Regulation by Ca^{2+} and Inositol 1,4,5-Trisphosphate (InsP$_3$) of Single Recombinant Type 3 InsP$_3$ Receptor Channels: Ca^{2+} Activation Uniquely Distinguishes Types 1 and 3 InsP$_3$ Receptors

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ABSTRACT   The inositol 1,4,5-trisphosphate (InsP$_3$) receptor (InsP$_3$R) is an endoplasmic reticulum–localized Ca^{2+}-release channel that controls complex cytoplasmic Ca^{2+} signaling in many cell types. At least three InsP$_3$Rs encoded by different genes have been identified in mammalian cells, with different primary sequences, subcellular locations, variable ratios of expression, and heteromultimer formation. To examine regulation of channel gating of the type 3 isoform, recombinant rat type 3 InsP$_3$R (r-InsP$_3$R-3) was expressed in *Xenopus* oocytes, and single-channel recordings were obtained by patch-clamp electrophysiology of the outer nuclear membrane. Gating of the r-InsP$_3$R-3 exhibited a biphasic dependence on cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$). In the presence of 0.5 mM cytoplasmic free ATP, r-InsP$_3$R-3 gating was inhibited by high $[Ca^{2+}]_i$ with features similar to those of the endogenous *Xenopus* type 1 InsP$_3$R (*X*-InsP$_3$R-1). Ca^{2+} inhibition of channel gating had an inhibitory Hill coefficient of $\sim$3 and half-maximal inhibiting $[Ca^{2+}]_i$ (K_{inh}) = 39 μM under saturating (10 μM) cytoplasmic InsP$_3$ concentrations ($[InsP_3]$). At $[InsP_3] < 100$ nM, the r-InsP$_3$R-3 became more sensitive to Ca^{2+} inhibition, with the InsP$_3$ concentration dependence of K_{inh} described by a half-maximal $[InsP_3]$ of 55 nM and a Hill coefficient of $\sim$4. InsP$_3$ activated the type 3 channel by tuning the efficacy of Ca^{2+} to inhibit it, by a mechanism similar to that observed for the type 1 isoform. In contrast, the r-InsP$_3$R-3 channel was uniquely distinguished from the *X*-InsP$_3$R-1 channel by its enhanced Ca^{2+} sensitivity of activation (half-maximal activating $[Ca^{2+}]_i$ of 77 nM instead of 190 nM) and lack of cooperativity between Ca^{2+} activation sites (activating Hill coefficient of 1 instead of 2). These differences endow the InsP$_3$R-3 with high gain InsP$_3$–induced Ca^{2+} release and low gain Ca^{2+}–induced Ca^{2+} release properties complementary to those of InsP$_3$R-1. Thus, distinct Ca^{2+} signals may be conferred by complementary Ca^{2+} activation properties of different InsP$_3$R isoforms.

KEY WORDS: single-channel electrophysiology • patch-clamp • *Xenopus* oocyte • nucleus • Ca^{2+} release channel

INTRODUCTION

Modulation of cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) in response to the second messenger inositol 1,4,5-trisphosphate (InsP$_3$)[1] provides a ubiquitous signaling system. InsP$_3$-mediated Ca^{2+} signals are often complex, being precisely controlled in both time and space as repetitive spikes or oscillations and as propagating waves that initiate at specific locations in the cell (Boitano et al., 1992; Lechleiter and Clapham, 1992; Amundson and Clapham, 1993; Atri et al., 1993; Berridge, 1993; Rooney and Thomas, 1993; Bootman and Berridge, 1995; Clapham, 1995; Toescu, 1995). A family of InsP$_3$ receptors (InsP$_3$Rs) with different primary sequences derived from different genes has been identified (Furuichi et al., 1989; Mignery et al., 1989; Sudhof et al., 1991; Blondel et al., 1993; De Smedt et al., 1994; Maranto, 1994) with alternatively spliced isoforms (Danoff et al., 1991; Nakagawa et al., 1991; Ferris and Snyder, 1992). The InsP$_3$Rs are $\sim$2,700 amino acid integral membrane proteins (Furuichi et al., 1994) that exist as tetramers (Supattapone et al., 1988; Maeda et al., 1991) in the endoplasmic reticulum (ER). Full-length sequences of cDNAs for three distinct isoforms (InsP$_3$R-1, InsP$_3$R-2, and InsP$_3$R-3) are 60–80% homologous (Furuichi et al., 1989; Mignery et al., 1989; Sudhof et al., 1991; De Smedt et al., 1994; Maranto, 1994; Joseph, 1995). The different isoforms have distinct and overlapping patterns of expression in different tissues (Maranto, 1994; Fujino et al., 1995; Furuichi and Mikoshiba, 1995). Most cells express more than one isoform (Bush et al., 1994; De Smedt et al., 1994; Newton et al., 1994; Sugiyama et al., 1994; Fujino et al., 1995; Joseph et al., 1995; Nucifora et al., 1996), and expression levels, both absolute and relative to other isoforms, can be modified during cell differentiation (Nakagawa et al., 1991; Kume et al., 1993) and by use-dependent degra-

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[1]*Abbreviations used in this paper:* CICR, Ca^{2+}-induced Ca^{2+} release; InsP$_3$, inositol 1,4,5-trisphosphate; ER, endoplasmic reticulum; IICR, InsP$_3$-induced Ca^{2+} release; PIP$_2$, phosphatidylinositol 4,5-bisphosphate; P_o, open probability; r-InsP$_3$R-3, rat type 3 InsP$_3$R; *X*-InsP$_3$R-1, *Xenopus* type 1 InsP$_3$R.

dation (Magnusson et al., 1993; Wojcikiewicz et al., 1994; Honda et al., 1995; Wojcikiewicz, 1995). In tissues that express more than one type of InsP$_3$R, isoform-specific antibodies immunoprecipitate others, suggesting that receptors may associate in heteroligomeric complexes (Joseph et al., 1995; Monkawa et al., 1995; Wojcikiewicz and He, 1995; Nucifora et al., 1996).

The diversity of InsP$_3$R expression in mammalian cells is impressive, suggesting that cells require distinct InsP$_3$Rs to provide unique Ca^{2+} signals and to regulate specific functions. Nevertheless, the functional correlates and physiological implications of this diversity are still unclear. Electrophysiological observations of all three isoforms have now been reported. The single-channel properties of the type 1 InsP$_3$R have been examined by reconstitution of mammalian channels in lipid bilayer membranes (Bezprozvanny et al., 1991, 1994; Watras et al., 1991; Bezprozvanny and Ehrlich, 1994), as well as by patch-clamp of the outer nuclear membrane of *Xenopus* oocytes (Mak and Foskett, 1994, 1997, 1998; Stehno-Bittel et al., 1995). The type 2 receptor was recently examined by bilayer reconstitution (Perez et al., 1997; Ramos-Franco et al., 1998). To date, there have been two sets of reports of type 3 channel activity. In one, bilayer reconstitution of membranes from a cell type which expressed more type 3 relative to other isoforms was used (Hagar et al., 1998; Hagar and Ehrlich, 2000); the other used patch-clamp electrophysiology of the outer nuclear membrane of *Xenopus* oocytes engineered to express the recombinant rat type 3 receptor (Mak et al., 2000). Together, these studies have demonstrated that the ion permeation properties of the different InsP$_3$R isoforms are highly conserved, even across species (Mak et al., 2000). These results have therefore suggested that distinctions among channel isoforms may instead reside in their differential regulation or intracellular localization. To begin to address this issue, we describe here the regulation by [Ca^{2+}]$_i$ and cytoplasmic InsP$_3$ concentration ([InsP$_3$]) of the gating of recombinant rat type 3 InsP$_3$R (r-InsP$_3$R-3) expressed in *Xenopus* oocytes. We used patch-clamp electrophysiology to study single recombinant channels in the outer membrane of the nuclear envelope of isolated *Xenopus* oocyte nuclei. In addition, the results obtained in this study have been compared with those obtained from the *Xenopus* type 1 InsP$_3$R (*X*-InsP$_3$R-1) in the same physiologically relevant membrane system (Mak et al., 1998). Our results reveal that, in addition to sharing similar permeation and gating properties, the two isoforms have highly similar responses to InsP$_3$ and inhibition by high [Ca^{2+}]$_i$. However, important differences exist in the responses of the two isoforms to activation by [Ca^{2+}]$_i$. Distinct Ca^{2+} activation responses confer on these channels unique Ca^{2+}-induced Ca^{2+} release (CICR) properties, which likely contribute to dis-

tinct spatial and temporal Ca^{2+} signals in cells expressing different and multiple InsP$_3$R isoforms.

MATERIALS AND METHODS

Selection and Microinjection of Xenopus Oocytes

Maintenance of *Xenopus laevis* and surgical extraction of ovaries were carried out as described previously (Mak and Foskett, 1994, 1997, 1998). Because oocytes have endogenous InsP$_3$R (*X*-InsP$_3$R-1), it was necessary to distinguish them from expressed channels in our patch-clamp experiments (Mak et al., 2000). Endogenous *X*-InsP$_3$R-1 channel activity detected in patch-clamp studies of oocyte nuclei is highly variable from batch to batch of oocytes, although the activity level among oocytes from the same batch is very consistent (Mak and Foskett, 1994). Therefore, for each new batch of oocytes, a day of patch-clamping of isolated nuclei (at least 6 nuclei, 6–10 patches from each) was performed, to determine the endogenous channel expression level. Only batches with extremely low channel activity (<1 out of 15 patches exhibited InsP$_3$R channel activity) were used for subsequent cRNA injections. Out of 496 nuclear patches from uninjected oocytes in selected batches, only 19 channels were detected in 11 patches. The mean number of InsP$_3$R channels per nuclear patch was 0.038.

Oocytes selected for microinjection were defolliculated as described previously (Jiang et al., 1998). 23 nl of rat InsP$_3$R-3 cRNA (1 µg/µl) was injected into the cytoplasm of defolliculated oocytes as described previously (Mak et al., 2000). cRNA-injected and uninjected but defolliculated control oocytes were placed 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 sodium 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 performed at various times after surgical extraction of the oocytes using control, uninjected oocytes maintained under identical conditions as cRNA-injected oocytes demonstrated that the probability of detection of endogenous InsP$_3$R channel in a nuclear patch never increased over time after oocyte isolation (Mak et al., 2000). Furthermore, Western blot analysis (Fig. 1) showed that injection of the recombinant r-InsP$_3$R-3 cRNA induced the expression of type 3 InsP$_3$R in oocytes (Fig. 1, lanes I and J) without causing any detectable increase in the level of expression of the endogenous type 1 InsP$_3$R (Fig. 1, lanes A, C, E, and G) compared with uninjected control oocytes (Fig. 1, lanes B, D, F, and H). The Western blots also showed that the InsP$_3$R-1– and InsP$_3$R-3–specific antibodies used in our experiments had very little cross-reactivity.
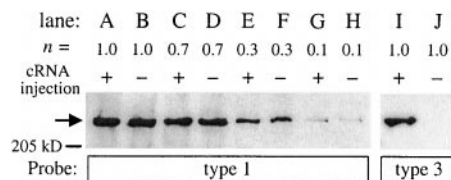

FIGURE 1. Expression of endogenous *X*-InsP$_3$R-1 and recombinant r-InsP$_3$R-3 in cRNA-injected (+) and control, uninjected (−) *Xenopus* oocytes. Western analysis was performed as described in Mak et al. (2000). (A–H) Immunoblotted with InsP$_3$R-1-specific antibody (Joseph and Samanta, 1993; Joseph et al., 1995); (I–J) immunoblotted with InsP$_3$R-3-specific antibody (Transduction Labs.). Aliquots equivalent to *n* oocytes from the same lysate sample were used in the lanes.

Patch-clamp studies were performed 4–5 d after cRNA microinjection. The mean number of InsP$_3$R per nuclear patch for the cRNA-injected oocytes increased dramatically by 47-fold, from 0.038 to 1.80. In 1,020 experiments, 1,831 channels were detected in 518 patches, with 354 of the patches exhibiting multiple InsP$_3$R channels. If we assume a random, binomial association of X-InsP$_3$R-1 and r-InsP$_3$R-3 to form tetrameric channels, most (91.8%) of the channels detected in cRNA-injected oocytes were homotetrameric r-InsP$_3$R-3 channels. This value probably underestimates the percentage of homotetrameric r-InsP$_3$R-3 channels because of the higher probability of heterologously expressed channels to associate with other heterologously expressed channel monomers during protein biogenesis rather than with endogenous channels, due to the pronounced mismatch of the protein translation rates of expressed versus endogenous channels (Joseph et al., 2000).

Patch-clamping the Oocyte Nucleus

Patch-clamp experiments were performed as described (Mak and Foskett, 1994, 1997, 1998). In brief, stage V or VI oocytes were opened mechanically just before use, and the nucleus was separated from the cytoplasm for patch-clamping. Like the X-IP$_3$R-1 (Mak and Foskett, 1994, 1997), the r-InsP$_3$R-3 inactivated under constant [Ca^{2+}]$_i$, [InsP$_3$], and applied potential (mean channel activity duration $\sim$2 min). Thus, experiments were done in the "on-nucleus" configuration, with the solution in the perinuclear lumen between the outer and inner nuclear membranes in apparent equilibrium with the bath solution (Mak and Foskett, 1994). As inactivation was generally abrupt, with no detectable change in channel kinetics up to the disappearance of channel activity (Mak and Foskett, 1997), kinetic measurements were made during the entire period the channels were active. All experiments were performed at room temperature with the pipet electrode at +20 mV relative to the reference bath electrode. Each experiment recorded the InsP$_3$R channel activity at a specific [Ca^{2+}]$_i$ and [InsP$_3$], with no change of the pipet solution. Data acquisition was performed as described previously (Mak et al., 1998), with currents recorded with a filtering frequency of 1 kHz and a digitizing frequency of 5 kHz.

Analyses of Patch-clamp Current Traces

The patch-clamp current traces were analyzed using MacTac software (Bruxton) to identify channel opening and closing events using a 50% threshold (Mak et al., 1999). 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 open probability (P_o) evaluation, whereas only current traces with a single InsP$_3$R channel were used for dwell time analysis. 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. Assuming that there are n identical and independent channels in the membrane patch, and each of the channels is Markovian with open probability of P_o and open duration distribution characterized by a single exponential component of time constant τ, the mean dwell time of highest channel current level is τ/n (Eq. 1). If T is the minimum duration of an open event that is detectable in the experimental system, i.e., only events with duration longer than T will have amplitudes greater than the 50% threshold after filtering, then the rate of detection of the highest current level:

$$R_n = \frac{n(P_o)^n}{\tau}\left[\exp\left(-\frac{nT}{\tau}\right)\right]. \qquad (1)$$

In our patch-clamp set up, T was empirically determined to be 0.2 ms using test pulses of variable duration. τ of InsP$_3$R channels is $\sim$3–15 ms over the range of experimental conditions used. In experimental conditions with $P_o > 0.1$, only current records with longer than 10 s of InsP$_3$R channel activities were used. 10 s $\gg 1/R_3$, 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 record duration $>5/R_2$ were used, to ensure that they were truly single-channel records.

Multiple conductance states were observed for recombinant r-InsP$_3$R-3 (Mak et al., 2000). Only current traces exhibiting only the predominant main (M) conductance state were used for analyses. Each data point shown is the mean of results from at least four separate patch-clamp experiments performed under the same conditions. Error bars indicate the SEM.

Solutions for Patch-clamp Experiments

All patch-clamp experiments were performed with pipet solutions containing 140 mM KCl, 10 mM HEPES, and 0.5 mM Na$_2$ATP, pH adjusted to 7.1 with KOH. By using K$^+$ as the current carrier and appropriate quantities of the high affinity Ca^{2+} chelator, BAPTA (1,2-bis(O-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid [100–500 μM]; Molecular Probes), or the low affinity Ca^{2+} chelator, 5,5′-dibromo BAPTA (100–160 μM; Molecular Probes), or ATP (0.5 mM) alone to buffer Ca^{2+} in the experimental solutions, Ca^{2+} concentration was tightly controlled in our experiments. Total Ca^{2+} content (64–306 μM) in the solutions was determined by induction-coupled plasma mass spectrometry (Mayo Medical Laboratory). Free [Ca^{2+}] were calculated using the Maxchelator software (C. Patton, Stanford University, Stanford, CA). The free [Ca^{2+}] of all solutions was also directly measured, using Ca^{2+}-selective minielectrodes (Baudet et al., 1994), and found to agree with the calculated [Ca^{2+}] to within the accuracy of the electrode measurement (10%). Pipet solutions contained various concentrations of InsP$_3$ (Molecular Probes) used with no further purification. The bath solutions used in all experiment had the same composition as the pipet solutions, except they lacked Na$_2$ATP and had a free Ca^{2+} concentration of 220 nM.

R E S U L T S

Ca^{2+} Dependence of the Kinetic Properties of r-InsP$_3$R-3 Gating

Gating of the InsP$_3$R is sensitive to [Ca^{2+}]$_i$ as well as [InsP$_3$] (Bezprozvanny and Ehrlich, 1995; Joseph, 1995; Taylor and Traynor, 1995). Low [Ca^{2+}]$_i$ stimulate InsP$_3$-activated channels, whereas higher [Ca^{2+}]$_i$ are inhibitory (Taylor and Marshall, 1992; Iino and Tsukioka, 1994; Mak et al., 1998). The biphasic effects of [Ca^{2+}]$_i$ on InsP$_3$-mediated Ca^{2+} release are believed to underlie oscillations, waves, and transitions from localized to global cellular responses (Berridge, 1993; Putney and St. J. Bird, 1993; Toescu, 1995). Whereas it is generally agreed that the type 1 isoform is inhibited by high [Ca^{2+}]$_i$, it has been suggested that the types 2 (Ramos-Franco et al., 1998) and 3 (Hagar et al., 1998) isoforms are not. In the X-InsP$_3$R-1, Ca^{2+} is a true agonist (it gates the channel directly by binding to the channel), whereas InsP$_3$ allosterically activates the Ca^{2+}-liganded channel by reducing the Ca^{2+} affinity of the inhibitory binding sites on the channel (Mak et al., 1998). Because of the central role of [Ca^{2+}]$_i$ in regulating the channel, we systematically in-

FIGURE 2. Typical single-channel current traces of the r-InsP₃R-3 at various $[Ca^{2+}]_i$ in the presence of 10 μM InsP₃. Arrows indicate closed channel current level in all current traces.

FIGURE 4. Ca^{2+} dependence of r-InsP₃R-3 channel open probability under various $[InsP_3]$. Different symbols denote data for various $[InsP_3]$ as tabulated. The curves are theoretical fits using the Hill equation (Eq. 2), with K_{inh} varying with $[InsP_3]$ as listed in the graph, whereas P_{max}, K_{act}, H_{act}, and H_{inh} remained independent of $[InsP_3]$ with values tabulated in the graph.

vestigated the effects of $[Ca^{2+}]_i$ on the kinetic properties of the r-InsP₃R-3 in native ER membrane.

To examine specifically the effects of $[Ca^{2+}]_i$ on r-InsP₃R-3 channel gating, a functionally saturating concentration of InsP₃ (10 μM) was applied to the cytoplasmic (pipet) side of the channel to stimulate it fully at all experimental $[Ca^{2+}]_i$ (Fig. 2). At $[Ca^{2+}]_i$ corresponding to resting levels in cells (10–100 nM), the P_o of the r-InsP₃R-3 channel was moderate (<0.5; Fig. 4), with the channel evidently active (Fig. 2 A). The P_o increased to ~0.8 when $[Ca^{2+}]_i$ was raised from 100 nM to 1 μM, which was associated with decreasing mean closed duration (τ_c) (Figs. 3 and 4). Between $[Ca^{2+}]_i$ of 1 and 25 μM, P_o remained high (~0.8; Figs. 3 and 4), with the channel exhibiting long sustained bursts of activities lasting up to several seconds, during which it only closed briefly (Fig. 2 C). As $[Ca^{2+}]_i$ was increased beyond 25 μM, P_o dropped precipitously, as a result of an increase in τ_c to >200 ms (Figs. 3 and 4). Within the more than three orders of magnitude range of $[Ca^{2+}]_i$ examined (24.7 nM–82.8 μM), the mean open dura-

tion (τ_o) of the r-InsP₃R-3 channel lay within a narrow range (4–16 ms) with no systematic dependence on $[Ca^{2+}]_i$ (Fig. 3 A). In contrast, τ_c changed about two orders of magnitude (from 3 to 210 ms; Fig. 3 B) over the same range of $[Ca^{2+}]_i$, accounting for most of the strong dependence of channel P_o on $[Ca^{2+}]_i$ (Fig. 4).

Detailed analyses of the r-InsP₃R-3 channel dwell time histograms revealed that the channel has at least two distinguishable open kinetic states (Fig. 5): a long kinetic state with time constant (τ) of ~8 ms and a short kinetic state with τ ~1 ms. The relative weight of the short kinetic state decreased as the channel was activated by increasing $[Ca^{2+}]_i$, and then increased as high $[Ca^{2+}]_i$ inhibited channel activity. The channel closed dwell time histograms revealed at least three distinguishable closed kinetic states (Fig. 5): (1) a long state with τ > 10 ms; (2) a medium state with 3.5 ms < τ < 10 ms; and (3) a short kinetic state with τ < 1 ms. The decrease in τ_c and, therefore, increase in channel P_o, as the channel was activated by increases in $[Ca^{2+}]_i$, was achieved by both a decrease in the relative weights and the time constants of the long and medium closed kinetic states. Reversal of this trend increased τ_c and decreased P_o when the channel was inhibited by $[Ca^{2+}]_i$ > 25 μM (Fig. 5 D).

The r-InsP₃R-3 P_o versus $[Ca^{2+}]_i$ response in 10 μM InsP₃ could be well fitted to a biphasic Hill equation (Fig. 4) so that:

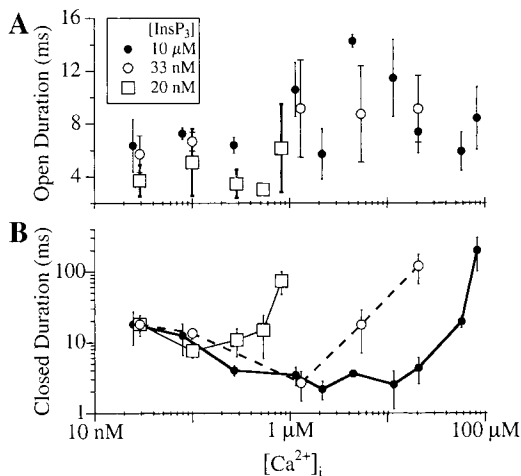**FIGURE 3.** Ca^{2+} dependencies of mean open channel duration (A) and closed channel duration (B) of the r-InsP₃R-3 activated by various concentrations of InsP₃ as tabulated. In the closed channel duration graph, data points obtained with the same InsP₃ concentration are connected with a line for clarity.
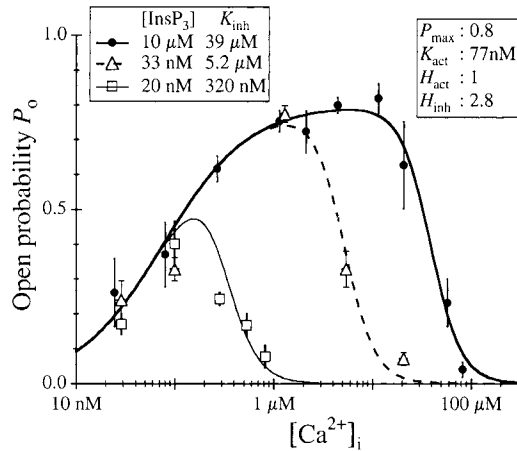
$$P_o = P_{max}\{1 + (K_{act}/[Ca^{2+}]_i)^{H_{act}}\}^{-1}\{1 + ([Ca^{2+}]_i/K_{inh})^{H_{inh}}\}^{-1}.$$
$$(2)$$

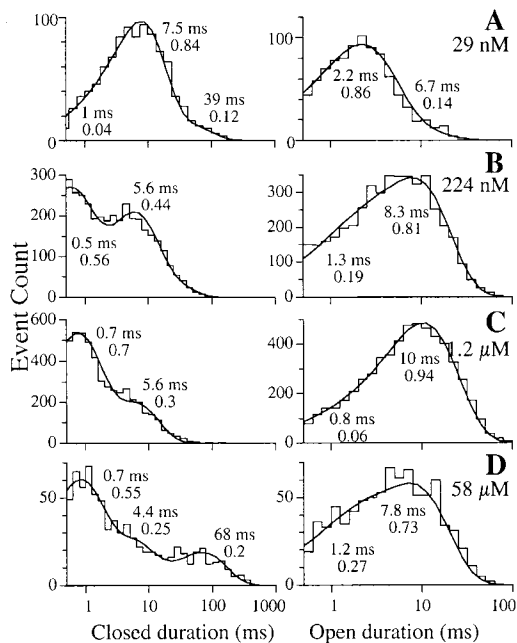This suggests that the tetrameric InsP₃R-3 channel can achieve a maximum open probability P_{max} of 0.80 ±

FIGURE 5. Single-channel open and closed channel duration histograms of r-InsP$_3$R-3 in 10 μM InsP$_3$, with 29 nM, 224 nM, 1.2 μM, and 57.5 μM Ca^{2+}, respectively, as listed in the graphs. The open and closed duration histograms shown for each [Ca^{2+}]$_i$ were derived from one patch-clamp experiment performed under the stated [Ca^{2+}]$_i$. Similar histograms were obtained from two additional experiments at each set of experimental conditions. The smooth curves are theoretical probability density functions consisting of two to four exponential components fitted to the histograms, as outlined in Sigworth and Sine (1987). The time constant and relative weight of each exponential component is labeled beside the corresponding peak in the curves. Duration histograms obtained with the same set of experimental conditions are fitted with the same number of exponential components and the time constants and relative weights of corresponding exponential components lie within ~30% of the values shown.

0.03, with two distinct types of functional Ca^{2+} binding sites: activating sites with a half-maximal activating [Ca^{2+}]$_i$, K_{act}, of 77 ± 10 nM and a Hill coefficient H_{act} of 1.0 ± 0.1; and inhibitory sites with half-maximal inhibitory [Ca^{2+}]$_i$, K_{inh}, of 39 ± 7 μM and Hill coefficient H_{inh} of 2.8 ± 0.4. The Hill coefficient H_{act} of ~1 indicates that Ca^{2+} activation of the InsP$_3$R-3 is not cooperative under our experimental conditions, whereas the large Hill coefficient H_{inh} of 2.8 indicates that inhibition of the InsP$_3$R by Ca^{2+} is a highly cooperative process.

InsP$_3$ Sensitivity of the Ca^{2+} Dependence of r-InsP$_3$R-3

The Ca^{2+} dependence of the gating of the X-InsP$_3$R-1 is regulated by [InsP$_3$] (Mak et al., 1998), and the different receptor isoforms are believed to differ in their affinities for InsP$_3$ (Sudhof et al., 1991; Newton et al., 1994) and in the efficacy of InsP$_3$ to activate them (Ramos-Franco et al., 1998). Therefore, we systematically investigated the InsP$_3$ sensitivity of the Ca^{2+} dependence of r-InsP$_3$R-3 gating. At [InsP$_3$] < 100 nM, the

r-InsP$_3$R-3 became more sensitive to Ca^{2+} inhibition: at 33 nM, K_{inh} decreased from 39 μM (determined at 10 μM InsP$_3$) to 5.2 ± 0.8 μM (Fig. 4), whereas P_{max}, H_{inh}, K_{act}, and H_{act} were not affected. K_{inh} further decreased to 320 ± 50 nM, in response to 20 nM InsP$_3$. As in the case in saturating [InsP$_3$], Ca^{2+} inhibition of P_o in the presence of subsaturating (<100 nM) [InsP$_3$] was caused mainly by an increase in the mean closed dwell time whereas the mean open dwell time remained relatively constant (Fig. 3). These results indicate that InsP$_3$ activates the type 3 channel by tuning the efficacy of Ca^{2+} to inhibit it. No r-InsP$_3$R-3 channel activities were observed in the absence of InsP$_3$ in the pipet (in 25 nM Ca^{2+}). Therefore, as in the case for X-InsP$_3$R-1, InsP$_3$ is a necessary agonist for r-InsP$_3$R-3.

These data can be described by a simple model similar to one derived for the X-InsP$_3$R-1 (Mak et al., 1998), in which the InsP$_3$-concentration dependence of K_{inh} was fitted with a simple Hill equation (Eq. 3):

$$K_{inh} = K_\infty \{ 1 + (K_{IP3}/[InsP_3])^{H_{IP3}} \}^{-1}. \tag{3}$$

The results imply that the InsP$_3$R has a single class of functional InsP$_3$ binding sites with a half-maximal activating [InsP$_3$], K_{IP3}, of 55 ± 6 nM, a Hill coefficient H_{IP3} of 4.5 ± 1, and a maximum half-maximal inhibitory [Ca^{2+}]$_i$, K_∞, of 39 ± 7 μM at saturating [InsP$_3$]. The large Hill coefficient H_{IP3} of ~4 indicates that InsP$_3$ activation of the InsP$_3$R is highly cooperative, requiring InsP$_3$ binding to perhaps all four monomers of the channel to relieve the Ca^{2+} inhibition and gate the channel open.

DISCUSSION

We recently described the first functional expression of the r-InsP$_3$R-3, and examined its detailed permeation properties by patch-clamp electrophysiology of single channels in the *Xenopus* oocyte outer nuclear membrane (Mak et al., 2000). Because the endogenous *Xenopus* InsP$_3$R, which is a type 1 isoform, had been studied in this same preparation (Mak et al., 1998), the channel properties of the two isoforms in the same environment could be compared. Despite the divergent isoform types and species differences, remarkably similar single-channel permeation properties of the two isoforms were observed. Both channels have nearly identical permeabilities to divalent and monovalent cations and gate to similar main and rarer subconductance states, both undergo use-dependent inactivation with similar kinetics, and they share a propensity to exist in channel clusters (Mak et al., 1998, 2000). Because three distinct InsP$_3$R genes have been identified (Furuichi et al., 1989; Mignery et al., 1989; Sudhof et al., 1991; Blondel et al., 1993; Maranto, 1994) and most cell types express more than one isoform (Bush et al., 1994; De Smedt et al., 1994; Newton et al., 1994; Sugi-

yama et al., 1994; Fujino et al., 1995; Joseph et al., 1995; Nucifora et al., 1996; De Smedt et al., 1997), it has been assumed that each isoform must possess unique properties. Thus, it was suggested that distinctions among isoforms might reside in channel regulation and/or localization (Mak et al., 2000). Therefore, in this study we examined the effects of $[Ca^{2+}]_i$ and $[InsP_3]$, the major regulators of $InsP_3R$ channel activity, on the gating kinetics of the r-$InsP_3$R-3 channel in native ER membrane.

Gating Properties and Ca^{2+} Inhibition of the r-$InsP_3$R-3 and X-$InsP_3$R-1 Channels Are Similar

Our results indicate that many gating properties of the main conductance state M of the X-$InsP_3$R-1 and r-$InsP_3$R-3 channels are similar in their responses to $[Ca^{2+}]_i$. Both channels have a P_{max} of 0.8. Like the X-$InsP_3$R-1 channel, the r-$InsP_3$R-3 channel displays two distinct types of functional Ca^{2+} binding sites: (1) activating sites whose properties and their implications will be discussed in detail below; and (2) inhibitory sites, which have similar K_{inh} (54 $\pm$ 3 μM for X-$InsP_3$R-1 and 39 $\pm$ 7 μM for r-$InsP_3$R-3) and H_{inh} (3.9 $\pm$ 0.7 for X-$InsP_3$R-1 and 2.8 $\pm$ 0.4 for r-$InsP_3$R-3) in 10 μM $InsP_3$. In both channels, the mean open channel durations remain within a narrow range (3–16 ms) over the whole range of $[Ca^{2+}]_i$ examined. Thus, Ca^{2+}-induced changes in P_o of both channels were mainly due to large changes in the mean closed channel durations, which decreased as the channels became activated, and increased as P_o decreased due to inhibition by high $[Ca^{2+}]_i$ (Fig. 3; Mak et al., 1998). Analyses of the channel dwell time distributions revealed that the open channel dwell time distribution was not significantly affected by $[Ca^{2+}]_i$, as expected. The increase in mean closed channel durations as $[Ca^{2+}]_i$ increased at inhibitory $[Ca^{2+}]_i$ was mainly caused by an increase in the relative weight of a long closed kinetic state in both the r-$InsP_3$R-3 (Fig. 5, C and D) and the X-$InsP_3$R-1 (data not shown).

Our results indicate that the recombinant r-$InsP_3$R-3, when expressed and recorded in the oocyte outer nuclear membrane, exhibits inhibition of channel activity by high $[Ca^{2+}]_i$. This behavior agrees with that observed in recent studies demonstrating a biphasic dependence on $[Ca^{2+}]_i$ of type 3 $InsP_3R$ activities measured by either $^{45}Ca^{2+}$ efflux from loaded Ca^{2+} stores in permeabilized cells—16HBE14o- bronchial mucosal cells in Missiaen et al. (1998, 2000) and RINm5F cells in Swatton et al. (1999)—that express the type 3 $InsP_3R$ as the major $InsP_3R$ isoform, or reduction of $^{45}Ca^{2+}$ influx into microsomal vesicles isolated from COS-7 cells overexpressing exogenous type 3 $InsP_3R$ and sarco/ER ATPase by transient transfection (Boehning and Joseph, 2000). However, these flux studies indicated that $InsP_3$-induced Ca^{2+} flux was inhibited at a lower $[Ca^{2+}]_i$

($\sim$1 μM) than we observed in our single-channel patch-clamp experiments (Fig. 4). The discrepancy between the results from cell-based assays and our single-channel measurements might possibly be explained by the difficulty in attaining sufficient control of the concentrations of $InsP_3$ and Ca^{2+} in the microdomains around the $InsP_3R$ in the Ca^{2+} flux measurements. $InsP_3$ concentrations used in the flux experiments— 200 nM in Swatton et al. (1999), 1.5 μM in Missiaen et al. (2000), 3 μM in Missiaen et al. (1998), and 1 μM in Boehning and Joseph (2000)—were lower than we used in our experiments (10 μM $InsP_3$). Whereas such $InsP_3$ concentrations were saturating in our patch-clamp experiments ($K_{IP3} \sim$ 55 nM), they might not be sufficient to ensure that the $[InsP_3]$ in the microenvironment around the $InsP_3R$ was saturating. Because $InsP_3$ activates the channel by decreasing the sensitivity to Ca^{2+} inhibition (this study), Ca^{2+} release responses observed in the presence of a subsaturating concentration of $InsP_3$ will be predicted to be associated with a lower half-maximal inhibitory $[Ca^{2+}]_i$, as observed in those studies. Alternately, there may be factors in the permeabilized cells and isolated microsomal vesicles associated with the $InsP_3R$, for example phosphatidylinositol 4,5-bisphosphate (PIP$_2$; Lupu et al., 1998), which were absent in our nuclear patch-clamp studies, that reduced its sensitivity to $InsP_3$ activation or increased its sensitivity to Ca^{2+} inhibition in those studies.

These observations of biphasic Ca^{2+} dependence of the type 3 $InsP_3R$ are in contrast with observations in another recent study (Hagar et al., 1998), which suggested that Ca^{2+} activation was similar for the types 1 and 3 channels, and that high $[Ca^{2+}]_i$ were not inhibitory to type 3 $InsP_3R$ gating. The reasons for these contradictory results are not clear. Different recording solutions and voltages were used in the two studies, and the numbers of channels and range of $[InsP_3]$ studied were significantly greater in this study. Nevertheless, it is difficult to understand how these variables could affect the channel gating response to Ca^{2+}. Whereas recombinant channels of known subunit stoichiometry (nearly all predicted to be homotetramers) in native ER membrane were studied here, membrane proteins from cells (RIN-5F) that expressed multiple $InsP_3R$ isoforms (although type 3 was qualitatively the dominant isoform, from 77% [Swatton et al., 1999] to 96% [Wojcikiewicz and He, 1995]) reconstituted in artificial bilayers were examined in the other study (Hagar et al., 1998). It is important to note that the biphasic Ca^{2+} dependence of the recombinant type 3 channel observed in our study is very reminiscent of that of the endogenous type 1 channel. Thus, it is highly unlikely that the observed behavior of the type 3 channel is conferred upon it by heterologous expression. It seems possible that reconstitution procedures affect the channel, or that the reconstituted channels recorded were

not homotetrameric type 3 channels. The maximum P_o of the recombinant type 3 channel recorded in nuclear patch-clamp experiments was 0.8 in this study, but only 0.05 for the reconstituted channels recorded in the bilayer study. A similar disparity between the high P_{max} observed for type 1 channels in nuclear membrane patches (0.8; Mak and Foskett, 1997; Mak et al., 1998) compared with those reconstituted from cerebellar microsomes (0.05; Bezprozvanny et al., 1991; Kaftan et al., 1997) may be caused by PIP_2 binding tightly to the $InsP_3$ binding sites of reconstituted channels (Lupu et al., 1998), thus, preventing their effective activation by $InsP_3$. It is possible that the channels observed in the RIN-5F membrane reconstitution study (Hagar et al., 1998) was similarly affected by their association with some cytosolic factor, absent in our nuclear patching studies, which reduced both their P_{max} and sensitivity to Ca^{2+} inhibition.

It has been suggested (Michikawa et al., 1999) that an interaction between calmodulin and the type 1 $InsP_3R$ (Yamada et al., 1995; Cardy et al., 1997; Patel et al., 1997; Hirota et al., 1999) mediates Ca^{2+}-dependent inhibition of type 1 $InsP_3R$ activity. Although there is no evidence at present to suggest that calmodulin directly interacts with the type 3 $InsP_3R$ (Yamada et al., 1995; Cardy et al., 1997), calmodulin has been also reported to influence Ca^{2+} inhibition of the type 3 $InsP_3R$ (Missiaen et al., 2000). However, in preliminary experiments, we have been unable to obtain any evidence linking calmodulin to Ca^{2+} inhibition of either the types 1 or 3 channels (our unpublished data). Nevertheless, it is possible that a cellular factor (e.g., calmodulin or a calmodulin-like molecule) is present in our nuclear patching system but not in the reconstituted bilayer system, which may influence the Ca^{2+} sensitivity of the type 3 channel and account for the distinct behaviors observed in our experiments and those in Hagar et al. (1998). Therefore, although the preponderance of the data at this time suggest that the Ca^{2+} inhibition properties of the types 1 and 3 isoforms are not significantly dissimilar, it remains to be determined whether this property of either or both channels is intrinsic to the channels themselves or conferred by regulatory interactions with other proteins.

InsP_3 Activates the r-InsP_3R-3 Channel by Tuning Ca²⁺ Inhibition

InsP$_3$ Activates the r-InsP$_3$R-3 Channel by Tuning Ca^{2+} Inhibition

All the observed gating properties of the r-$InsP_3R$-3 channel over wide ranges of [$InsP_3$] and [Ca^{2+}]$_i$ could be well fitted with a biphasic Hill equation with the half-maximal inhibitory [Ca^{2+}]$_i$, K_{inh}, being the only $InsP_3$ concentration-sensitive parameter. Thus, the effect of $InsP_3$ binding is not to enable activation of the r-$InsP_3R$-3 by Ca^{2+}, as expected for coagonist ligands and which has been generally assumed (Taylor and Richardson, 1991; Mauger et al., 1994; Joseph, 1995; Taylor and Traynor,

1995), but rather it is to ameliorate inhibition of the channel by Ca^{2+}. A similar analysis of the X-$InsP_3R$-1 reached the same conclusion (Mak et al., 1998). The nearly identical behaviors of the types 1 and 3 channels from two different species strongly suggest that this is the mechanism by which $InsP_3$ regulates all $InsP_3$ receptors. The Hill coefficient H_{IP3} for both channels was ~4, indicating that this process of $InsP_3$ activation of $InsP_3R$ channel activity is highly cooperative (Meyer et al., 1988; Carter and Ogden, 1997; Dufour et al., 1997), requiring $InsP_3$ binding to probably all four monomers of the channel to relieve the Ca^{2+} inhibition and gate the channel open. Because $InsP_3$ binding to the $InsP_3R$ is not cooperative (Taylor and Richardson, 1991; Mauger et al., 1994; Joseph, 1995; Taylor and Traynor, 1995), the cooperative effects of [$InsP_3$] on Ca^{2+} release that are observed in cells (Meyer et al., 1988; Schrenzel et al., 1995; Carter and Ogden, 1997), therefore, are likely intrinsic to individual $InsP_3R$ channels.

Analysis of the effects of $InsP_3$ on channel gating indicates that the functional half-maximal activating [$InsP_3$], K_{IP3}, of the r-$InsP_3R$-3 is ~55 nM. A similar analysis for the X-$InsP_3R$-1 indicated that $K_{IP3} \sim 50$ nM (Mak et al., 1998). For single reconstituted type 2 receptor channels, $K_{IP3} \sim 58$ nM (Ramos-Franco et al., 1998). Therefore, these single-channel studies suggest that the functional $InsP_3$ affinities of the three channel types are quite similar. Although these functional affinities are within the range of those determined in binding studies (~2–200 nM; Taylor and Richardson, 1991), the binding studies have suggested differential $InsP_3$ sensitivities among the three isoforms (Cardy et al., 1997; Welch et al., 1997). Nevertheless, the published binding data are quite variable, possibly reflecting different experimental protocols involved in the binding and single-channel studies, regulation of $InsP_3$ affinity (Benevolensky et al., 1994; Yoneshima et al., 1997), or selection for "activatable" receptors in single-channel studies. The latter may be relevant in light of the distinction in some binding studies between a high affinity (~2 nM) inactive state and low affinity (20–60 nM) active state (Hingorani and Agnew, 1992; Benevolensky et al., 1994; Marshall and Taylor, 1994; Mauger et al., 1994). Importantly, the functional Hill coefficient for $InsP_3$ binding to both X-$InsP_3R$-1 and r-$InsP_3R$-3 observed in nuclear patch-clamping is ~4, suggesting a general requirement for all four monomers in a tetramer to bind $InsP_3$.

These functional and binding affinities of the various $InsP_3R$ isoforms for $InsP_3$ (~50 nM) are very different from the functional $InsP_3$ EC_{50} of 3.2 μM reported in a recent study of the regulation by $InsP_3$ of the type 3 $InsP_3R$ reconstituted into lipid bilayers (in 160 nM Ca^{2+}; Hagar and Ehrlich, 2000). That study used the same experimental system as used in Hagar et al.

(1998). Both reconstitution studies recorded a significantly lower maximum P_o for both the type 3 (0.05 in Hagar et al. [1998] and 0.08 in Hagar and Ehrlich [2000]) and type 1 (0.04 in Kaftan et al. [1997]) channels than observed in our nuclear patching experimental system (0.8 for both types 1 and 3 InsP$_3$R). Although we are again not certain about the causes of the discrepancies between the two experimental systems, it is possible that cellular factors like PIP$_2$ may be associated with the InsP$_3$R reconstituted into the planar lipid bilayer system and reduce the sensitivity of the InsP$_3$R to InsP$_3$ activation (Lupu et al., 1998).

The analyses of the kinetics of channel gating and the responses to InsP$_3$ for both the types 1 and 3 InsP$_3$R channels studied in native ER membrane now suggest a unifying model for channel activation by InsP$_3$. Because [Ca^{2+}]$_i$ affects the gating of the InsP$_3$R primarily by regulating the closed state duration, and InsP$_3$ regulates gating by tuning the channel sensitivity to Ca^{2+} inhibition, it follows that the kinetic basis for channel activation by InsP$_3$ is the destabilization of the closed kinetic state(s). Consequently, InsP$_3$ activates Ca^{2+} signaling by increasing the frequency of relatively stereotypic channel openings of (on average) ~10 ms, each of which is the fundamental Ca^{2+} release event in Ca^{2+} signaling. InsP$_3$ enhances the frequency of fundamental release events by reducing the Ca^{2+} affinity of the inhibitory binding site of the monomer to which it binds, in a process that is highly cooperative.

Ca^{2+} Activation of the r-InsP$_3$R-3

The activating Ca^{2+} binding sites of the r-InsP$_3$R-3 had a half-maximal activating [Ca^{2+}]$_i$, K_{act}, of 77 nM and H_{act} of ~1. These values contrast markedly with those obtained for the X-InsP$_3$R-1 (Mak et al., 1998), where K_{act} ~ 210 nM and H_{act} ~ 2 (Fig. 6). Thus, in addition to a higher intrinsic sensitivity of the activating sites for Ca^{2+}, the type 3 receptor lacks the apparent cooperativity among these sites that is observed in the type 1 receptor. This result agrees well with the flatter Ca^{2+} dependence of InsP$_3$-induced Ca^{2+} release observed in B cells genetically engineered to express only InsP$_3$R-3 compared with that observed in cells expressing InsP$_3$R-1 only (Miyakawa et al., 1999). In light of the similarities between the two isoforms in their permeation and gating properties (Mak et al., 2000), and Ca^{2+} inhibition and regulation by InsP$_3$ (this study), these differences in Ca^{2+} activation of the InsP$_3$R channels represent the major distinguishing features between the two channels in our studies. Importantly, neither K_{act} nor H_{act} is affected by [InsP$_3$] in either channel.

The differences in the Ca^{2+} dependencies of the activation of the types 1 and 3 InsP$_3$R are reflected in the dwell time distributions of the two isoforms at activating [Ca^{2+}]$_i$ (<1 μM). Whereas the relative weight of
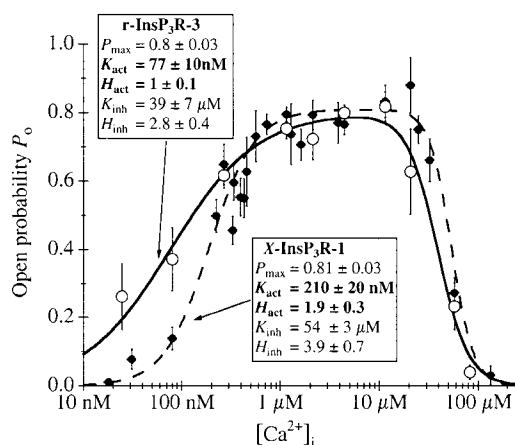

FIGURE 6. Comparison of the Ca^{2+} dependencies of P_o of r-InsP$_3$R-3 and X-InsP$_3$R-1 in 10 μM InsP$_3$ and 0.5 mM ATP. Open circles represent data for r-InsP$_3$R-3 from this study, fitted with the solid curve; closed circles represent data for X-InsP$_3$R-1 taken from (Mak et al., 1998), fitted with the dashed curve. The curves are calculated using the Hill equation (Eq. 2) with the tabulated parameters. Higher affinity and lack of cooperativity of the Ca^{2+} activation sites of the type 3 channel endow it with high gain IICR and low gain CICR. In contrast, lower affinity and presence of cooperativity of the Ca^{2+} activation sites of the type 1 channel confer low gain IICR and high gain CICR. Under resting [Ca^{2+}]$_i$, low levels of stimulation will trigger release of Ca^{2+} by IICR from the type 3 channel, which in turn will trigger further release by CICR from the type 1 channel.

the long open state ($\tau \sim 8$ ms) of both type 1 and type 3 InsP$_3$R channels increased with [Ca^{2+}]$_i$ during activation, most of the change in P_o was the result of changes in the closed channel dwell time distribution. When [Ca^{2+}]$_i$ was raised from 30 to 224 nM, there was a much more dramatic change in the closed channel dwell time distribution of the X-InsP$_3$R-1 channel compared with that of the r-InsP$_3$R-3 channel (Figs. 5 and 7). The rise in [Ca^{2+}]$_i$ caused the predominant long closed state ($\tau > 10$ ms), as well as the longest closed state ($\tau > 100$ ms), of the X-InsP$_3$R-1 channel to disappear (Fig. 7), resulting in the steep increase in channel P_o with $H_{act} \sim 2$. In contrast, the long closed state ($\tau > 10$ ms) of the r-InsP$_3$R-3 had a low relative weight in 31 nM Ca^{2+}, giving the channel a lower K_{act} compared with the X-InsP$_3$R-1. The disappearance of the long closed state with the rise in [Ca^{2+}]$_i$ only caused a gentle increase in channel P_o with $H_{act} \sim 1$.

Importantly, both the lower K_{act} and lack of cooperativity confer upon the type 3 channel the ability to remain active even at low [Ca^{2+}]$_i$ (<100 nM) when stimulated by InsP$_3$, where the type 1 receptor would be nearly quiescent (Fig. 6). For example, at [Ca^{2+}]$_i$ ~ 50 nM, a resting level measured in many cell types, the P_o of the type 3 channel in the presence of low concentrations of InsP$_3$ (10 nM say) is ~0.3, whereas the type 1 channel P_o is nearly 10-fold lower (Fig. 6). At ~25 nM Ca^{2+}, gating of the type 3 receptor is relatively robust
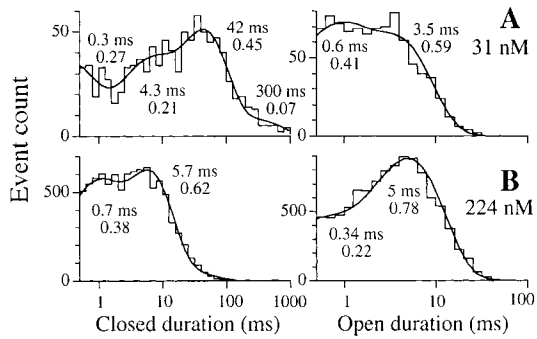
FIGURE 7. Single-channel open and closed channel duration histograms and the fitted theoretical probability density functions of X-InsP$_3$R-1 in 10 μM InsP$_3$ with 31 and 224 nM Ca^{2+}, respectively, obtained as described in the legend to Fig. 5.

($P_o \sim 0.2$), whereas type 1 channel activities can hardly be detected (Fig. 6).

Physiological Significance of Different Ca^{2+} Activation Properties of InsP$_3$R-1 and InsP$_3$R-3

The distinct properties of the Ca^{2+} activation sites of the InsP$_3$R isoforms are likely to be of important physiological significance. Ca^{2+} released by the type 3 channel will serve to trigger release from other type 3 channels in a process of CICR. However, the results of our study now demonstrate that the type 3 receptor is designed to respond with only a limited dynamic range of CICR at resting [Ca^{2+}]$_i$ (50 nM) and moderate stimulation ([InsP$_3$] > 20 nM), as CICR can increase the frequency of fundamental release events by only ~2.6-fold (P_o increases from ~0.3 at resting [Ca^{2+}]$_i$ to a maximum of ~0.8). The narrow dynamic range of CICR in the type 3 receptor is a consequence of its relatively robust channel activity at resting [Ca^{2+}]$_i$, which in turn derives from the high affinity of its Ca^{2+} activation sites, as well as their lack of cooperativity. In contrast, the type 3 receptor is poised to respond to low levels of stimulation that would be insufficient to activate the type 1 receptor. The same properties that confer the type 3 receptor with a low gain CICR, confer on it an exquisite sensitivity to weak stimuli at resting [Ca^{2+}]$_i$, as its Ca^{2+} release activity can increase from $P_o \sim 0$ to $P_o \sim 0.3$ when [InsP$_3$] rises from 0 to <10 nM. Thus, in response to weak stimuli, i.e., low levels of InsP$_3$, the InsP$_3$R-3 behaves as a "switch", imparting high gain to InsP$_3$-induced Ca^{2+} release (IICR).

Although there is little difference in the functional InsP$_3$ sensitivities of the types 1 and 3 InsP$_3$Rs, their differential Ca^{2+} activation properties result in an apparent higher InsP$_3$ sensitivity in vivo of the type 3 release channel under conditions of resting levels of Ca^{2+} in the cytoplasm. The high gain IICR property of the type 3 receptor will enable it to provide a "trigger" release of Ca^{2+} that could recruit other release channel types. In

contrast, the type 1 receptor is relatively insensitive to low levels of InsP$_3$ at resting [Ca^{2+}]$_i$ (low gain IICR). However, it is well designed to provide a wide dynamic range of Ca^{2+} release activity at resting [Ca^{2+}]$_i$ (50 nM) and moderate stimulation ([InsP$_3$] > 20 nM) by CICR, which can increase the frequency of fundamental release events by ~20-fold (P_o increases from 0.05 to 0.8 as [Ca^{2+}]$_i$ increases from 50 to 1,000 nM) (Fig. 6). Thus, the type 1 channel displays high gain CICR and low gain IICR. Because this behavior is complementary to the behavior of the type 3 channel, the presence of the two channels would be predicted to confer a distinct "[Ca^{2+}]$_i$ repertoire" in response to stimulation, in contrast to the behaviors expected if either was the sole expressed isoform.

The efficacy of Ca^{2+} released through type 3 channels to trigger type 1 channel activity by CICR will depend on spatial proximity of the two channels and [InsP$_3$], due to the limited range of Ca^{2+} diffusion in the cytoplasm. Functional X-InsP$_3$R-1 and r-InsP$_3$R-3 channels in the ER membrane have a high propensity to exist in clusters of up to 10 channels (Parker et al., 1996; Mak and Foskett, 1997; Mak et al., 2000). Immunostaining has revealed overlapping as well as distinct (Lee et al., 1997; Yule et al., 1997; Monkawa et al., 1998) localization for the isoforms in different cell types. In several epithelial cell types, the type 3 InsP$_3$R is exclusively and intimately associated with the apical plasma membrane. Electrophysiological and optical imaging experiments have demonstrated that low levels of stimulation are associated with Ca^{2+} release events in close proximity to the apical membrane ("trigger zone"; Kasai and Petersen, 1994; Petersen, 1996). With more intense stimulation, [Ca^{2+}]$_i$ rises first in the trigger zone and then propagates as a wave to the basal pole of the cell (Kasai and Petersen, 1994; Petersen, 1996). We suggest, based on the results of this study, that the spatial restriction of the type 3 InsP$_3$R to the apical region confers to this region an exquisite sensitivity to InsP$_3$ and, therefore, provides a basis for these physiological observations. Our results can likely be generalized to predict that in cells which coexpress the types 1 and 3 receptors, the type 3 receptor will initiate the Ca^{2+} response, and the subsequent signal will be carried by CICR from the type 1 channel. Spatial restriction of the type 3 channel will enable these responses to be manifested as Ca^{2+} waves.

By regulating the affinity of the Ca^{2+} inhibition sites, the [InsP$_3$] will also determine the efficacy of "crosstalk" from the type 3 to the type 1 receptor, as it defines the extent to which Ca^{2+} can be released. At low [InsP$_3$] (<30 nM), CICR from the InsP$_3$R-1 is limited by highly efficacious negative feedback by Ca^{2+}. Because InsP$_3$ binding to the InsP$_3$R reduces Ca^{2+} inhibition of the channel, [Ca^{2+}]$_i$ that can inhibit channel ac-

tivity at low [InsP$_3$] will be insufficient to inhibit it when [InsP$_3$] is increased. In addition to enabling graded Ca^{2+} release from InsP$_3$-sensitive stores, this mechanism enables more intense stimuli to promote greater diffusive spread of the local Ca^{2+} signal to other sites.

In summary, our results indicate that the types 1 and 3 InsP$_3$R isoforms are functionally similar in terms of their permeation and gating properties, regulation by InsP$_3$, and inhibition by cytoplasmic Ca^{2+}. However, the isoforms are uniquely distinguished by their sensitivities to activation by Ca^{2+}. Differential Ca^{2+} sensitivity of Ca^{2+} activation sites confers on each InsP$_3$R isoform distinct and complementary release properties in response to cellular stimulation. The relative expression level and spatial localization of different InsP$_3$R types will enable these properties to interact to generate complex Ca^{2+} signals, including graded release, oscillations, and waves.

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