a mutant $InsP_3R$-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 $[Ca^{2+}]_i$ (Zhang and Joseph, 2001; Nosyreva et al., 2002). Furthermore, whereas the $InsP_3R$-3 lacks the CaM binding site present in the $InsP_3R$-1 and no interaction between $InsP_3R$-3 and CaM has been detected (Yamada et al., 1995; Cardy and Taylor, 1998; Lin et al., 2000), electrophysiological studies of the recombinant rat $InsP_3R$-3 in its native membrane environment demonstrated that it is nevertheless inhibited by high $[Ca^{2+}]_i$ (Mak et al., 2001a) with quantitative features similar to those of inhibition of the $InsP_3R$-1 in the same membrane (Mak et al., 1998).

Here, we investigated the possible effects of CaM on high $[Ca^{2+}]_i$ inhibition of the gating of single endogenous $InsP_3R$-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 $[Ca^{2+}]_i$ inhibition of $InsP_3R$-1 channel gating. Inhibition of $InsP_3R$-1 gating by high $[Ca^{2+}]_i$ can be reversibly abrogated by exposure of the channel to a bathing solution containing ultra-low $[Ca^{2+}]$ (<5 nM). Our observations indicate that inhibition of $InsP_3R$-1 channel gating by high $[Ca^{2+}]_i$ can be disrupted by environmental conditions experienced by the channel, and therefore may not be an invariant property of a specific $InsP_3R$ isoform. Furthermore, these observations support an allosteric model in which Ca^{2+} inhibition of the $InsP_3R$ 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 μg/μl) 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 d 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 μl 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 μg/ml gentamycin, and 100 μM N-acetyl-Leu-Leu-Norleucinal; Sigma-Aldrich). 80 μl of ASOS in each well was changed daily. Nuclear patch clamp experiments and immunoprecipitations were performed 2–4 d after c-RNA 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 uninjected), 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 $InsP_3R$ (type 1) and CaM was performed using oocyte lysates, as described in (Mak et al., 2000), with a specific type 1 $InsP_3R$ 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 ($[Ca^{2+}]_i$) of the pipette solutions (to which the cytoplasmic side of the $InsP_3R$ is exposed in patch-clamp experiments) was tightly controlled by buffering various amounts of added $CaCl_2$ (40–400 μM) with 500 μM of the high-affinity Ca^{2+} chelator, BAPTA (1,2-bis(*O*-aminophenoxy) ethane-*N,N,N′,N′*-tetraacetic acid; Molecular Probes) and 0.5 mM Na_2ATP (100 nM $< [Ca^{2+}]_i <$ 2.5 μM); or 500 μM of the low-affinity Ca^{2+} chelator, 5,5′-dibromo BAPTA (Molecular Probes) and 0.5 mM Na_2ATP (5 μM $< [Ca^{2+}]_i <$ 15 μM); or 0.5 mM Na_2ATP alone (15 μM $< [Ca^{2+}]_i <$ 300 μM). Solutions with $[Ca^{2+}]_i >$ 300 μM contained no Ca^{2+} chelator for buffering. The normal Ca^{2+} bath solution (NCaS) contained 500 μM BAPTA and 250 μM $CaCl_2$ (free $[Ca^{2+}] \approx$ 400–500 nM), and the physiological Ca^{2+} bath solution (PCaS) contained 500 μM BAPTA and 70 μM $CaCl_2$ (free $[Ca^{2+}] = 48 \pm 5$ nM). The free $[Ca^{2+}]$ 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 ~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 ~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 material. 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 (±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 (~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 the 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 $InsP_3R$ channel activity at a specific $[Ca^{2+}]_i$ 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 $InsP_3R$ channel, or two $InsP_3R$ 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 $InsP_3R$ 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 $InsP_3R$ 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 $InsP_3R$ Gating in Endoplasmic Reticulum Membrane

Previous single-channel patch-clamp studies of the endogenous *Xenopus* type 1 $InsP_3R$ (*X*-$InsP_3R$-1) in its native ER membrane environment revealed a biphasic regulation by $[Ca^{2+}]_i$ 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 $InsP_3R$-1 gating by high $[Ca^{2+}]_i$ (Michikawa et al., 1999). We therefore investigated the possibility that the high $[Ca^{2+}]_i$ inhibition of *X*-$InsP_3R$-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 $InsP_3R$ channel activities in membrane patches (P_d) 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 $InsP_3R$ was exposed) alternately containing either

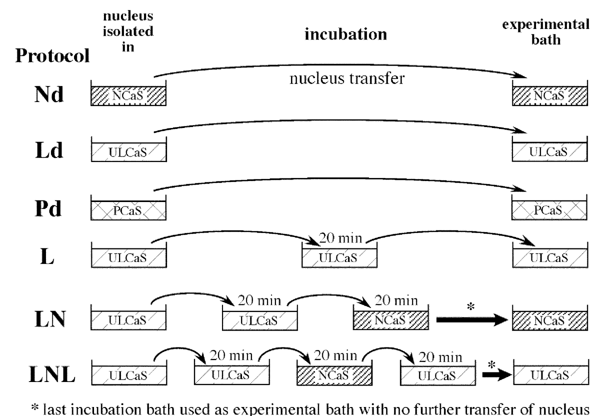

FIGURE 1. Schematic diagram showing the various protocols used to isolate oocyte nuclei for nuclear patch clamp experiments.

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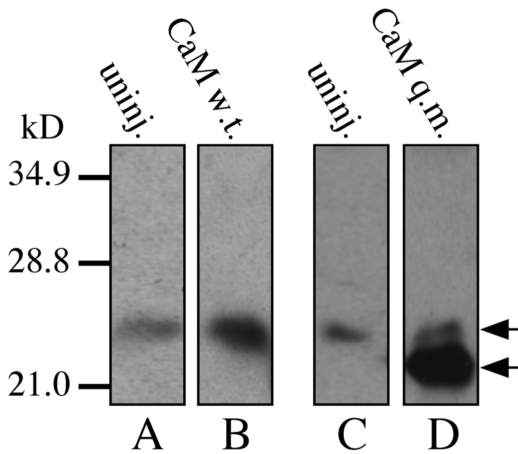

FIGURE 2. Western blots of oocyte lysates probed with a CaM antibody (both w.t. and q.m.). Lysates from oocytes injected with CaM cRNA (w.t. or q.m.) or uninjected oocytes were used as labeled. Oocytes used for lanes A and B or C and D were from the same batches, respectively. Top arrow indicates wild-type CaM and the bottom arrow indicates the quadruple mutant CaM. The slightly faster mobility of q.m. CaM is likely a reflection of the known Ca^{2+}-binding dependence of CaM mobility in gels (Xia et al., 1998).

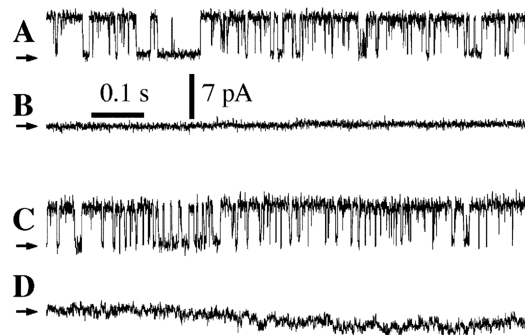

FIGURE 3. Typical current traces from nuclei in NCaS bath with pipette solutions containing 10 μM $InsP_3$. Arrows indicate closed channel current levels. (A and B) Uninjected oocytes were used. $InsP_3R$ channel activity was observed with $[Ca^{2+}]_i$ of 755 nM (A, $n = 3$), whereas no channel activity was observed in a membrane patch obtained from the same region of the same nucleus with $[Ca^{2+}]_i$ of 290 μM and the pipette solution containing 500 μM W-7 (B, $n = 5$). (C and D) Oocytes injected with CaM q.m. cRNA were used. $InsP_3R$ channel activity was observed with $[Ca^{2+}]_i$ of 2.1 μM (C, $n = 4$), whereas no channel activity was observed in a membrane patch obtained from the same region of the same nucleus with $[Ca^{2+}]_i$ of 290 μM (D, $n = 9$).

$[Ca^{2+}]_i$ = 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 $InsP_3R$ channels in the regions selected during the series of experiments. In contrast, the latter solution has $[Ca^{2+}]_i$ sufficiently high to inhibit $InsP_3R$ 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 $InsP_3R$ 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 $InsP_3R$ 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 $InsP_3R$ 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 $InsP_3R$ 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 $InsP_3R$ 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 $InsP_3R$ and not free to diffuse away into the large experimental bath. We therefore explored the possibility that Ca^{2+} inhibition of $InsP_3R$ 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 $InsP_3R$.

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 $InsP_3R$ channel gating was still inhibited by high $[Ca^{2+}]_i$: $InsP_3R$ channel activities were detected in 11 out of 11 patches with pipette solutions containing $[Ca^{2+}]_i$ = 2.1

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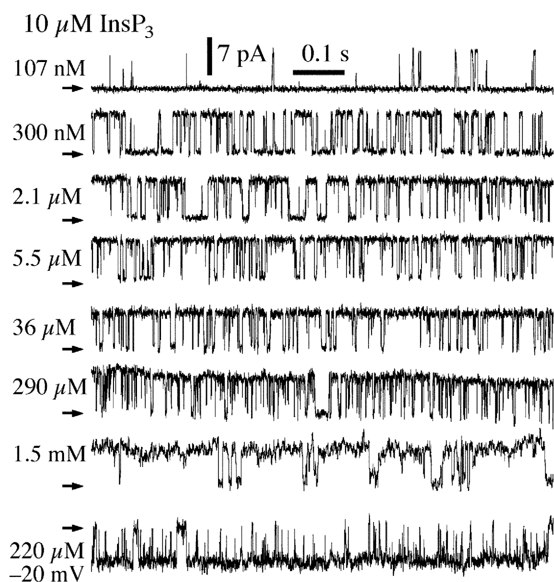

FIGURE 4. Typical current traces from nuclei in ULCaS bath isolated by protocol L. Arrows indicate closed channel current levels. Pipette solutions contained 10 μM $InsP_3$ and $[Ca^{2+}]_i$ as tabulated. The last current trace was obtained with −20 mV applied transmembrane potential. Other current traces were obtained with +20 mV applied potential.

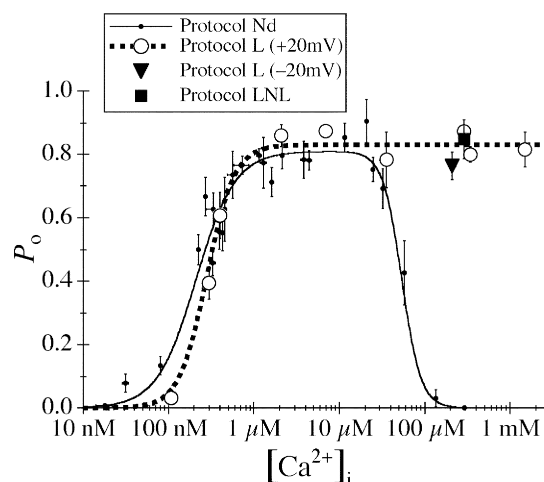

FIGURE 5. $[Ca^{2+}]_i$ dependencies of the channel P_o of the $InsP_3R$ in oocyte nuclei isolated using various protocols (Nd, L, and LNL) and applied potentials (±20 mV) as tabulated. All pipette solutions used contained 10 μM $InsP_3$. The dashed curve is a simple activating Hill equation fit for 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 $InsP_3R$ channel P_o was lower in ULCaS than in NCaS at $[Ca^{2+}]_i \approx 100$ nM. It is possible that this reflects some intrinsic properties of the $InsP_3R$ after exposure to the low bath $[Ca^{2+}]$. Alternately, this may only be an artifact as a result of the movement of free Ca^{2+} ion across the open channel. With pipette $[Ca^{2+}]_i \approx 100$ nM, when the oocyte nucleus was in NCaS ($[Ca^{2+}] = 400–500$ nM), the Nernst reversal potential for Ca^{2+} ions was ∼35 mV so Ca^{2+} ions moved across the open $InsP_3R$ channel from the lumenal side to the cytoplasmic side despite an applied transmembrane voltage of 20 mV. This could cause the effective $[Ca^{2+}]_i$ at the activating Ca^{2+}-binding sites on the cytoplasmic side of the channel to be higher than the free $[Ca^{2+}]$ in the bulk of the pipette solution if the Ca^{2+}-binding sites are close enough to the ion conducting pore. Conversely, when the nucleus was in ULCaS ($[Ca^{2+}] < 5$ nM), Ca^{2+} ions moved across the open $InsP_3R$ channel in the opposite direction, down the electrical and chemical gradients, possibly lowering the effective $[Ca^{2+}]_i$ at the Ca^{2+}-binding sites. In $[Ca^{2+}]_i < 250$ nM, the mean open channel duration ($\langle\tau_o\rangle$) of the $InsP_3R$ increases with $[Ca^{2+}]_i$ (Mak and Foskett, 1998). Therefore, if Ca^{2+} flux across the open $InsP_3R$ channel caused the effective $[Ca^{2+}]_i$ at the activating Ca^{2+}-binding sites to deviate from the free $[Ca^{2+}]$ in the bulk pipette solution, then channels in NCaS bath would have longer $\langle\tau_o\rangle$ and higher channel P_o than those in ULCaS bath, as observed. On the other hand, in $[Ca^{2+}]_i > 300$ nM, $\langle\tau_o\rangle$ does not exhibit any dependence on $[Ca^{2+}]_i$ although the mean closed channel duration ($\langle\tau_c\rangle$) is still affected by $[Ca^{2+}]_i$ (Mak and Foskett, 1998). Deviation of effective $[Ca^{2+}]$ at the Ca^{2+}-binding sites from the bulk free $[Ca^{2+}]$ would dissipate quickly by diffusion once the channel closed and therefore would not affect $\langle\tau_c\rangle$. Thus, there would be no difference between the observed P_o of $InsP_3R$ in ULCaS and NCaS bath in $[Ca^{2+}]_i > 300$ nM, as observed.

μM (Fig. 3 C), but no channel activity was detected in any of 9 patches with pipette solutions containing 290 μM $[Ca^{2+}]_i$ (Fig. 3 D). These results therefore also did not support the hypothesis that Ca^{2+} inhibition of $InsP_3R$ channel gating is mediated by CaM.

The lack of effect of overexpression of the q.m. CaM on Ca^{2+} inhibition of gating may suggest that endogenous CaM is not normally associated with the $InsP_3R$. We examined the biochemical association between the $InsP_3R$ 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 $InsP_3R$ 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 $InsP_3R$ ($n = 4$; unpublished data). These results therefore do not provide evidence of an association between CaM and the $InsP_3R$.

In summary, our single-channel patch clamp experiments revealed that neither high concentrations of a CaM antagonist, nor overexpression of a Ca^{2+}-insensitive q.m. CaM had any effect on $[Ca^{2+}]_i$ inhibition of $InsP_3R$ channel gating. In addition, coimmunoprecipitation failed to demonstrate an association between CaM and the $InsP_3R$. Thus, our investigations did not provide any evidence supporting the hypothesis that high $[Ca^{2+}]_i$ inhibition of $InsP_3R$ 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).

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Abrogation of Ca^{2+}-dependent Inhibition of $InsP_3R$ Channel Gating

Our experimental results suggested that CaM is not involved in the inhibition of $InsP_3R$ channel gating by high $[Ca^{2+}]_i$. 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 $InsP_3R$ in the isolated nucleus, thereby rendering the $InsP_3R$ insensitive to Ca^{2+} inhibition. We reasoned that the putative effector, as a sensor of $[Ca^{2+}]_i$, might be dependent on normal $[Ca^{2+}]_i$ for its association with the $InsP_3R$. 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 $InsP_3R$ 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 μM cytoplasmic (pipette) $[InsP_3]$ and $[Ca^{2+}]_i < 20$ μM, gating of the $InsP_3R$ exposed to the ULCaS was very similar to that of $InsP_3R$ 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 $[Ca^{2+}]_i < 150$ nM, it increased dramatically to 0.8 as $[Ca^{2+}]_i$ was increased from 150 nM to 1μM, and then P_o remained at the maximum level of 0.8 when $[Ca^{2+}]_i$ was further increased from 1 to 20 μM (Fig. 5). The $InsP_3R$ in nuclei isolated by protocol Nd were inhibited by $[Ca^{2+}]_i > 20$ μM (Mak et al., 1998) but, remarkably, $InsP_3R$ in nuclei isolated into ULCaS by protocol L exhibited robust channel activities in $[Ca^{2+}]_i$ 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 $InsP_3R$ channel to be no longer inhibited by high $[Ca^{2+}]_i$. All of the $InsP_3R$ 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 $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ inhibition of the $InsP_3R$ and thus its P_o. We performed patch clamp experiments with −20 mV applied potential, using high $[Ca^{2+}]_i$ pipette solution ($[Ca^{2+}]_i = 221$ μM) with nuclei isolated with protocol L. The $InsP_3R$ 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 $[Ca^{2+}]_i$ 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

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

with maximum channel open probability (P_{max}) = 0.81 ± 0.02, half-maximal activating $[Ca^{2+}]_i$ (K_{act}) = 210 ± 20 nM, activation Hill coefficient (H_{act}) = 1.9 ± 0.3, half-maximal inhibitory $[Ca^{2+}]_i$ (K_{inh}) = 54 ± 3 μM, and inhibitory Hill coefficient (H_{inh}) = 3.9 ± 0.7 (Mak et al., 1998). Our new data indicated that the $InsP_3R$ in nuclei isolated by protocol L into ULCaS exhibited no inhibition by high $[Ca^{2+}]_i$, so that the Ca^{2+} dependence of channel P_o can be fitted by a simple activating Hill equation

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

with maximum open probability P_{max} of 0.84 ± 0.01, half-maximal activating $[Ca^{2+}]_i$ (K_{act}) of 280 ± 30 nM, and activation Hill coefficient (H_{act}) of 2.7 ± 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 $[Ca^{2+}]_i$ inhibition of $InsP_3R$ gating. We found that channel activities could be detected with a pipette solution containing 10 μM $InsP_3$ and 290 μM $[Ca^{2+}]_i$ 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 $InsP_3R$ 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 $[Ca^{2+}]_i$ inhibition of $InsP_3R$ 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_d, using pipette solutions with 10 μM $InsP_3$ and 0.5 mM ATP, containing alternately 630 nM or 221 μM $[Ca^{2+}]_i$, $InsP_3R$ channels were observed in seven out of seven patches with 630 nM $[Ca^{2+}]_i$, but no $InsP_3R$ channel activity was observed in any of 11 patches with 221 μM $[Ca^{2+}]_i$, 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 $InsP_3R$ 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 $[Ca^{2+}]_i$, with no effect on the values of the channel Ca^{2+} activation parameters (K_{act}, H_{act}) or P_{max} in Eq. 1. It seemed likely that this mode of $InsP_3$ activation cannot operate if the channel is not inhibited by high $[Ca^{2+}]_i$ 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 $InsP_3R$ 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_d, with pipette solutions alternately containing either 10 μM $InsP_3$ and $[Ca^{2+}]_i$ = 755 nM, or no $InsP_3$ and $[Ca^{2+}]_i$ between 60 nM and 290 μM. Again, the former solution was used to ascertain the presence of functional $InsP_3R$ channels in the regions of the isolated nuclei selected for our experiments for the entire duration of the series. $InsP_3R$ 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 $InsP_3R$ was no longer inhibited by high $[Ca^{2+}]_i$ 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 $InsP_3R$ channels that exhibit no high $[Ca^{2+}]_i$ inhibition still require $InsP_3$ for gating, we examined the effects of subsaturating $[InsP_3]$ on channel gating under conditions that abolish high $[Ca^{2+}]_i$ inhibition. It was shown previously that in the presence of a subsaturating concentration of $InsP_3$ (10–33 nM), $InsP_3R$ 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 μM $[Ca^{2+}]_i$, a normally inhibiting $[Ca^{2+}]_i$, as well as in 4.2 μM $[Ca^{2+}]_i$ (Fig. 6 B) with similar channel P_o (Fig. 6 C). Importantly, the maximum P_o observed in subsaturating $[InsP_3]$ was lower than that observed in saturating $[InsP_3]$ (c.f. Fig. 4, $[Ca^{2+}]_i$ = 5.5 and 340 μM, and Fig. 6 B). Within the subsaturating range, i.e., $[InsP_3]$ <100 nM, increasing $[InsP_3]$ affected the channel activity mainly by tuning the value of P_{max} in the simple activating Hill equation (Eq. 2) (Fig. 6 C), instead of affecting the Ca^{2+} inhibitory parameters (K_{inh} or H_{inh}), but not P_{max} in the biphasic Hill equation (Eq. 1), as normally observed in the $InsP_3R$ channel exposed to NCaS (Mak et al., 1998). Thus, the effect of $InsP_3$ on the $InsP_3R$ channel in ULCaS was dramatically different from that observed in NCaS.

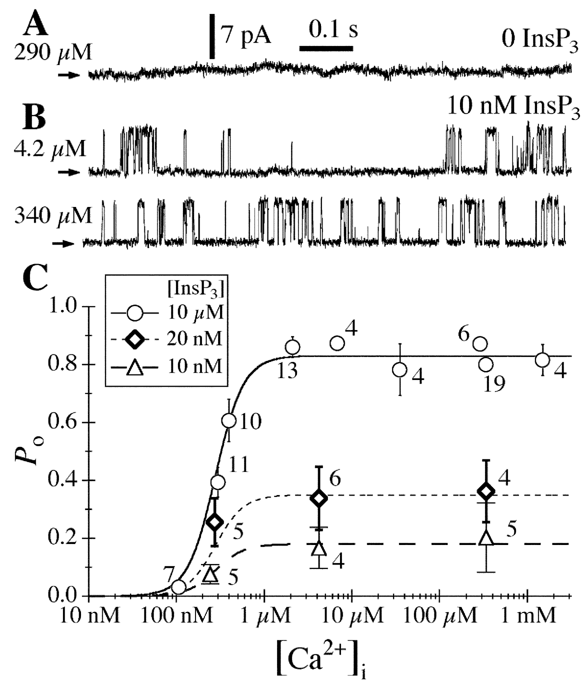

FIGURE 6. (A and B) Typical current traces from nuclei isolated with protocol L. Arrows indicate closed channel current levels. (A) The pipette solutions contained no $InsP_3$ and 290 μM $[Ca^{2+}]_i$ as tabulated. (B) The pipette solutions contained 10 nM $InsP_3$ and $[Ca^{2+}]_i$ as tabulated. (C) $[Ca^{2+}]_i$ dependence of the channel P_o of the $InsP_3R$ 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_{act} = 280 nM and H_{act} = 2.7. The dashed, dotted, and continuous curves have P_{max} = 0.18, 0.35, and 0.84 for $[InsP_3]$ = 10 nM, 20 nM, and 10 μM, respectively.

Reversibility of the Regulation by Bath $[Ca^{2+}]$ of Ca^{2+} Inhibition of the $InsP_3R$ Channel

It is possible, as we stated before, that the inhibition of $InsP_3R$ gating by high $[Ca^{2+}]_i$ is mediated by some molecule that is tightly bound to the $InsP_3R$ in the NCaS bath, and that dissociates from the channel in the presence of extremely low $[Ca^{2+}]$ in the ULCaS bath. Dissociation of this putative effector from the $InsP_3R$ channel can then render the channel insensitive to inhibition by high $[Ca^{2+}]_i$. 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-

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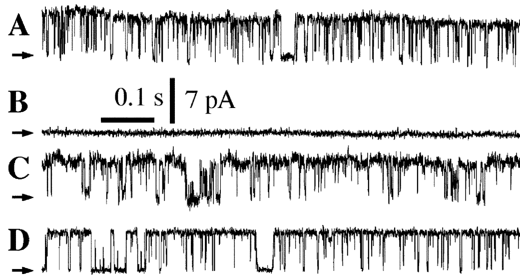

FIGURE 7. Typical current traces obtained from nuclei isolated using different protocols, all from the same batch of oocytes. Arrows indicate closed channel current levels. All pipette solutions contained 10 μM $InsP_3$. (A) $InsP_3R$ channel activity in 290 μM $[Ca^{2+}]_i$ in nuclei isolated by protocol L into ULCaS, $n = 5$. (B) Absence of $InsP_3R$ channel activity in 290 μM $[Ca^{2+}]_i$ in nuclei isolated by protocol LN, $n = 11$. (C) $InsP_3R$ channel gating in 5.5 μM $[Ca^{2+}]_i$ ($n = 2$) in the same nucleus as used in B. (D) $InsP_3R$ channel activity in 290 μM $[Ca^{2+}]_i$ in nuclei isolated by protocol LNL, $n = 4$.

tocol L (Fig. 7 A). However, when the nuclei were returned to the NCaS bath for 20 min before patch clamping (protocol LN, Fig. 1), no $InsP_3R$ channel activities were detected at $[Ca^{2+}]_i$ = 290 μM (Fig. 7 B) in any of the 11 patches obtained, even though channel gating was observed in 4 out of 5 patches using pipette solutions with $[Ca^{2+}]_i$ = 5.5 μM (Fig. 7 C). Thus, despite prior exposure to ULCaS, normal Ca^{2+} inhibition of $InsP_3R$ channel gating was restored when the nuclei were transferred back into NCaS. This restoration of normal Ca^{2+} inhibition was in turn reversible. Reexposure of the nuclei to ULCaS (protocol LNL, Fig. 1) again eliminated normal Ca^{2+} inhibition of gating (Fig. 7 D). The $InsP_3R$ channels in nuclei isolated by protocol LNL exhibited the same P_o (Fig. 5, filled square) as those in nuclei isolated into ULCaS by protocol L without ever being exposed to NCaS (Fig. 5, open circles). These experiments indicated, first, that abolition of Ca^{2+} inhibition of channel gating by exposure of nuclei to ultra-low bath $[Ca^{2+}]$ was fully reversible, and second, that it was affected only by the $[Ca^{2+}]$ of the bathing solution in which the patch-clamp experiments were performed, independent of the history of bath $[Ca^{2+}]$ to which the nuclei were previously exposed. These results suggest either that the sensitivity of Ca^{2+} inhibition of the $InsP_3R$ to the bath $[Ca^{2+}]$ is an intrinsic property of the $InsP_3R$ channel, or that it is mediated by some molecule that remains in a stable complex with the channel throughout the multiple transfers of the nucleus into various baths containing different $[Ca^{2+}]$.

Is CaM Involved in the Regulation by Bath $[Ca^{2+}]$ of Ca^{2+} Inhibition of $InsP_3R$ Channel?

We explored the possible role of CaM in mediating the novel regulation of Ca^{2+} inhibition of $InsP_3R$ gating by the bath $[Ca^{2+}]$. The working hypothesis was that addition of CaM would restore normal inhibition of channel gating by high $[Ca^{2+}]_i$ after it had been relieved by exposure to the low $[Ca^{2+}]$ 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 $InsP_3$ and high $[Ca^{2+}]_i$ (290 μM). Nevertheless, $InsP_3R$ 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, $[Ca^{2+}]_i$ = 290 μM). Thus, addition of CaM did not reconstitute normal high $[Ca^{2+}]_i$ inhibition of channel gating.

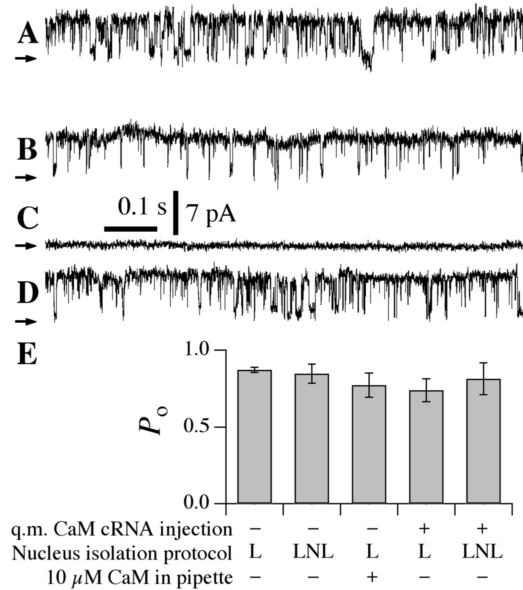

FIGURE 8. (A) Typical current traces obtained from nuclei isolated from uninjected oocytes using protocol L with pipette solution containing 10 μM $InsP_3$, 290 μM $[Ca^{2+}]_i$, and 10 μM purified CaM, $n = 4$. (B–D) Typical current traces obtained from nuclei isolated from oocytes expressing q.m. CaM with pipette solution containing 10 μM $InsP_3$ and 290 μM $[Ca^{2+}]_i$. $InsP_3R$ channel activity was observed in 290 μM $[Ca^{2+}]_i$ 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 $InsP_3R$ channel P_o at 10 μM $InsP_3$ and 290 μM $[Ca^{2+}]_i$ observed in various nuclei under experimental conditions as tabulated.

We also performed a series of patch-clamp experiments on nuclei isolated from oocytes expressing the Ca^{2+}-insensitive q.m. CaM. When the nuclei were isolated by protocol L into ULCaS bath, $InsP_3R$ channel activities were observed in high (290 μM) $[Ca^{2+}]_i$ (six of eight patches, Fig. 8 B) as frequently as in the normally "permissive" $[Ca^{2+}]_i$ between 500 nM and 5.5 μM (seven of eight patches). Furthermore, the channel P_o was the same as that observed in the channels in nuclei isolated by protocol L from uninjected oocytes (P >

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0.05, Fig. 8 E). In addition, expression of the q.m. CaM had no effect on the reversibility of the low $[Ca^{2+}]$ bath effect. Thus, no $InsP_3R$ 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 $[Ca^{2+}]_i$ inhibition of the channel was still completely abrogated in nuclei isolated from mutant CaM-expressing oocytes by protocol LNL. Thus, channel activities were observed in 290 μM $[Ca^{2+}]_i$ (Fig. 8 D) with P_d (8 of 13 patches) similar ($P > 0.05$) to that in $[Ca^{2+}]_i$ between 500 nM and 5.5 μM (seven of eight patches). $InsP_3R$ channel P_o 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 $InsP_3R$ channel gating observed in nuclei isolated by 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 $[Ca^{2+}]_i$ of $InsP_3R$-1 Gating?

Numerous investigations have explored the interactions between the $InsP_3R$ 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 $[Ca^{2+}]_i$ inhibition of the $InsP_3R$-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 $[Ca^{2+}]_i$ inhibition of single-channel $InsP_3R$-1 gating using the nuclear patch clamp method (Mak and Foskett, 1994). This approach enables single-channel recording of endogenous and recombinant $InsP_3R$ channels in their native membrane environment. Similar biphasic regulation by $[Ca^{2+}]_i$ of both the endogenous *Xenopus* type 1 channel and the recombinant rat type 3 $InsP_3R$ 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 $[Ca^{2+}]_i$ inhibition of $InsP_3R$ channel gating. We have found no evidence to support the hypothesis that inhibition of $InsP_3R$-1 channel activities by high $[Ca^{2+}]_i$ is mediated by direct interaction between the $InsP_3R$ channel and CaM. First, in the presence of 500 μM W-7, a CaM antagonist that was previously reported to alleviate Ca^{2+} inhibition of $InsP_3R$-1 channels reconstituted into bilayers (Michikawa et al., 1999), the *X*-$InsP_3R$-1 was still inhibited by high $[Ca^{2+}]_i$ 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*-$InsP_3R$-1 in the oocyte nuclear envelope (Fig. 3, C and D). Third, addition of CaM (10 μM) to the pipette solution did not reconstitute normal Ca^{2+} inhibition of $InsP_3R$ 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, coimmunoprecipitation experiments did not detect any association between CaM and $InsP_3R$-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 $InsP_3R$-1 channel gating by high $[Ca^{2+}]_i$.

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 $InsP_3R$ protein itself. Many regions of the protein have been shown to bind Ca^{2+} in 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 $InsP_3R$ 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 $[Ca^{2+}]_i$ Inhibition of $InsP_3R$-1 Channel Gating

Our investigations have revealed a novel CaM-independent regulation of the $InsP_3R$-1 channel: abrogation of high $[Ca^{2+}]_i$ inhibition of $InsP_3R$-1 channel gating by

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exposure of the channel to ultra-low bath [Ca^{2+}] (<5 nM). The physical location of the low [Ca^{2+}]$_{bath}$-sensing mechanism on the $InsP_3R$ 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 lumenal side of the $InsP_3R$-1 channel is exposed. In this case, exposure to the ultra-low bath [Ca^{2+}] causes the lumenal [Ca^{2+}] to fall to low levels due to uncompensated Ca^{2+} leak; and the [Ca^{2+}] sensing mechanism responsible for switching high [Ca^{2+}]$_i$ inhibition of $InsP_3R$-1 channel on and off is located on the lumenal side of the $InsP_3R$ channel. The existence of a Ca^{2+}-binding site on the lumenal side of the $InsP_3R$-1 channel has been reported (Sienaert et al., 1996). Our previous studies indicated that the ionic composition of the solution in the perinuclear space of the isolated oocyte nucleus 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 $InsP_3R$ channel activities that could not be inhibited by high [Ca^{2+}]$_i$ 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 lumenal Ca^{2+}-binding sites of the $InsP_3R$ channel to dissociate from the sites after the drop in lumenal [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+}]$_i$ 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, $InsP_3R$ channel activities were nevertheless observed for typically >10 s when the channel was exposed to [Ca^{2+}]$_i$ ~290 μM 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+}]$_i$ 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 ≈0.003 s^{-1} and the reverse rate constant (k_r) is such that 0.1 s^{-1} ≈ k_r × 290 μM. If the [Ca^{2+}]-sensing mechanism is a simple Ca^{2+} binding site, then the equilibrium constant K (k_f/k_r) for Ca^{2+} dissociation from the site should then be ≈10 μM. 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+}]$_{bath}$-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 lumenal [Ca^{2+}] is being sensed in the disruption of high [Ca^{2+}]$_i$ inhibition of the $InsP_3R$, and to determine the molecular mechanisms involved in that process.

Mechanism of Regulation of High [Ca^{2+}]$_i$ Inhibition of $InsP_3R$-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+}]$_i$ inhibition as well as the effect of $InsP_3$ to modulate maximum channel P_o, rather than K_{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+}]$_i$ conditions, Ca^{2+} binding to this $InsP_3$-insensitive site provides the observed high [Ca^{2+}]$_i$ inhibition (K_{inh} ~50 μM) 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+}]$_i$ 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+}]$_i$ a Fundamental Distinguishing Feature among the $InsP_3R$ Isoforms?

The three isoforms of $InsP_3R$ 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 $InsP_3R$ isoforms are very similar (Mak et al., 2000; Ramos-Franco et al., 2000), differences among the iso-

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forms in localization and channel gating and its regulation are likely to be reasons for the existence of $InsP_3R$ diversity. A review of published single-channel studies of various $InsP_3R$ isoforms suggests that different sensitivities to inhibition by high $[Ca^{2+}]_i$ may be one distinguishing functional feature among the various $InsP_3R$ 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 $InsP_3R$ channel activity.

In the presence of ~1 μM $InsP_3$, native and recombinant $InsP_3R$-1 channels (including various splice variants) reconstituted into lipid bilayers exhibited similar strong inhibition by $[Ca^{2+}]_i$ with half-maximal inhibitory $[Ca^{2+}]_i$ of 0.1–2 μM (Bezprozvanny et al., 1991; Ramos-Franco et al., 1998a,b; Tu et al., 2002), whereas native *Xenopus* and recombinant rat $InsP_3R$-1 channels studied in their native membrane environment using nuclear patch clamp techniques exhibited inhibition by high $[Ca^{2+}]_i$, but with a significantly higher half-maximal inhibitory $[Ca^{2+}]_i$ of ~50 μM (Mak et al., 1998; Boehning et al., 2001). When reconstituted into planar bilayers, Ca^{2+} inhibition of $InsP_3R$-1 could be alleviated by very high $[InsP_3]$ (180 μM) (Kaftan et al., 1997; Moraru et al., 1999), whereas Ca^{2+} inhibition of $InsP_3R$-1 studied in the native membrane environment was not further affected by $[InsP_3]$ once the channel was saturated with $[InsP_3]$ >100 nM (Mak et al., 1998).

$InsP_3R$-2 channels reconstituted in lipid bilayers exhibited variable but low sensitivity to inhibition by high $[Ca^{2+}]_i$, with a half-maximal $[Ca^{2+}]_i$ of ~400 μM for recombinant $InsP_3R$-2 channels (Ramos-Franco et al., 2000) and >1 mM for native channels (Ramos-Franco et al., 1998b, 2000) in 1 μM $InsP_3$.

Native type 3 $InsP_3R$ channels reconstituted into lipid bilayers exhibited no detectable inhibition by high $[Ca^{2+}]_i$ and its P_o remained at its maximum value (~0.05) in $[Ca^{2+}]_i$ between 1 and 100 μM in the presence of 2 μM $InsP_3$ (Hagar et al., 1998). In marked contrast, recombinant r-$InsP_3R$-3 in the nuclear membrane of oocytes is inhibited by high $[Ca^{2+}]_i$ in an $InsP_3$-dependent manner very similar to that for *X*-$InsP_3R$-1 under identical experimental conditions (Mak et al., 2001a).

How can we account for such divergent results? Our studies here demonstrate that Ca^{2+} inhibition of the *X*-$InsP_3R$-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 (Mak et al., 1998), whereas after ULCaS 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 $InsP_3R$-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 $InsP_3R$ 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 $InsP_3R$-1 and $InsP_3R$-3 channel isoforms exhibited very similar inhibition by high $[Ca^{2+}]_i$ 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 the $InsP_3R$-3 used in (Hagar et al., 1998) disrupted the normal high $[Ca^{2+}]_i$ inhibition of the $InsP_3R$-3, causing the observed lack of Ca^{2+} inhibition, in very much the same way that exposure to a ULCaS bath abrogated the high $[Ca^{2+}]_i$ inhibition of $InsP_3R$-1 observed in this study. Whereas the procedures used in the isolation and reconstitution and recording of $InsP_3R$-1 by themselves did not eliminate high $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ inhibition (Kaftan et al., 1997; Moraru et al., 1999). By the same token, it is possible that the very low sensitivity to high $[Ca^{2+}]_i$ inhibition of the $InsP_3R$-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 $InsP_3R$ channels in the native ER membrane environment, under the same experimental conditions as those used for the types 1 and 3 $InsP_3R$ isoforms; and of the sensitivities of Ca^{2+} inhibition of the other $InsP_3R$ isoforms to exposure to ultra-low bath $[Ca^{2+}]$. Nevertheless, our identification in this study of conditions that can radically alter the $[Ca^{2+}]_i$ inhibition properties of the channel suggests that careful consideration of the isolation protocols and other conditions to which $InsP_3R$ channels are exposed before they are examined will be warranted in future studies.

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