

3-1-2000

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Recommended Citation

Mak, D O; McBride, S; Raghuram, V; Yue, Y; Joseph, Suresh K.; and Foskett, J K, "Single-channel properties in endoplasmic reticulum membrane of recombinant type 3 inositol trisphosphate receptor." (2000). *Department of Pathology, Anatomy and Cell Biology Faculty Papers*. Paper 134. <http://jdc.jefferson.edu/pacbfp/134>

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Single-Channel Properties in Endoplasmic Reticulum Membrane of Recombinant Type 3 Inositol Trisphosphate Receptor

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abstract The inositol 1,4,5-trisphosphate receptor (InsP₃R) is an intracellular Ca²⁺-release channel localized in endoplasmic reticulum (ER) with a central role in complex Ca²⁺ signaling in most cell types. A family of InsP₃Rs encoded by several genes has been identified with different primary sequences, subcellular locations, variable ratios of expression, and heteromultimer formation. This diversity suggests that cells require distinct InsP₃Rs, but the functional correlates of this diversity are largely unknown. Lacking are single-channel recordings of the recombinant type 3 receptor (InsP₃R-3), a widely expressed isoform also implicated in plasma membrane Ca²⁺ influx and apoptosis. Here, we describe functional expression and single-channel recording of recombinant rat InsP₃R-3 in its native membrane environment. The approach we describe suggests a novel strategy for expression and recording of recombinant ER-localized ion channels in the ER membrane. Ion permeation and channel gating properties of the rat InsP₃R-3 are strikingly similar to those of *Xenopus* type 1 InsP₃R in the same membrane. Using two different two-electrode voltage clamp protocols to examine calcium store-operated calcium influx, no difference in the magnitude of calcium influx was observed in oocytes injected with rat InsP₃R-3 cRNA compared with control oocytes. Our results suggest that if cellular expression of multiple InsP₃R isoforms is a mechanism to modify the temporal and spatial features of [Ca²⁺]_i signals, then it must be achieved by isoform-specific regulation or localization of various types of InsP₃Rs that have relatively similar Ca²⁺ permeation properties.

key words: calcium • calcium release channel • electrophysiology • expression • *Xenopus*

INTRODUCTION

Modulation of cytoplasmic free Ca²⁺ concentration ([Ca²⁺]_i) is a ubiquitous signaling system involved in the regulation of numerous cell physiological processes, in which the second messenger inositol 1,4,5-trisphosphate (InsP₃)¹ plays a central role in most cell types. Analysis of InsP₃-mediated [Ca²⁺]_i signals in single cells has revealed an unexpected complexity in numerous cell types. In the temporal domain, this complexity is manifested as repetitive spikes or oscillations, the frequencies of which are often tuned to the degree of stimulation. Observations of [Ca²⁺]_i spiking have suggested that [Ca²⁺]_i signals may be transduced not only by amplitude detection, but by frequency encoding as well (Lechleiter and Clapham, 1992; Berridge, 1993; Bootman and Berridge, 1995; Clapham, 1995; Toescu, 1995). In the spatial domain, [Ca²⁺]_i signals may initiate at specific locations in the cell and propa-

gate as waves, at velocities ranging from 10 to 50 μm/s (Boitano et al., 1992; Amundson and Clapham, 1993; Atri et al., 1993; Rooney and Thomas, 1993). Thus, [Ca²⁺]_i signals are often organized to provide different signals to discrete parts of the cell.

InsP₃-mediated [Ca²⁺]_i signals are therefore precisely controlled in both time and space, resulting in a signaling system at once ubiquitous, versatile, and specific. A family of InsP₃ receptors (InsP₃Rs) with different primary sequences derived from different genes has been identified (Furuichi et al., 1989; Sudhof et al., 1991; Blondel et al., 1993; De Smedt et al., 1994; Maranto, 1994; Mignery et al., 1989) with alternatively spliced isoforms (Danoff et al., 1991; Nakagawa et al., 1991; Ferris and Snyder, 1992). The InsP₃Rs are ~2,700–2,800 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). The type 1 InsP₃R (InsP₃R-1) was originally cloned from rat (Mignery et al., 1989) and mouse (Furuichi et al., 1989) cerebellum where it is expressed at high levels. Full-length InsP₃R-1 cDNAs have also been cloned from *Caenorhabditis elegans* (Dal Santo et al., 1999), *Drosophila* (Yoshikawa et al., 1992), *Xenopus* (Kume et al., 1993), and human (Yamada et al., 1994). The predicted sequences are 50–70% homologous.

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¹Abbreviations used in this paper: Ab-1, InsP₃R-1-specific antibody; ER, endoplasmic reticulum; InsP₃, inositol 1,4,5-trisphosphate; InsP₃R, InsP₃ receptor; SOC, store-operated Ca²⁺; TEV, two-electrode voltage-clamp.

Full-length sequences of cDNAs for two distinct isoforms, type 2 (InsP₃R-2) and type 3 (InsP₃R-3), have also been determined (Sudhof et al., 1991; De Smedt et al., 1994; Maranto, 1994). The three full-length sequences are 60–80% homologous (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, if not all, mammalian cells examined outside the central nervous system 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 degradation (Magnusson et al., 1993; Wojcikiewicz et al., 1994; Honda et al., 1995; Wojcikiewicz, 1995). In tissues that express more than one InsP₃R isoform, isoform-specific antibodies immunoprecipitate others, suggesting that receptors may associate in heterologomeric complexes (Joseph et al., 1995; Monkawa et al., 1995; Wojcikiewicz and He, 1995; Nucifora et al., 1996).

This diversity of InsP₃R expression is impressive, and suggests that cells require distinct InsP₃Rs to regulate specific functions. Nevertheless, the functional correlates and physiological implications of this diversity are virtually unknown. That the InsP₃R is a ligand-gated ion channel was originally demonstrated by tracer Ca²⁺ fluxes after reconstitution of purified cerebellar type 1 receptors into liposomes (Ferris et al., 1989). The intracellular location of the InsP₃Rs has restricted studies of their single-channel properties (Bezprozvanny et al., 1991; Bezprozvanny and Ehrlich, 1995; Kaftan et al., 1997), largely necessitating the use of indirect measurements to infer their channel activities; e.g., studies involving populations of InsP₃Rs using measurements of [Ca²⁺] or fluxes in intact or permeabilized cells or membrane vesicles (Taylor and Richardson, 1991; Putney and Bird, 1993; Berridge, 1995). The single-channel properties of the type 1 InsP₃R have been examined by reconstitution of mammalian channels in lipid bilayer membranes (Bezprozvanny et al., 1991; Watras et al., 1991; Bezprozvanny et al., 1994; Bezprozvanny and Ehrlich, 1994) and more recently by patch clamp of the outer nuclear membrane of *Xenopus* oocytes (Stehno-Bittel et al., 1995; Mak and Foskett, 1994, 1997, 1998). The type 2 receptor was recently examined by bilayer reconstitution (Perez et al., 1997; Ramos-Franco et al., 1998b). To date, there has been only one report of putative type 3 channel activity, using bilayer reconstitution of membranes from a cell type that expressed more type 3 relative to other isoforms (Hagar et al., 1998). Notably, there have been no single-channel recordings of the recombinant type 3 receptor. The

InsP₃R-3 is a widely expressed InsP₃R isoform (De Smedt et al., 1997; Newton et al., 1994) and it has been implicated in roles in addition to Ca²⁺ release, including plasma membrane Ca²⁺ influx in response to depletion of intracellular Ca²⁺ stores (DeLisle et al., 1996; Putney, 1997) and in apoptosis (Khan et al., 1996). Here, we describe the first functional expression and single-channel recording of recombinant rat InsP₃R-3 (r-InsP₃R-3) in its native membrane environment. The approach we describe suggests a novel general strategy for successful expression and recording of recombinant ER-localized ion channels in the ER membrane. Use of conditions that were similar to those previously employed in studies of the endogenous type 1 InsP₃R channel in the outer nuclear membrane has now enabled the first comparison of the permeation and gating properties of different InsP₃R isoforms in the same native membrane environment. Our results demonstrate that the ion permeation and channel gating properties of the r-InsP₃R-3 channel in the ER membrane are remarkably similar in most respects to those of the *Xenopus* InsP₃R-1 (*X*-InsP₃R-1) in the same membrane. The similarities of permeation and gating properties of InsP₃Rs between isoforms and species suggest that if cellular expression of multiple InsP₃R isoforms is a mechanism to modify the temporal and spatial features of [Ca²⁺]_i signals, then it must be achieved by isoform-specific regulation and/or localization of various types of InsP₃Rs that have relatively similar channel gating and permeation properties.

MATERIALS AND METHODS

Synthesis of r-InsP₃R-3 cRNA for Expression in Xenopus Oocytes

Rat InsP₃R-3 cDNA was introduced into a modified pSP64 vector by digesting pSP-CFTR (provided by Dr. B. Skach, Oregon State University of the Health Sciences, Corvallis, OR) with *Ava*I and *Xba*I to remove the CFTR cDNA. The vector was blunt-ended with T₄ DNA polymerase, religated with T₄ ligase, linearized with *Eco*R1, and ligated by T₄ DNA ligase to a *Not*I/*Eco*R1 adapter sequence. The new vector was linearized by *Not*I and dephosphorylated by calf intestinal alkaline phosphatase. InsP₃R-3 cDNA was excised from pCB6-InsP₃R-3 (provided by Dr. G. Bell, University of Chicago, Chicago, IL) by *Not*I, purified (GeneClean III kit; Bio 101, Inc.), and introduced into pSP64 to create pSP-InsP₃R-3. Plasmid DNA of selected clones of transfected *Escherichia coli* was digested to verify the orientation and sequence of subcloned cDNAs. The final selected clones were amplified, and plasmid DNA was extracted and purified (plasmid purification kit; QIAGEN Inc.). In vitro transcribed cRNA was synthesized using the RibOMAX Large Scale RNA Production System (Promega Corp.).

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 an endogenous InsP₃R-1 (*X*-InsP₃R-1), it was necessary to distinguish them from expressed channels in our patch-clamp experiments using isolated oocyte

nuclei. Endogenous channel activity detected in patch-clamp experiments using 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). It has been common to find batches of oocytes that fail to exhibit any channel activity when their nuclei are patched. Although this has been a significant problem in our studies of the *X*InsP₃R-1, we reasoned that it would enable the use of this system for expression studies by providing a null background. Therefore, for each new batch of oocytes, a day of patch clamping of isolated nuclei (at least six nuclei, 6–10 patches from each) was performed to determine the endogenous channel expression. Only batches with extremely low channel activity (<1 of 15 patches exhibited InsP₃R channel activity) were used for subsequent cRNA injections.

Oocytes selected for microinjection were defolliculated (Jiang et al., 1998). *Xenopus* ovaries were mechanically separated into pieces containing five or fewer oocytes each, and treated with 2 mg/ml solution of collagenase type IV (Sigma Chemical Co.) in Ca²⁺-free SOS (100 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 5 mM HEPES, pH adjusted to 7.6 with NaOH) at room temperature for 1 h. Defolliculated oocytes were washed four to five times and kept in SOS (same as Ca²⁺-free SOS except with 1.8 mM CaCl₂), and then used for injection <24 h after defolliculation.

23 nl of cRNA (1 μg/μl) was injected into the cytoplasm of defolliculated oocytes using a nanoliter injector (World Precision Instruments) (Goldin, 1992). cRNA-injected and uninjected but defolliculated control oocytes were placed in individual wells in 96-well plates containing 200 μl of ASOS [SOS with 3 mM Na pyruvate, 100 μg/ml gentamycin, and 100 μM ALLN (*N*-acetyl-Leu-Leu-Norleucinal; Sigma Chemical Co.)]. 80 μl of ASOS in each well was changed daily. Patch clamp and two-electrode voltage clamp studies using injected oocytes were performed 4–5 d after cRNA microinjection.

Western Analysis

For each sample, 10 oocytes were placed in 200 μl of ice-cold oocyte extraction buffer [OEB, containing 150 mM NaCl, 100 mM TrisHCl, 10 mM EDTA, 1% Triton X100 (wt/vol), pH 8.0] with protease inhibitor mix (1 mM PMSF, 1 μM pepstatin A, 1 μM leupeptin, 10 μM E-64; Sigma Chemical Co.) and homogenized. The homogenate was placed on ice for 30 min, and then spun at top speed for 15 min on a bench-top centrifuge (Eppendorf). 150 μl of the clear supernatant, excluding the top emulsion layer from yolk, was extracted. 15 μl of the clear extract (approximately equivalent to one oocyte) was separated by 5% SDS-PAGE, transferred to nitrocellulose, and analyzed by blotting with an InsP₃R-1-specific antibody (Ab-1; Joseph and Samanta, 1993; Joseph et al., 1995), or an InsP₃R-3-specific antibody (Ab-3; Transduction Laboratories) and detected by enhanced chemiluminescence (Amersham Pharmacia Biotechnology).

Immunoprecipitation

The protocol used was modified from that used for immunoprecipitation of InsP₃Rs from WB cell extracts (Joseph et al., 1995). For each sample, 15 oocytes were placed in 300 μl of ice-cold OEB with protease inhibitor mix and homogenized. 225 μl of clear oocyte extract was obtained as described for Western analyses. The extract was precleared for 2 h with 40 μl of protein A agarose [50% slurry (vol/vol); GIBCO BRL], and then treated overnight with Ab-1. Immune complexes of Ab-1 and *X*InsP₃R-1 were precipitated with protein A agarose pretreated with OEB containing 1% bovine serum albumin. The immunoprecipitated complexes were washed once using ice-cold OEB with an additional 350 mM NaCl and 1% SDS and twice using ice-cold OEB,

separated by 5% SDS-PAGE, transferred to nitrocellulose, immunoblotted with Ab-3, and detected by enhanced chemiluminescence. Similar oocyte samples were precleared with protein G agarose [50% slurry (vol/vol); GIBCO BRL], treated overnight with Ab-3, immunoprecipitated with BSA-pretreated protein G agarose, washed, and immunoblotted with Ab-1.

Patch Clamping the Oocyte Nucleus

Patch-clamp experiments were performed as described previously (Mak and Foskett, 1994, 1997, 1998). In brief, stage V or VI oocytes were opened mechanically just before use. The nucleus was separated from the cytoplasm and transferred to a dish on the stage of a microscope for patch clamping. Due to channel inactivation (Mak and Foskett, 1994, 1997), unless stated otherwise, experiments were done in "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). Following standard conventions, the applied potential is that of the pipette electrode minus the reference bath electrode (positive current flows from pipette outward). Experiments were performed at room temperature.

Single-channel currents were amplified with an Axopatch-1D amplifier (Axon Instruments) with anti-aliasing filtering at 1 kHz, transferred to a Power Macintosh 8100 via an ITC-16 interface (Instrutech Corp.), digitized at 5 kHz, and written directly onto hard disk by Pulse+PulseFit software (HEKA Elektronik). Data were analyzed and fitted with theoretical curves using Mac-Tac 3.0 (Bruyton), and Igor Pro 3 (WaveMetrics).

Solutions for Patch-clamp Experiments

Unless stated otherwise, the pipette solutions used in patch-clamp experiments contained (mM): 140 KCl, 10 HEPES, 0.5 Na₂ATP, pH adjusted to 7.1 with KOH with 0 or 3 MgCl₂. Because of chelation of Mg²⁺ by ATP, the actual free Mg²⁺ in the solutions was 0 or 2.5 mM. By using K⁺ as the current carrier and appropriate quantities of the high-affinity Ca²⁺ chelator, BAPTA [100–500 μM 1,2-bis(*O*-aminophenoxy) ethane-*N,N,N',N'*-tetraacetic acid; Molecular Probes, Inc.], or the low-affinity Ca²⁺ chelator, 5,5'-dibromo BAPTA (100–400 μM; Molecular Probes, Inc.), or just ATP (0.5 mM) to buffer [Ca²⁺] in the experimental solutions, [Ca²⁺] was tightly controlled in our experiments. Total Ca²⁺ content in the solutions (5–350 μM) was determined by induction-coupled plasma mass spectrometry (Mayo Medical Laboratory, Rochester, MN). Free [Ca²⁺] were calculated using the Maxchela software (C. Patton, Stanford University, Stanford, CA). Pipette solutions contained various concentrations of InsP₃ (Molecular Probes, Inc.) as stated. Unless stated otherwise, all patch-clamp experiments used a bath solution with the same composition as the pipette solutions except with no Na₂ATP or MgCl₂, and calculated free Ca²⁺ concentration of 250 nM.

In ion selectivity determinations, the low K⁺ solution contained 110 mM *N*-methyl-d-glucamine Cl, 30 mM KCl, 10 mM HEPES, 1.27 mM BAPTA, pH adjusted to 7.1 by KOH. [Ca²⁺] was calculated to be 2 μM. The high Ca²⁺ solution contained 50 mM CaCl₂, 45 mM *N*-methyl-d-glucamine Cl, 30 mM KCl, 10 mM HEPES, pH adjusted to 7.1 by KOH.

Oocyte Two-Electrode Voltage-clamp Experiments

Conventional two-electrode voltage-clamp (TEV) methods (Stühmer, 1992) were used to measure membrane currents in either InsP₃R-3-expressing oocytes (InsP₃R cRNA injected, with functional expression confirmed by patch clamping of isolated nuclei), or uninjected control oocytes, using an OC-725C oocyte clamp amplifier (Warner Instrument Corp.) connected to a Pow-

erMac 7100 via an ITC-16 interface. Single oocytes were placed in a chamber (0.6 ml vol) containing a low-Ca²⁺ solution LCa96 (96 mM NaCl, 1 mM KCl, 0.2 mM CaCl₂, 5.8 mM MgCl₂, 10 mM HEPES, pH 7.5 by NaOH). Pulse+PulseFit software was used to ramp the applied transmembrane potential (V_m) at regular intervals. V_m was clamped at the prestimulation reversal potential in between the voltage ramps. Transmembrane current (I_m) and V_m were digitized at 1 kHz during the voltage ramps and written directly onto hard disk. The whole-oocyte membrane conductance was evaluated as the slope (dI_m/dV_m) of a fifth-order polynomial (Jiang et al., 1998) fitted to the raw, monotonically increasing I_m – V_m curve from each voltage ramp using Igor Pro 3 software.

In experiments that monitored changes in [Ca²⁺]_i by measuring the plasma membrane Ca²⁺-activated Cl⁻ current (I_{Cl1} described by Hartzell, 1996), V_m was ramped from -60 to 20 mV in 550 ms, repeated at 1-s intervals. The membrane conductance was evaluated at V_m = 10 mV. To provide sustained stimulation to activate the InsP₃R maximally, 23 nl of 10 μM InsP₃ was repeatedly (90-s intervals) microinjected into the voltage-clamped oocytes using a nanoliter injector (Hartzell, 1996). After relaxation of the InsP₃-induced membrane current to a steady level (usually 250–300 s after first injection of InsP₃), 6 ml of high-Ca²⁺ solution HCa96 (same composition as LCa96 except with 5.8 mM CaCl₂ and 0.2 mM MgCl₂) was perfused into the oocyte chamber, and the Cl⁻ current induced by Ca²⁺ influx through the store-operated Ca²⁺ channel, I_{SOc} described in Hartzell (1996), was recorded.

In experiments designed to measure I_{SOc} directly, protocols previously described in Yao and Tsien (1997) were followed. Ca²⁺ store depletion was accomplished by extracellular application of fetal bovine serum (1:100) in LCa96 (Miledi and Parker, 1989). V_m was ramped from -100 to 50 mV in 700 ms, repeated at 3-s intervals. Contributions to the membrane current by the Ca²⁺-activated Cl⁻ channels were minimized by microinjection of Ca²⁺ chelator—41.4 nl of 100 mM BAPTA for a final concentration of ~4 mM (Yao and Tsien, 1997); and evaluating the conductance at V_m = -80 mV, where the Ca²⁺-activated Cl⁻ channels (I_{Cl1} and I_{Cl2}) were least active (Hartzell, 1996). The bathing LCa96 solution was replaced with HCa96 by perfusion of 6 ml of HCa96 into the oocyte chamber to observed I_{SOc} directly.

RESULTS

Expression of r-InsP₃R-3 in *Xenopus* Oocytes

Multiple isoforms of InsP₃R exist in mammalian cells, coded by different genes and expressed in distinct and overlapping patterns in various tissues. To determine whether different isoforms exhibit distinct single-channel properties, we used *Xenopus* oocytes because we have demonstrated that it is possible to record InsP₃R channel activity in their isolated nuclei (Mak and Foskett, 1994, 1997, 1998), and oocytes can efficiently express mammalian ion channel proteins from injected cRNAs and cDNAs (Soreq and Seidman, 1992). Oocytes microinjected with the cRNA of r-InsP₃R-3 were sampled at various times after microinjection for biochemical determinations of InsP₃R-3 expression. Western blot analysis using a specific InsP₃R-3 antibody (Ab-3) revealed a band at the appropriate molecular weight (~250 kD) in cRNA-injected oocytes (Fig. 1 B) that was not present in uninjected control oocytes (A), confirming expression of r-InsP₃R-3 in oocytes by cRNA injection.

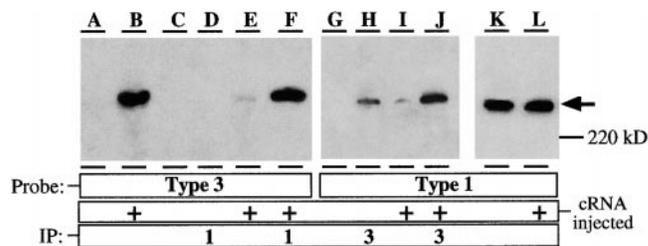


Figure 1. Expression of endogenous type 1 and recombinant type 3 InsP₃ receptors in *Xenopus* oocytes. (A–F) Immunoblotted with Ab-3; (G–L) immunoblotted with Ab-1. (A) Western blot of lysate from an equivalent of one control oocyte, demonstrating lack of endogenous type 3 InsP₃R expression; (B) Western blot of lysate from an equivalent of one r-InsP₃R-3 cRNA-injected oocyte, demonstrating expression of the recombinant r-InsP₃R-3 protein; (C) Western blot of type 3 InsP₃R adsorbed nonspecifically to agarose beads, from lysate from fifteen control oocytes; (D) Western blot of type 3 InsP₃R in InsP₃R-1 immunoprecipitate, from lysate from fifteen control oocytes; (E) Western blot of type 3 InsP₃R adsorbed nonspecifically to agarose beads, from lysate from fifteen cRNA-injected oocytes; (F) Western blot of type 3 InsP₃R in InsP₃R-1 immunoprecipitate from lysate, from fifteen cRNA-injected oocytes. Difference between F and E represents the amount of recombinant r-InsP₃R-3 specifically immunoprecipitated with X-InsP₃R-1. (G) Western blot of X-InsP₃R-1 adsorbed nonspecifically to agarose beads, from lysate from fifteen control oocytes; (H) Western blot of X-InsP₃R-1 in InsP₃R-3 immunoprecipitate, from lysate from fifteen control oocytes. The InsP₃R detected is most likely caused by cross-interaction of the immunoprecipitating Ab-3 with the large amount of endogenous X-InsP₃R-1 present in the lysate from fifteen oocytes. (I) Western blot of X-InsP₃R-1 adsorbed nonspecifically to agarose beads, from lysate from fifteen cRNA-injected oocytes; (J) Western blot X-InsP₃R-1 in InsP₃R-3 immunoprecipitate, from lysate from fifteen cRNA-injected oocytes. Difference in protein amounts between H/I and J represents the amount of X-InsP₃R-1 specifically immunoprecipitated with recombinant r-InsP₃R-3. (K) Western blot of X-InsP₃R-1 in lysate from an equivalent of one control oocyte; (L) Western blot of X-InsP₃R-1 in lysate from an equivalent of one cRNA-injected oocyte. Comparisons of K and L indicate that r-InsP₃R-3 expression does not induce changes in the expression levels of the endogenous X-InsP₃R-1. Western blots of type 1 InsP₃R in InsP₃R-1 immunoprecipitates or type 3 InsP₃R in InsP₃R-3 immunoprecipitates are not shown because too much protein was present in those lanes for the protein bands to be properly detected with the same exposure as the other lanes.

tion. Expression was detectable as early as 7 h after injection, and increased to a steady level by 48 h after injection. Expression level then remained constant up to 5 d after injection, before declining (not shown).

Functional Detection of r-InsP₃R-3 Expressed in Oocytes

To reduce the likelihood of detecting endogenous X-InsP₃R-1 channels in patch-clamp experiments using r-InsP₃R-3 cRNA-injected oocytes, we determined the level of functional expression of the endogenous X-InsP₃R-1 channel in the outer nuclear envelope by patch-clamp experiments on isolated nuclei from freshly procured oocytes (uninjected). As described in materials and methods, batch-to-batch variability among oocytes

in the functional expression of endogenous *X*-InsP₃R-1 enabled suitable oocytes with sufficiently low endogenous channel activity to be chosen for expression studies of recombinant r-InsP₃R-3. We selected for cRNA microinjection only batches of oocytes in which the probability of detecting *X*-InsP₃R-1 activity in a nuclear patch-clamp experiment (with 10 μM InsP₃ in the pipette) was extremely low: <1 of 15 patches exhibited endogenous InsP₃R channel activity. Of 336 nuclear patches from uninjected oocytes in such selected batches, only 17 channels were detected in nine patches. The mean number of InsP₃R channels per nuclear patch ($\langle n \rangle$) was 0.051. The probability of detection of endogenous InsP₃R in nuclear envelopes of uninjected control oocytes maintained under identical conditions as cRNA-injected oocytes never increased over time. On the other hand, the probability of detecting InsP₃R-like channel activities increased dramatically in injected oocytes measured 4–5 d after cRNA microinjection. In 767 experiments, 1,546 channels were detected in 381 patches, with 286 patches exhibiting multiple InsP₃R channels. $\langle n \rangle$ for the cRNA-injected oocytes increased to 2.02. Thus, the r-InsP₃R-3 cRNA injection into appropriately selected batches of oocytes resulted in a >40-fold increase in the mean number of InsP₃R channels detected by nuclear patch-clamp experiments.

The channel activities detected in the nuclei from the cRNA-injected oocytes (Fig. 2 A) were highly reminiscent of those displayed by the *X*-InsP₃R-1 (B) (Mak and Foskett, 1994, 1997). Two sets of experiments were conducted to confirm their identities as InsP₃-sensitive channels. First, in multiple nuclear patches with the pipette solutions alternately containing either 10 μM or no InsP₃, the channels were only detected in the presence of InsP₃ (59 channels in 13 patches from three nuclei), but not in its absence (zero channels in seven patches). Second, the channel activities were completely suppressed by the competitive InsP₃ inhibitor heparin (100 μg/ml) in all nine patches obtained from five nuclei, which showed 55 channels in 22 patches without heparin. These pharmacological results, together with the statistical data above, strongly suggested that the channel activities observed in nuclei from cRNA-injected oocytes were recombinant r-InsP₃R-3 expressed due to the cRNA injection.

Two additional sets of evidence further indicated that the InsP₃R detected in patch-clamp experiments using nuclei from cRNA-injected oocytes were not contributed by the endogenous *X*-InsP₃R-1. First, a series of Western blot analyses of cRNA-injected oocytes conducted using a type 1 isoform-specific antibody (Ab-1) demonstrated that there was no detectable increase in the expression levels of the endogenous *X*-InsP₃R-1 in the oocytes injected with r-InsP₃R-3 cRNA compared with the uninjected ones (Fig. 1, K and L). Thus, the 40-fold greater

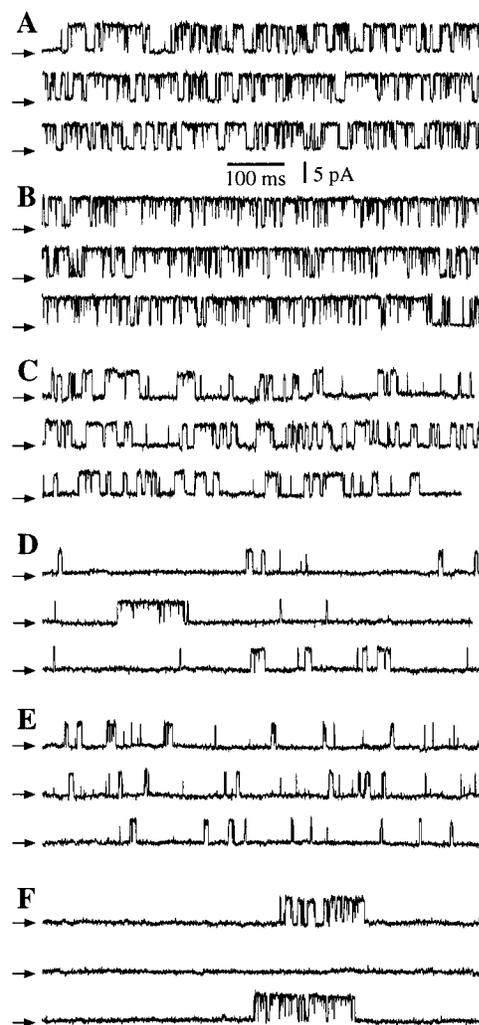


Figure 2. Current traces obtained by patch clamping the outer membrane of isolated oocyte nuclei in symmetric 0-mM Mg²⁺ solutions. All current records shown were obtained with $V_{app} = 20$ mV, and with 0.5 mM free ATP and 10 μM InsP₃ in the pipette solution. The arrows indicate the closed channel current level. (A, C, and E) Nuclei from r-InsP₃R-3 cRNA-injected oocytes. (B, D, and F) Nuclei from uninjected oocytes. (A and B) $[Ca^{2+}]_i = 1,150$ nM. (C and D) $[Ca^{2+}]_i = 80$ nM. (E and F) $[Ca^{2+}]_i = 57.5$ μM. The average P_o (\pm SEM) evaluated for n experiments under the shown experimental conditions are: (A) 0.75 ± 0.03 ($n = 16$); (B) 0.80 ± 0.02 ($n = 8$); (C) 0.45 ± 0.07 ($n = 4$); (D) 0.14 ± 0.03 ($n = 7$); (E) 0.23 ± 0.07 ($n = 4$); (F) 0.27 ± 0.11 ($n = 6$).

number of InsP₃R channels observed in cRNA-injected oocyte nuclei was not caused by upregulation of *X*-InsP₃R-1 expression. Second, although the InsP₃R channel activities observed in cRNA-injected oocyte nuclei when $[Ca^{2+}]_i > 1$ μM were similar to those previously observed in uninjected oocyte nuclei (Mak and Foskett, 1994, 1997, 1998) (Fig. 2, A and B), and the channel activities in both injected and uninjected oocyte nuclei exhibited dramatic inhibition by $[Ca^{2+}]_i > 50$ μM (Fig. 2, E and F), when $[Ca^{2+}]_i$ was lowered to <500 nM, InsP₃R

channels from r-InsP₃R-3 cRNA-injected oocytes gated with a significantly higher open probability (P_o) than those from uninjected oocytes (Fig. 2, C and D). Thus, the channels observed upon expression of recombinant r-InsP₃R-3 were distinguishable from the endogenous channels. Taken together, these results strongly suggest that the channel activities we recorded in the present work were those of recombinant r-InsP₃R-3.

Heterologous Interactions of r-InsP₃R-3 and X-InsP₃R-1

Since types 1 and 3 InsP₃R isoforms can form heterotetrameric complexes in cells that coexpress them both (Joseph et al., 1995; Monkawa et al., 1995; Wojcikiewicz and He, 1995; Nucifora et al., 1996), we also considered whether some of the r-InsP₃R-3 channel activities we recorded might be contributed by heterotetramers formed with the X-InsP₃R-1. As indicated above, the mean number of InsP₃R channels detected by nuclear patch clamping increased 40-fold after r-InsP₃R-3 cRNA injection. Assuming that InsP₃R channels are tetramers and that they are formed by random, binomial associations of X-InsP₃R-1 and r-InsP₃R-3, the large majority (90.3%) of the channels detected in cRNA-injected oocytes are predicted to be homotetrameric r-InsP₃R-3 channels, and most of the rest of the channels (9.4%) are predicted to be composed of one X-InsP₃R-1 monomer associated with three r-InsP₃R-3 monomers. To more directly investigate the extent of heterotetramer formation in our studies, we undertook a series of coimmunoprecipitation experiments. Using lysates from oocytes microinjected with r-InsP₃R-3 cRNA, immunoprecipitation of X-InsP₃R-1 with Ab-1 coimmunoprecipitated the expressed r-InsP₃R-3, as evidenced by its mobility in SDS-PAGE and detection by immunoblotting with Ab-3 (Fig. 1 F). Two sets of control experiments demonstrated the specificity of the coimmunoprecipitation. First, r-InsP₃R-3 was not detected in similar protocols using uninjected oocytes (Fig. 1, C and D). Second, for both the control and injected oocytes, nonspecific binding to the agarose beads was also evaluated (Fig. 1, C and E). Converse experiments were similarly performed: immunoprecipitation by Ab-3 of r-InsP₃R-3 from cRNA-injected oocyte lysates coimmunoprecipitated X-InsP₃R-1 (Fig. 1 J). Again, similar controls, for nonspecific, antibody-independent binding to beads (Fig. 1, G and I), and using uninjected oocyte lysates (Fig. 1, G and H), were performed. These experimental results suggest that the *Xenopus* type 1 and rat type 3 InsP₃R isoforms may form heterotetrameric complexes in oocytes. The amount of r-InsP₃R-3 that coimmunoprecipitated with the X-InsP₃R-1 from the pooled lysates of 15 cRNA-injected oocytes (Fig. 1 F) was roughly equivalent to the amount of r-InsP₃R-3 protein present in the lysate of one cRNA-injected oocyte (Fig. 1 B). In control experiments, the amount of X-InsP₃R-1 immu-

noprecipitated by Ab-1 from the pooled lysates of 15 uninjected oocytes and detected by immunoblotting with Ab-1 was over an order of magnitude greater than the amount of X-InsP₃R-1 detected by Ab-1 in the lysate of one uninjected oocyte (not shown), indicating an efficiency of immunoprecipitation of X-InsP₃R-1 by Ab-1 of >67%. Therefore, the coimmunoprecipitation result indicates that ~7–10% of the r-InsP₃R-3 expressed in the cRNA-injected oocytes was involved in heteroligomer formation with the endogenous X-InsP₃R-1. This biochemical result compares well with the ~9% predicted from the binomial statistics. The good agreement between these completely independent determinations suggests that >90% of the channel activities observed in r-InsP₃R-3-injected oocyte nuclei in the present studies were contributed by homotetramers of r-InsP₃R-3 monomers, and <10% of them were contributed by tetramers that contained one X-InsP₃R-1 monomer.

Characterization of the Single-channel Conductance and Gating Properties of r-InsP₃R-3 in Xenopus Oocytes

Because these results strongly suggested that the channel activities we measured in oocytes expressing r-InsP₃R-3 were overwhelmingly contributed by type 3 homotetramers, we proceeded to use this expression system to characterize the properties of the r-InsP₃R-3 channel. The major rationale for developing this expression system was to determine whether different InsP₃R isoforms have similar or distinct permeation and gating properties when their channel activities are measured in the same physiological membrane system. To facilitate comparisons of the permeation and gating behaviors of the two isoforms, we used conditions that were previously employed to characterize the X-InsP₃R-1 in this same nuclear membrane (Mak and Foskett, 1994, 1998). In symmetric KCl solution containing 2.5 mM Mg²⁺, the r-InsP₃R-3 channel has a stable conductance (Fig. 3 A), with a channel current–voltage relation that is nonlinear and asymmetric with respect to the origin, with $I(-V_{app}) = -0.7 \times (V_{app})$, where V_{app} is the applied voltage ($V_{app} > 0$ mV in this equation) (Fig. 3 B). The slope conductance near 0 mV was ~119 pS, increasing to ~360 pS at +50 mV. We previously demonstrated that nonlinearity of the X-InsP₃R-1 I-V relation was caused by Mg²⁺ permeation in the channel (Mak and Foskett, 1998). We therefore investigated the I-V relation in the absence of free Mg²⁺. In symmetric KCl solution with 0 mM Mg²⁺, the InsP₃R-3 I-V relation became linear with a conductance of 358 ± 8 pS (see Fig. 5 B). Thus, Mg²⁺ is a permeant ion blocker, reducing channel conductance and causing rectification of the I-V relation in the r-InsP₃R-3, as it does in the X-InsP₃R-1 (Mak and Foskett, 1998). Mg²⁺ also plays an important role in stabilizing the conductance of X-InsP₃R-1 channel. A similar role for Mg²⁺ was observed for the

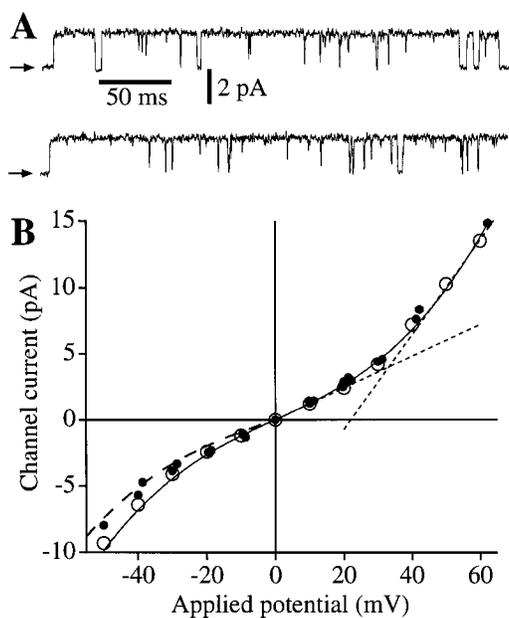


Figure 3. Conductance of the recombinant r-InsP₃R-3 in the nuclear membrane. (A) Typical patch clamp current traces for r-InsP₃R-3 channels in symmetric solutions containing 2.5 mM Mg²⁺ and [Ca²⁺]_i = 940 nM. (B) I-V relation for the r-InsP₃R-3 (●) in symmetric 2.5 mM Mg²⁺ solutions. Channel currents were averaged from all channel opening and closing events from five current records (over 100 events in each record). SEM bars are smaller than the symbols. I-V relation for the X-InsP₃R-1 channels in the same ionic conditions (○) obtained previously (Mak and Foskett, 1998) is also plotted for comparison. Solid curve is a fifth-order odd polynomial [$I = a_1V_{app} + a_3(V_{app})^3 + a_5(V_{app})^5$] fitted to the r-InsP₃R-3 data under positive V_{app} . (Dotted line) The slope conductances (dI/dV) of 119 pS at 0 mV and 360 pS at 50 mV. Dashed curve: 0.7× the value of the solid curve.

r-InsP₃R-3 channel. Thus, in the absence of Mg²⁺ on both (Fig. 4 A) or just one (B) side of the r-InsP₃R-3 channel, the channel conductance occasionally fluctuated under constant V_{app} during the course of a recording (~2 min), or even during single-channel openings (~10 ms). In patches with multiple channels, their conductances fluctuated independently (Fig. 4 A) or in concert (Fig. 4 B). However, most records showed channels with stable conductances (Fig. 2). To avoid possible effects of channel conductance fluctuations, a voltage ramp protocol (Fig. 5) was used to determine the channel conductance of the r-InsP₃R-3.

Conductance states other than the predominant main (M) state were also observed in either the presence or absence of Mg²⁺. Most noticeably, a small conductance state (S) with conductance ~0.7 that of the M state (Fig. 6 A) was frequently observed (>20% of channel open time). A half (H) conductance state with conductance 0.5 that of state M was observed occasionally (<1% of channel open time) (Fig. 6 B). Besides the normal kinetic mode, the InsP₃R-3 channel was sometimes (<5% of the patches) observed in a

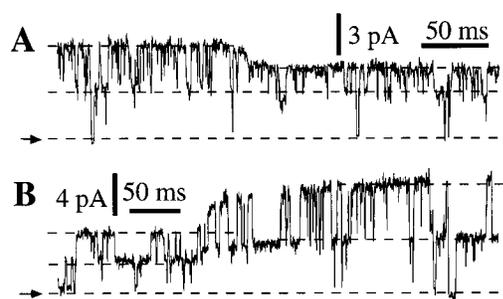


Figure 4. r-InsP₃R-3 channel conductance fluctuates in the absence of Mg²⁺. (A) Current trace from a patch with two InsP₃R-3 channels, one of which had fluctuating conductance, in symmetric solutions containing 0 mM Mg²⁺; [Ca²⁺]_i = 221 nM. (B) Current trace showing two InsP₃R-3 channels with conductance fluctuating in concert, with 2.5 mM Mg²⁺ on the pipette side and 0 mM Mg²⁺ on the bath side of the channel; [Ca²⁺]_i = 1,260 nM.

“flicker” kinetic mode, in which the channel rapidly alternated between two conductance states (F_1 and F_2), with conductances ~0.25 and 0.75 that of the M state. Similar flicker kinetics have been observed in the X-InsP₃R-1 channel (Mak and Foskett, 1997). The conductance substates (S and H) and flicker kinetic mode were observed in both the presence and absence of Mg²⁺. From statistical considerations based on the frequency of observance of the substate and the probability of detecting heteroligomeric InsP₃R channels, the more frequently observed substate (S) must be a substate of the r-InsP₃R-3 homotetramer, but we cannot ascertain whether the less frequently observed substates (H, F_1 , and F_2) occurred in r-InsP₃R-3 homotetramers or in heterotetramers of X-InsP₃R-1 and r-InsP₃R-3.

To determine the ion selectivity of the r-InsP₃R-3 channel, asymmetric ionic conditions were used. In the presence of a [K⁺] gradient, in the absence of Mg²⁺, the channel I-V relation is linear (Fig. 7 A) with a reversal potential (V_{rev}) of 25.2 mV. Using the Goldman-Hodgkin-Katz (GHK) equations (Hille, 1992), the relative permeabilities of the channel to K⁺ and Cl⁻ (P_K/P_{Cl}) were calculated to be 4.6. In the absence of Mg²⁺, with a [Ca²⁺] gradient, the channel I-V relation is nonlinear with $V_{rev} = 20.6$ mV (Fig. 7 B). Using the GHK equations and the value of P_K/P_{Cl} , the relative permeabilities of Ca²⁺ to K⁺ (P_{Ca}/P_K) were determined to be 11. Thus, the channel is a Ca²⁺-selective cation channel.

In all experimental conditions, inactivation of the r-InsP₃R-3 channel was consistently observed. In >90% of the experiments, channel activities terminated within 2 min of formation of the seal. In some of the patches (~10%), the inactivation could be reversed by an increase of 20–40 mV in V_{app} . In some patches, the channels could be repeatedly reactivated by jumps in V_{app} before they inactivated irreversibly. These results indicate that the r-InsP₃R-3 channel, like the X-InsP₃R-1 (Mak and Foskett, 1997), has two inactivated states: one

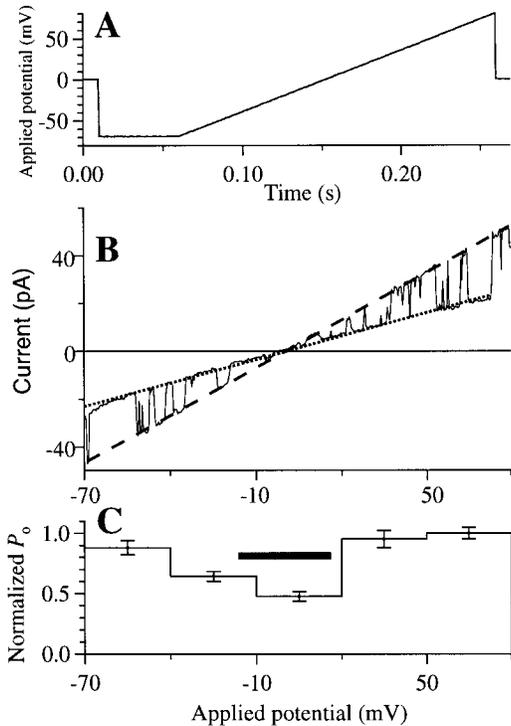


Figure 5. Channel properties of the r-InsP₃R-3 in symmetric 0 mM Mg²⁺ solutions ([Ca²⁺]_i = 80 nM) under a voltage ramp. (A) The applied voltage ramp used to obtain the current traces. (B) I-V relation obtained from a typical current trace using the voltage ramp. Dotted and dashed lines are the fitted closed and open channel I-V relations, respectively. The channel conductance was evaluated as the difference between the slopes of the two fitted lines. (C) Voltage dependence of P_o of the r-InsP₃R-3 channel. The P_o of the channel observed at various V_{app} during the voltage ramp was binned and averaged over 28 traces to generate the histogram. SEM of the P_o is also plotted. The horizontal bar indicates the voltage range -15 to 15 mV in which channel openings and closings were difficult to discern, so P_o was underestimated.

(I_{Vdep}) from which it can be reactivated by a jump in V_{app}, and one (I_{irr}) from which it cannot exit in the presence of the steady [InsP₃] and [Ca²⁺]_i conditions used in our experiments.

Analysis of the InsP₃R-3 P_o in experiments in which V_{app} was altered by ramps indicates that P_o between -70 and -40 mV was not significantly different from that between 20 and 80 mV (Fig. 5). The decrease in P_o observed between -40 and 20 mV is likely an artifact caused by the difficulty in discerning channel openings due to the small channel current under those potentials.

Clustering of the Expressed r-InsP₃R-3

We previously reported that the X-InsP₃R-1 exists in clusters in the outer membrane of the nuclear envelope (Mak and Foskett, 1997). To determine whether channel clustering was also a property of the expressed r-InsP₃R-3, we analyzed the distribution of channel densities among the nuclear patches from cRNA-injected oocytes. The number of channels in each patch was es-

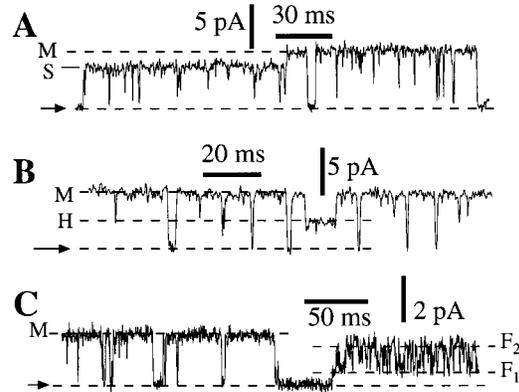


Figure 6. Conductance substates of the InsP₃R channels observed in nuclear patches obtained from r-InsP₃R-3 cRNA-injected oocytes. (A) Current trace showing a transition of an InsP₃R channel from the small (S) conductance state to the main (M) conductance state in symmetric solutions containing 0 mM Mg²⁺; [Ca²⁺]_i = 1,150 nM. (B) Current trace showing an InsP₃R channel in the rare half (H) conductance state in symmetric solutions containing 0 mM Mg²⁺; [Ca²⁺]_i = 255 nM. (C) Current trace showing an InsP₃R channel in the normal and flicker kinetic modes in symmetric 2.5 mM Mg²⁺; [Ca²⁺]_i = 940 nM. F₁ and F₂ denote the conductance states in the flicker kinetic mode.

timated by determining the number of InsP₃R channel current levels observed. Histogram analysis of the distribution of patches exhibiting various numbers of InsP₃R channel current levels was undertaken using the results from 767 nuclear patches (Fig. 8). Analysis of these results and comparisons with the Poisson distribution (Fig. 8) suggests that the expressed r-InsP₃R-3 are not evenly distributed over the surface of the outer nuclear membrane, but are localized in clusters. The similar clustering observed for both the endogenous X-InsP₃R-1 and recombinant expressed X-InsP₃R-3 suggests that the ability of the InsP₃R tetramers to associate into clusters is a general property of InsP₃R_s.

Role of the r-InsP₃R-3 in Ca²⁺ Release-activated Plasma Membrane Ca²⁺ Influx

Two studies have previously concluded that the r-InsP₃R-3 plays a role in mediating Ca²⁺ flux into the cell across the plasma membrane. In lymphocytes, the r-InsP₃R-3 was localized at or near the plasma membrane in apoptotic cells, and antisense inhibition of its expression diminished apoptosis, presumably by diminishing Ca²⁺ influx (Khan et al., 1996). In *Xenopus* oocytes, expression of r-InsP₃R-3 was without effect on InsP₃-mediated Ca²⁺ release, but enhanced the extent and duration of Ca²⁺ influx activated by InsP₃-induced Ca²⁺ release from intracellular stores (DeLisle et al., 1996). Ca²⁺ influx across the plasma membrane in response to depletion of intracellular Ca²⁺ stores, Ca²⁺-release-activated Ca²⁺ influx (DeLisle et al., 1996) or store-operated Ca²⁺ (SOC) influx (Parekh et al., 1997), has been observed in numerous cell types, but the mechanisms that

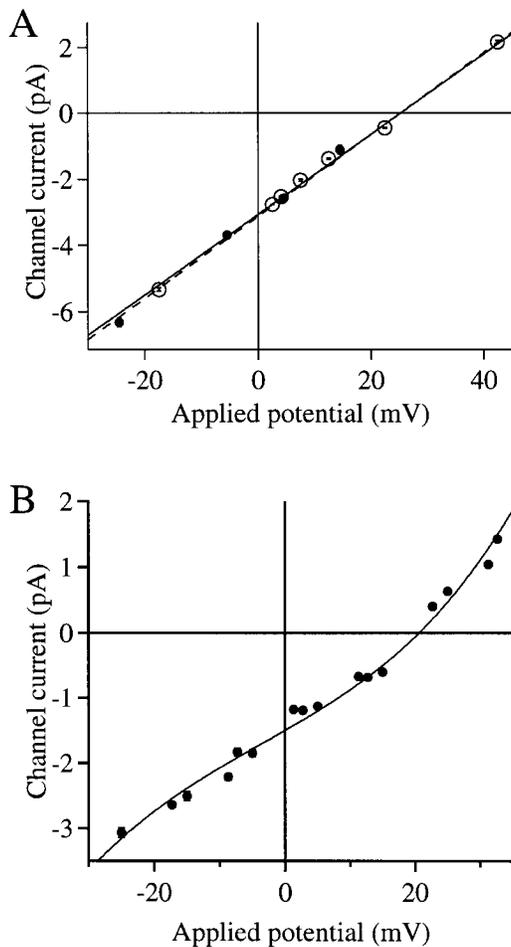


Figure 7. Ion selectivity of the r-InsP₃R-3. (A) The I-V curve of the InsP₃R-3 in the presence of solutions containing asymmetric K⁺ concentrations. The pipette contained the low K⁺ solution. The bath solution was the standard 140 mM KCl solution with 0 mM Mg²⁺. V_{app} values were corrected for the liquid junction potential (Neher, 1992) between the asymmetric solutions across the membrane patch. Channel currents were obtained as in Fig. 3. ● are data points for r-InsP₃R-3 fitted with the dashed line, giving $V_{rev} = 25.2 \pm 0.7$ mV. ○ are data points for X-InsP₃R-1 fitted with the solid line, giving $V_{rev} = 25.3 \pm 0.2$ mV. (B) The I-V curve of InsP₃R-3 in the presence of a [Ca²⁺]_i gradient. The pipette contained the standard 140 mM KCl solution with 0 mM Mg²⁺ and [Ca²⁺]_i = 24.7 nM. To avoid exposing the oocyte nucleus to high [Ca²⁺]_i, which interferes with formation of giga-ohm seals (Mak and Foskett, 1994), an excised patch with InsP₃R activities was first obtained from a nucleus in the standard 140 mM KCl solution (0 mM Mg²⁺). The bath solution was then replaced with the high Ca²⁺ solution by perfusion. V_{app} values were corrected for liquid junction potential (Neher, 1992) between the asymmetric solutions across the membrane patch. Channel currents were obtained as in Fig. 3. The solid curve is a fifth order polynomial fitted to the data points, giving $V_{rev} = 20.6 \pm 0.5$ mV.

couple the state of Ca²⁺ filling of the internal stores with the plasma membrane influx pathway has remained obscure. In a recent review, Putney (1997) suggested, based in part on the above studies, that the InsP₃R-3 may contribute to, or in fact be, a plasma

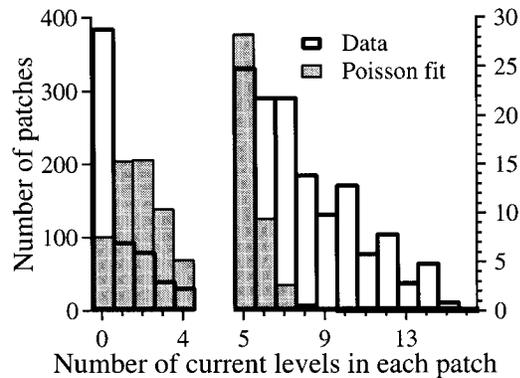


Figure 8. Histogram analysis of number of current levels (abscissa) observed in 767 nuclear patch clamp experiments using isolated nuclei from r-InsP₃R-3 cRNA-injected oocytes (unshaded histogram with thick outline). Also plotted is the Poisson distribution (shaded histogram with thin outline) with the same mean current level number ($\langle n \rangle = 2.016$) per patch. Note the different y-axis scales used in the histogram. The number of patches containing a large number of channels (6–15) exceeds that predicted from Poisson statistics, suggesting that the recombinant type 3 InsP₃R channel localizes in clusters.

membrane Ca²⁺ influx pathway involved in this process. The InsP₃R-3 has been localized near or at the plasma membrane in several cell types (Maranto, 1994; Nathanson et al., 1994; Lee et al., 1997; Yule et al., 1997). Our patch-clamp results suggest that the InsP₃R-3 expressed in oocytes localizes to the outer membrane of the nuclear envelope, and presumably to the ER as well, although they do not exclude a plasma membrane localization. We therefore examined whether expression of r-InsP₃R-3 in *Xenopus* oocytes conferred an enhanced SOC influx in this cell type. We employed two different published TEV protocols to examine SOC influx in oocytes injected with r-InsP₃R-3 cRNA. In one (Hartzell, 1996), [Ca²⁺]_i was monitored by recording the magnitude of the Ca²⁺-activated Cl⁻ current using TEV (Fig. 9 A). InsP₃ was injected into the cytoplasm to activate the InsP₃R, as evidenced by a rapid increase of the Ca²⁺-activated Cl⁻ current ($\Delta G_{IP_3}^{Cl}$). After depletion of the internal Ca²⁺ stores, as evidenced by the lack of Cl⁻ current responses to repeated subsequent InsP₃ injections (Fig. 9 A), the magnitude of Ca²⁺ influx was monitored indirectly by the Cl⁻ current response (ΔG_{SOC}^{Cl}) to an acute step increase in [Ca²⁺]_i in the bathing solution. Comparison of responses of control ($n = 14$ oocytes from five different batches) and r-InsP₃R-3-expressing ($n = 21$ oocytes from the same five batches) oocytes revealed no significant difference in the magnitudes of $\Delta G_{IP_3}^{Cl}$ (24.2 ± 2.6 μ S, mean \pm SEM, for controls oocytes; 22.9 ± 3.8 μ S for r-InsP₃R-3 expressing oocytes; $P = 0.77$) or ΔG_{SOC}^{Cl} (26.6 ± 5.0 μ S for control oocytes; 20.2 ± 3.9 μ S for expressing oocytes; $P = 0.32$). Functional expression of the r-InsP₃R-3 in these experiments was verified by high probability of detect-

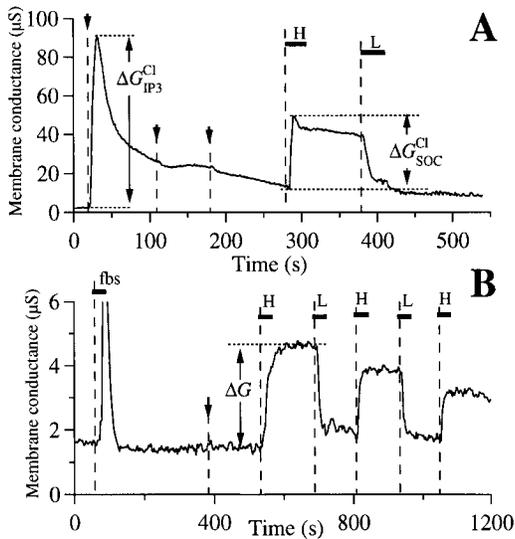


Figure 9. Store-operated calcium influx across the oocyte plasma membrane. Whole-cell transmembrane conductance of r-InsP₃R-3 cRNA-injected *Xenopus* oocytes was recorded in two types of TEV experiments. (A) Ca²⁺-activated plasma membrane Cl⁻ channel current after microinjection of InsP₃ in various bathing solutions. Arrows indicate injections of InsP₃ into the oocyte. Horizontal bars indicate exchange of bathing solution by perfusion with a high Ca²⁺ solution HCa96 (H) or a low Ca²⁺ solution LCa96 (L). The magnitudes of the changes in membrane conductance due to plasma membrane Ca²⁺-activated Cl⁻ current response to InsP₃ injection ($\Delta G_{IP_3}^{Cl}$) and due to Ca²⁺ influx through store-operated Ca²⁺ channels (ΔG_{SOC}^{Cl}) are marked. (B) Store-operated Ca²⁺ currents after activation of InsP₃R in various bathing solutions. Horizontal bars indicate exchange of bathing solution with high Ca²⁺ solution HCa96 (H), low Ca²⁺ solution LCa96 (L), and low Ca²⁺ LCa96 containing 1:100 fetal bovine serum (fbs). Arrow indicates microinjection of BAPTA. Magnitude of changes in membrane conductance due to Ca²⁺ influx through store-operated Ca²⁺ channels (ΔG) is indicated.

ing InsP₃R channel activities during subsequent patch clamping of nuclei from the oocytes used in the TEV experiments. Thus, we were unable to reproduce the results of DeLisle et al. (1996). A second protocol (Yao and Tsien, 1997) was also employed (Fig. 9 B). Membrane conductance (ΔG) due to the SOC influx pathway was not different ($P = 0.98$) in control ($n = 3$, mean = $2.4 \pm 0.5 \mu S$) compared with InsP₃R-3-expressing oocytes ($n = 4$, mean = $2.4 \pm 0.3 \mu S$). These studies also do not support the hypothesis that InsP₃R-3 plays a rate-limiting role in mediating SOC influx.

DISCUSSION

In this report, we have demonstrated the functional expression of the type 3 isoform of the InsP₃R, a widely expressed isoform in mammalian tissues, in a system which enabled recording of its single-channel activity in its normal ER membrane environment. Patch-clamp electrophysiology of the r-InsP₃R-3 in the *Xenopus* nu-

clear envelope represents the first description of the permeation and gating properties of the recombinant type 3 InsP₃R.

Use of *Xenopus* oocytes for Expression of Recombinant ER-localized Ion Channels

A major impediment to understanding [Ca²⁺]_i signaling has been the lack of robust systems to study the gating and permeation properties of the InsP₃R. As intracellular ion channels, they have been considered inaccessible to patch-clamp electrophysiological approaches. Most studies have therefore employed indirect measurements that can only infer channel activity, and which report the responses from populations of channels containing multiple receptor isoforms. Some recent studies have employed cultured cells that express predominantly one isoform (Kaznacheyeva et al., 1998), but these studies continue to suffer from the problems of receptor heterogeneity as well as the inability to understand Ca²⁺ release properties within the context of relevant ion channel parameters, including gating and permeation measures. As with other intracellular ion channels, reconstitution of the InsP₃R into planar lipid membranes has been used to record the single-channel properties (Bezprozvanny et al., 1991, 1994; Watras et al., 1991; Bezprozvanny and Ehrlich, 1994; Perez et al., 1997; Ramos-Franco et al., 1998b). Two strategies have been employed. In the first, membranes from cerebellum (Bezprozvanny et al., 1991; Watras et al., 1991), heart (Perez et al., 1997; Ramos-Franco et al., 1998b) and RIN (Hagar et al., 1998) cells have been used, because of their high expression levels of InsP₃R-1, InsP₃R-2, and InsP₃R-3, respectively. In the second, membranes were obtained from cells engineered to over-express recombinant InsP₃R: InsP₃R-1 in HEK-293 cells (Kaznacheyeva et al., 1998), and wild-type and mutant InsP₃R-1 in COS-1 cells (Ramos-Franco et al., 1998a, 1999). The latter approach has the potential advantage of enabling structure-function studies of the protein. However, the relevance of channel behavior in vitro in artificial bilayers for in vivo behavior in ER membrane remains unknown.

Since the ER is continuous with the outer membrane of the nuclear envelope (Dingwall and Laskey, 1992), we reasoned that ER-localized ion channels would also be present in the nuclear envelope outer membrane, and that single-channel properties of the InsP₃R in its native membrane environment could be examined by patch clamping isolated nuclei. Formation of seals with gigaohm resistance on the outer nuclear membrane, a prerequisite for studying single ion channel activity, was achieved with high frequency despite the high density of nuclear pores in the *Xenopus* oocyte nuclear envelope (Mak and Foskett, 1994, 1997, 1998; Stehno-Bittel

et al., 1995). We have used this system to study the endogenous InsP_3R channel (Mak and Foskett, 1994, 1997, 1998). The oocyte expresses only a single InsP_3R isoform (type 1) and lacks other (e.g., ryanodine receptor) Ca^{2+} release channels (Parys et al., 1992). Because the *Xenopus* oocyte has been used extensively to express functional recombinant mammalian plasma membrane ion channels, we reasoned that this system, combined with nuclear patch clamping, would enable the recording of recombinant InsP_3R channel activities in their native ER membrane. The results presented here demonstrate that this is the case. The major potential complication in this approach is that the oocyte nucleus may not present a null background for expression of recombinant channels, due to the presence of endogenous InsP_3R channels. Nevertheless, we have been able to minimize this problem by using batches of oocytes in which endogenous channel activity could not be detected by nuclear patch clamping. The basis for frog-to-frog variability in our ability to record the endogenous InsP_3R -1 in oocyte nuclei is not clear, although we believe there is a seasonal component, in addition to others. It has not been uncommon in our experience to examine oocytes from many different frogs over the course of a couple of months and not record the endogenous channel in any of hundreds of nuclear patches. In preliminary experiments, we determined that the lack of detectable endogenous InsP_3R channel activities immediately after oocyte isolation did not reverse over time in culture. Therefore, in the present study, we selected "null backgrounds" for expression of the type 3 channel by performing a day of screening patch-clamp experiments to ascertain that the endogenous channel was minimally present in the nuclei of the oocytes used for injection. In contrast, in oocytes injected to express the r- InsP_3R -3, the number of InsP_3R channels observed in nuclear patches was dramatically increased by nearly 40-fold. Based purely on statistical probabilities, there is therefore little likelihood of contamination of the data by InsP_3R -1 homotetramers in our studies. Nevertheless, it remained possible that expression of the recombinant r- InsP_3R -3 might have upregulated the endogenous *Xenopus* type 1 channel. However, Western blot analysis revealed that the amount of InsP_3R -1 remained constant in both control and injected oocytes. These results strongly indicate that channel activities recorded in the r- InsP_3R -3-expressing oocytes were contributed by the recombinant channels. Assuming random association of monomers to form tetrameric functional channels, binomial statistics indicates that >90% of the recorded channels in these studies are contributed by r- InsP_3R -3 homotetramers, whereas <10% of recorded channels represent tetramers containing three type 3 monomers with one type 1 monomer. The validity of these statistically based

conclusions was evaluated in coimmunoprecipitation experiments using control and injected oocytes. We determined that the endogenous type 1 InsP_3R coimmunoprecipitated with the expressed recombinant type 3 InsP_3R . Coimmunoprecipitation of different InsP_3R isoforms has previously been interpreted to indicate heterotetramer formation (Joseph et al., 1995; Monkawa et al., 1995; Wojcikiewicz and He, 1995; Nucifora et al., 1996). Of note, quantification of the amounts of coimmunoprecipitated protein suggests that <10% of the expressed recombinant type 3 channels were associated with the endogenous *Xenopus* channels. This biochemical result is in good agreement with the extent of isoform associations predicted from patch clamp statistics. Thus, the combined patch-clamp results and their statistical analysis, strongly corroborated by biochemical evidence, indicates that a large majority (>90%) of the patch-clamp data in the present experiments were contributed by r- InsP_3R -3 homotetramers.

The similar permeation properties of the expressed and endogenous channels, as we have now defined in the present work, necessitated this exhaustive approach to ensure the identities of the recorded channels. Nevertheless, we discovered that the gating properties of the two channels can be used to distinguish them, under conditions of low cytoplasmic Ca^{2+} concentration. Thus, whereas the P_o of both channels is equivalent (~ 0.8) when cytoplasmic Ca^{2+} concentration is $1 \mu\text{M}$ (Mak et al., 1998, and this study), the P_o of the type 3 channel is considerably higher than that of the endogenous channel when cytoplasmic Ca^{2+} concentration is $\sim 80 \text{ nM}$. Although more detailed studies are required to define the Ca^{2+} concentration dependence of InsP_3R -3 gating, this ability to distinguish the expressed channels from the endogenous ones provides additional strong support for the validity of this approach. Parenthetically, our results suggest that high $[\text{Ca}^{2+}]_i$ ($>50 \mu\text{M}$) inhibits the type 3 channel similarly to the endogenous type 1 channel. The conclusion that high $[\text{Ca}^{2+}]_i$ inhibition does not distinguish the types 1 and 3 channels contrasts with that reached in a bilayer study using microsomes isolated from a cell type expressing predominately the type 3 isoform (Hagar et al., 1998). The bases for the discrepant results in the two studies are not known. In the present study, homotetramers of the type 3 isoform were recorded, as discussed above, in a native membrane environment, whereas the bilayer study recorded channels of unknown isoform composition in artificial membranes after reconstitution. Further, more detailed studies are in progress to determine the complete $[\text{Ca}^{2+}]_i$ dependence of InsP_3R -3 gating, which may help to resolve these discrepancies. Our success in the approach described in the current work suggests that it will now be possible to express and record the properties of mutant InsP_3Rs in the ER in

future studies of structure–function relationships, and that it will be useful for studying other ER-localized ion channels, whether localized there normally or as a result of natural or engineered mutations. Of note, however, it may be necessary to perform a similar series of control experiments for each mutant expressed to ensure that the system behaves as we have described for the wild-type r-InsP₃R-3.

Permeation and Gating Properties of the r-InsP₃R-3 and X-InsP₃R-1 from Nuclear Patch Clamp Studies

We examined the basic channel properties of the r-InsP₃R-3 and found that they are remarkably similar, particularly in view of both the isoform and species differences, to those of the X-InsP₃R-1 in the same membrane system.

In terms of permeation, both channels are relatively nonselective cation channels: P_K/P_{Cl} is 4.6 for both X-InsP₃R-1 and r-InsP₃R-3 (Fig. 3). Our previously reported value of P_K/P_{Cl} for X-InsP₃R-1 (Mak and Foskett, 1994) was overestimated because Mg²⁺ present in the pipette and bath solutions in those experiments caused nonlinearity in the I-V relation of the channel (Mak and Foskett, 1998) and the use of the Goldman-Hodgkin-Katz equation to fit the experimental I-V relation and determine V_{rev} was inappropriate. Applying this revised value of P_K/P_{Cl} , $P_{Ca}:P_{Ba}:P_{Mg}:P_K$ for X-InsP₃R-1 is calculated to be 12.7:8.6:6.4:1 based on V_{rev} measured in Mak and Foskett (1998). Thus, the values for P_{Ca}/P_K for the two channels are very similar (12.7 for X-IP₃R-1; 11.0 for r-IP₃R-3). In both channels, the I-V relation is linear in the absence of Mg²⁺, and the absence of Mg²⁺ is associated with occasional conductance fluctuations under constant V_{app} during the course of a recording (~2 min), or even during single-channel openings (~10 ms). The conductances of both channels are stabilized and become rectified as [Mg²⁺] is increased on either side of the membrane. Mg²⁺ decreases single-channel conductance of both channels to a similar extent (Fig. 3 B), due to permeant ion block (Mak and Foskett, 1998).

Both channels display conductance states of S and H (Mak and Foskett, 1994, 1998), while both gate primarily in the main M state, though the frequency of finding the channel in state S was much higher in the r-InsP₃R-3. Both channels undergo two forms of InsP₃-dependent inactivation: one (relatively rare) that can be reversed by momentarily jumping the applied potential to 60 mV, and one (all channels) that is apparently irreversible under our steady state experimental conditions. Although we have not quantified the kinetics of irreversible inactivation for the InsP₃R-3, it is our general impression that they are similar to those of the X-InsP₃R-1 (time constant ~25 s) (Mak and Foskett, 1997). Both channels displayed flicker kinetics, although this was observed less frequently for the type 3 channel, and the

relative values of the two flicker state conductances is the same for both receptors (Mak and Foskett, 1997).

A difference between the two types of InsP₃R is their voltage dependencies. Whereas P_o of the X-InsP₃R-1 was reduced by an increase in V_{app} (Mak and Foskett, 1997), the P_o of r-InsP₃R-3 showed little voltage dependence, or was even slightly activated by high magnitude V_{app} regardless of polarity (this study).

Comparison of the Permeation and Gating Properties of Different InsP₃R Isoforms

Our results suggest that the permeation pathways and gating mechanisms are remarkably similar between the types 1 and 3 InsP₃R channels. Including the present data, single-channel recordings have now been obtained for all three known InsP₃R isoforms. A side-by-side comparison of the bovine type 1 and ferret type 2 receptors in proteoliposome-fused bilayers revealed that their conductance properties were nearly identical (Ramos-Franco et al., 1998b). Taken together with our results, these data indicate that the permeation pathways in all three InsP₃R channels are very similar. This result has implications for the structural properties of the conduction pores in the three isoforms. Of note, there is conservation among the isoforms of amino acid sequences in regions that are speculated to be involved in pore formation (Yamamoto-Hino et al., 1994).

Nevertheless, some differences have been reported in the gating behaviors of the three channel isoforms, although it is not clear whether they reflect isoform differences or are due to unknown variables associated with different experimental protocols. The type 1 channel has been studied in different systems, including reconstitution of cerebellar membranes (Bezprozvanny et al., 1991, 1994; Watras et al., 1991; Bezprozvanny and Ehrlich, 1994) and proteoliposomes (Kaznacheyeva et al., 1998) and patch clamp of the *Xenopus* nuclear envelope (Mak and Foskett, 1994, 1997, 1998; Stehno-Bittel et al., 1995), whereas the type 2 channel has been studied using reconstituted proteoliposomes (Perez et al., 1997; Ramos-Franco et al., 1998b), and the type 3 channel has been studied by reconstitution (Hagar et al., 1998) and nuclear patch clamping (this study). Furthermore, the channels have come from different species (type 1: canine, bovine, *Xenopus*, rat; type 2: ferret; type 3: rat). Reconstituted receptors in bilayers are distinguished by a maximum P_o of ~0.7 for the type 2 channel vs. ~0.2 for the type 1 isoform (Ramos-Franco et al., 1998b) and ~0.05 for the type 3 channel (Hagar et al., 1998). These differences were interpreted as reflecting isoform differences (Ramos-Franco et al., 1998b), but the fact that the maximum P_o observed for both the X-InsP₃R-1 (Mak et al., 1998) and r-InsP₃R-3 (Figs. 2 and 3 A) was nearly identical (~0.8) suggests that isoform differences may not be particularly rele-

vant. Indeed, other factors may be more important since the gating properties reported for the type 1 receptor from *Xenopus* in the nuclear membrane and rat/canine in artificial bilayers are quite distinct, and those for the rat type 3 channel in endoplasmic reticulum (this study) versus bilayers (Hagar et al., 1998) are similarly distinct. The *X*-InsP₃R-1 studied by nuclear patch clamping (Mak and Foskett, 1997) has a much higher maximum P_o , longer open time constant, and prominence of a different conductance state compared with the r-InsP₃R-1 studied by reconstitution into lipid bilayers (Watras et al., 1991), and demonstrates inactivation and voltage-dependent and flicker kinetics that have not been observed in the r-InsP₃R-1 studies. Similarly, the r-InsP₃R-3 studied by nuclear patch clamping (this study) has a maximum P_o of 80% vs. 5% for the reconstituted channel in bilayers (Hagar et al., 1998), and also displays the same rich repertoire of gating behaviors not observed in the bilayer studies. It is possible that the InsP₃R channel properties are sensitive to the membrane isolation, solubilization, and reconstitution protocols employed in bilayer studies. This explanation cannot, however, account for the differences observed between the types 1 and 2 receptors using similar reconstitution protocols (Ramos-Franco et al., 1998b) unless there is a differential sensitivity among isoforms or tissue sources to these procedures. In preliminary studies, we expressed the r-InsP₃R-1 in *Xenopus* oocytes and patch clamped the oocyte nuclear membrane, and have observed gating properties that are similar to those of the *X*-InsP₃R-1 and r-InsP₃R-3 in the same membrane (our unpublished data). This result suggests that the channels in the same native membrane environment gate similarly, and that the channel properties are sensitive to the membrane environment or to the protocols involved in reconstitution. Although further single-channel permeation and gating determinations are necessary to resolve these issues, the data available to date suggest that the three receptor isoforms share quite similar permeation properties. Taken together, these results therefore suggest that if cellular expression of multiple InsP₃R isoforms is a mechanism to modify the temporal and spatial features of [Ca²⁺]_i signals, then it must be achieved by isoform-specific regulation or localization of various types of InsP₃Rs that have relatively similar Ca²⁺ permeation properties.

We thank Dr. Graeme I. Bell and Dr. Bill Skach for providing expression vectors.

This work was supported by grants to J.K. Foskett from the National Institutes of Health (NIH) (GM56328) and the Department of Defense, and to S.K. Joseph from the NIH (DK34804).

Submitted: 10 November 1999

Revised: 7 January 2000

Accepted: 18 January 2000

Released online: 14 February 2000

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