

Single-Channel Recordings of Recombinant Inositol Trisphosphate Receptors in Mammalian Nuclear Envelope

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ABSTRACT Inositol 1,4,5-trisphosphate (InsP₃) receptors (InsP₃Rs) are intracellular Ca²⁺ channels gated by the second messenger InsP₃. Here we describe a novel approach for recording single-channel currents through recombinant InsP₃Rs in mammalian cells that applies patch-clamp electrophysiology to nuclei isolated from COS-7 cells transiently transfected with the neuronal (SII(+)) and peripheral (SII(−)) alternatively-spliced variants of the rat type 1 InsP₃R. Single channels that were activated by InsP₃ and inhibited by heparin were observed in 45% of patches from nuclei prepared from transfected cells overexpressing recombinant InsP₃Rs. In contrast, nuclei from cells transfected with the vector alone had InsP₃-dependent channel activity in only 1.5% of patches. With K⁺ (140 mM) as the permeant ion, recombinant SII(+) and SII(−) channels had slope conductances of 370 pS and 390 pS, respectively. The recombinant channels were 4-fold more selective for Ca²⁺ over K⁺, and their open probabilities were biphasically regulated by cytoplasmic [Ca²⁺]. This approach provides a powerful new methodology to study the permeation and gating properties of recombinant mammalian InsP₃Rs in a native mammalian membrane environment at the single-channel level.

INTRODUCTION

The second messengers inositol 1,4,5-trisphosphate (InsP₃) and diacylglycerol are generated in response to agonists that activate phospholipase C (Berridge and Irvine, 1989). InsP₃ diffuses through the cytoplasm and binds to its receptor (InsP₃R), which is an intracellular Ca²⁺ channel (Patel et al., 1999). Binding of InsP₃ to the InsP₃R gates the channel open. The resulting rapid release of stored Ca²⁺ from the endoplasmic reticulum generates elevations of the concentration of free Ca²⁺ in the cytoplasm ([Ca²⁺]_i), which is transduced into the modulation of a diverse array of cellular processes (Marks, 1997).

Structurally, the InsP₃R is a tetrameric channel composed of subunits derived from three separate genes (types 1, 2, and 3 InsP₃Rs) (Mignery et al., 1990; Südhof et al., 1991; Blondel et al., 1993). All three isoforms are capable of forming heterotetramers, adding to channel diversity (Joseph et al., 1995; Wojcikiewicz and He, 1995; Monkawa et al., 1995; Nucifora et al., 1996). Furthermore, the type 1 isoform is alternatively spliced at three separate locations in a tissue-specific manner (Mignery et al., 1990; Danoff et al., 1991; Newton et al., 1994). The resulting receptor heterogeneity is believed to contribute to the spatial and temporal complexity of [Ca²⁺]_i signals observed in many cell types (Miyakawa et al., 1999; Thomas et al., 1996). An important goal is to determine the functions, properties, and regulation of specific InsP₃R isoforms, but this has proved difficult due

to the presence of multiple isoforms in most tissues (Wojcikiewicz, 1995; Taylor et al., 1999). To address this problem, we have attempted to develop functional assays that measure the properties of recombinant InsP₃R channels specifically. We previously showed that ⁴⁵Ca²⁺ fluxes from microsomal vesicles prepared from COS-7 cells engineered to overexpress recombinant InsP₃Rs and sarcoplasmic- and endoplasmic-reticulum Ca²⁺-ATPase type 2b (SERCA-2b) could be attributed specifically to the recombinant InsP₃Rs (Boehning and Joseph, 2000). However, this approach cannot provide detailed information about gating or ion permeation through InsP₃R channels. We therefore sought to develop a method to measure the activities of recombinant InsP₃Rs in mammalian endoplasmic reticulum membranes at the single-channel level.

The intracellular location of InsP₃Rs once precluded the use of traditional patch-clamp electrophysiological approaches to measure single-channel currents, requiring that the channels be reconstituted into planar lipid bilayers. Bilayer reconstitution has recently been used to record the single-channel activities of recombinant types 1 and 2 InsP₃Rs (Kaznacheyeva et al., 1998; Ramos-Franco et al., 1998a, 2000). Nevertheless, this approach suffers from the uncertainty that the observed channel properties may not reflect those of the channel when it is in its normal membrane environment. The understanding that the outer membrane of the nuclear envelope is continuous with the endoplasmic reticulum membrane, and the ability to achieve gigohm seals on isolated nuclei with a patch pipette, has enabled single-channel recordings of the native *Xenopus laevis* type 1 InsP₃R by patch-clamp electrophysiology of isolated oocyte nuclei (Mak and Foskett, 1994). More recently, this system has been successfully developed to record the properties of recombinant InsP₃R channels (rat type 3) (Mak et al., 2000). Nevertheless, it is desirable to

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have a method to record the activities of recombinant mammalian channels in native mammalian membranes. Here, we have developed a novel methodology to study recombinant InsP₃R isoforms expressed in cultured mammalian cells. We have recorded single recombinant InsP₃R channels by patch-clamping nuclei isolated from COS-7 cells engineered to transiently overexpress recombinant rat InsP₃Rs. COS-7 cells were chosen for these studies because recombinant InsP₃Rs overexpressed in these cells do not associate with the endogenous InsP₃R channels (Boehning and Joseph, 2000). We describe, for the first, time, the single-channel properties of both the neuronal (SII(+)) and peripheral (SII(-)) splice variants of the rat type 1 InsP₃R in native mammalian membranes. Both channels required InsP₃ for gating and they had similar permeation properties and ionic selectivities. Examination of the [Ca²⁺]_i dependence of the open probability of the SII(+) channel showed it to be biphasic and remarkably similar to that of the *Xenopus* type 1 channel recorded in oocyte nuclear membranes. The experimental approach described here is advantageous over other methods because of the ability to record single recombinant InsP₃R channels in native mammalian membranes. The simplicity and reproducibility of expressing and detecting recombinant InsP₃R channels on COS-7 cell nuclei should significantly advance efforts to elucidate the structure-function relationships in this important intracellular Ca²⁺ channel.

EXPERIMENTAL PROCEDURES

Expression constructs

The neuronal rat type 1 INSP₃R SI(-), SIII(+), SII(+) splice variant was the kind gift of Dr. Thomas Südhof (University of Texas Southwestern Medical Center, Houston, TX). This cDNA was cloned into pcDNA3.1, as described elsewhere (Boehning and Joseph, 2000). The construct encoding the rat SII(-) splice variant lacking amino acids 1693–1732 has been described previously (Lin et al., 2000).

Cell culture and transfection

Maintenance and transfection of COS-7 cells with InsP₃R cDNA has been described in detail elsewhere (Boehning and Joseph, 2000). Briefly, 75-cm² flasks were seeded with 1.2×10^6 cells the day before transfection. Cells were transfected with LT1 transfection reagent (Mirus, Madison, WI) for 5 hours in serum-free Dulbecco's modified Eagle's medium (DMEM) before replacing the medium with DMEM supplemented with 10% fetal bovine serum. Cells were processed after 48 hours, as described below.

Preparation of cellular homogenates

The culture flasks were washed twice with phosphate-buffered saline (PBS) and the cells were released by exposure for 10 min to 5 mM EDTA, 0.5% bovine serum albumin in PBS. The cells were then centrifuged ($500 \times g$; 4°C), washed once with 5 ml of resuspension buffer (0.25 M sucrose, 0.15 M KCl, 3 mM β -mercaptoethanol, and 10 mM Tris, pH 7.5), pelleted again, and resuspended in 1 ml of the same buffer supplemented with 1 mM PMSF and $1 \times$ complete protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, Indiana). The cell suspension was then gently homogenized by 25 strokes in a motor-driven glass teflon homogenizer operating at 350 rpm, with cell integrity monitored by trypan blue staining. Homogenization was stopped when ~30% of cells showed nuclear trypan blue staining. Further homogenization resulted in a reduced probability of detecting InsP₃-gated channels. Cell lysates were stored in resuspension buffer on ice and used the same day in patch-clamp experiments.

Patch-clamping COS-7 cell nuclei

Approximately 10 μ l of cellular homogenate was added to a dish containing 1 ml of bath solution and 0.001% trypan blue (see Patch-clamp solutions) and transferred to the stage of a microscope. Isolated nuclei visually free of extraneous cellular debris were identified by trypan blue staining, and patch-clamped at room temperature. Because InsP₃R channel open probability (P_o) decreased after ~40 min in bath buffer at room temperature, nuclei were replaced regularly. Patch pipettes typically had resistances of 10–20 megohms; nuclear seals greater than 5 gigohms were routinely obtained. Most experiments were done in the on-nucleus configuration, although patches could be excised, exposing the luminal aspect of the InsP₃R to the bath, without degradation of seal quality or channel activity. Single-channel currents were amplified using an Axopatch-1D amplifier (Axon Instruments, Foster City, CA.) with anti-aliasing filtering at 1 kHz, and transferred to a Power Macintosh 8100 via an ITC-16 interface (Instrutech Corp, Port Washington, NY). Data were digitized at 5 kHz, and written directly to hard disk by Pulse + PulseFit software (HEKA Elektronik, Lambrecht/Pfalz, Germany). The applied potential is the pipette electrode potential minus the bath electrode reference potential, and positive current flows from the pipette to bath. Single-channel recordings were analyzed using TAC 3.03 (Bruxton, Seattle, WA) and plotted using Igor Pro 3 (WaveMetrics, Lake Oswego, OR) and SigmaPlot (SPSS science, Chicago, IL). Permeability ratios were calculated using the experimentally determined reversal potentials (Hille, 1992).

Patch-clamp solutions

The bath solution contained 140 mM KCl, 10 mM HEPES, 500 μ M BAPTA, 0.001% trypan blue, and 250 nM $[Ca^{2+}]_{free}$ (~ 220 μ M added Ca^{2+}) adjusted to pH 7.1 with KOH. Bath trypan blue concentrations $>0.002\%$ reduced InsP₃R single-channel P_o (data not shown), and were therefore avoided. Pipette solutions contained 140 mM KCl, 10 mM HEPES, 100 μ M BAPTA, 0.5 mM NaATP, 10 μ M InsP₃, and 200 nM $[Ca^{2+}]_{free}$ (unless otherwise noted), adjusted to pH 7.1 with KOH. To determine the effects of $[Ca^{2+}]_{free}$ on InsP₃R P_o , the free Ca^{2+} concentration in the patch pipette was varied. All P_o calculations were performed on recordings exhibiting only a single channel. Each P_o data point represents the average of at least three separate recordings obtained under identical experimental conditions. Free Ca^{2+} concentrations in all buffers were determined with a Ca^{2+} -selective mini-electrode (Sigel and Afholter, 1987). To determine relative ionic selectivity, we used a high- Ca^{2+} bath solution with 50 mM $CaCl_2$, 30 mM KCl, and 10 mM HEPES, adjusted to pH 7.1 with KOH; and a low K^+ (osmolality not adjusted) pipette solution with 14 mM KCl, 10 mM HEPES, 100 μ M BAPTA, 0.5 mM NaATP, 10 μ M IP₃ and 200 nM $[Ca^{2+}]_{free}$, adjusted to pH 7.1 with KOH. All ion selectivity determinations were corrected for the liquid junction potential (Neher, 1995).

RESULTS

Recording of recombinant InsP₃Rs on isolated nuclei

We have shown previously that InsP₃R isoforms expressed in COS-7 cells form tetramers, bind InsP₃, and localize to the endoplasmic reticulum (Joseph et al., 2000). Importantly, the recombinant channels do not hetero-multimerize with the endogenous receptor population, and their expression does not up-regulate the expression of the native type 2 and type 3 InsP₃Rs (Joseph et al., 2000; Boehning and Joseph, 2000). Recombinant InsP₃Rs transfected into COS-7 cells form functional Ca^{2+} release channels, as determined using a $^{45}Ca^{2+}$ flux assay (Boehning and Joseph, 2000). Previous studies demonstrated that native InsP₃R channel activities can be recorded by patch-clamping isolated *Xenopus laevis* oocyte nuclei (Mak and Foskett, 1994, 1997, 1998; Mak et al., 1998, 1999). The single channel activities of expressed recombinant mammalian InsP₃Rs can be similarly recorded, using batches of oocytes that express low levels of native InsP₃Rs (Mak et al., 2000). Nevertheless, it is technically challenging to discover such batches, and the system is not ideal because the recombinant mammalian channels are examined in an amphibian membrane. Because COS-7 cells express a relatively low density of endogenous InsP₃Rs (Wojcikiewicz, 1995; Joseph et al., 2000; Boehning and Joseph, 2000), we considered that this

cell type might provide a consistently low background level of endogenous InsP₃R channel activity in their isolated nuclei. We reasoned that nuclei isolated from transfected COS-7 cell nuclei might therefore provide a useful system for recording single recombinant mammalian InsP₃Rs in a native mammalian membrane.

COS-7 cell homogenates were added directly to a bath containing trypan blue on the stage of an inverted microscope equipped with video imaging. Isolated COS-7 cell nuclei were typically 10–30 μ m in diameter (Fig. 1 A). Nuclei suitable for patching were identified as those that

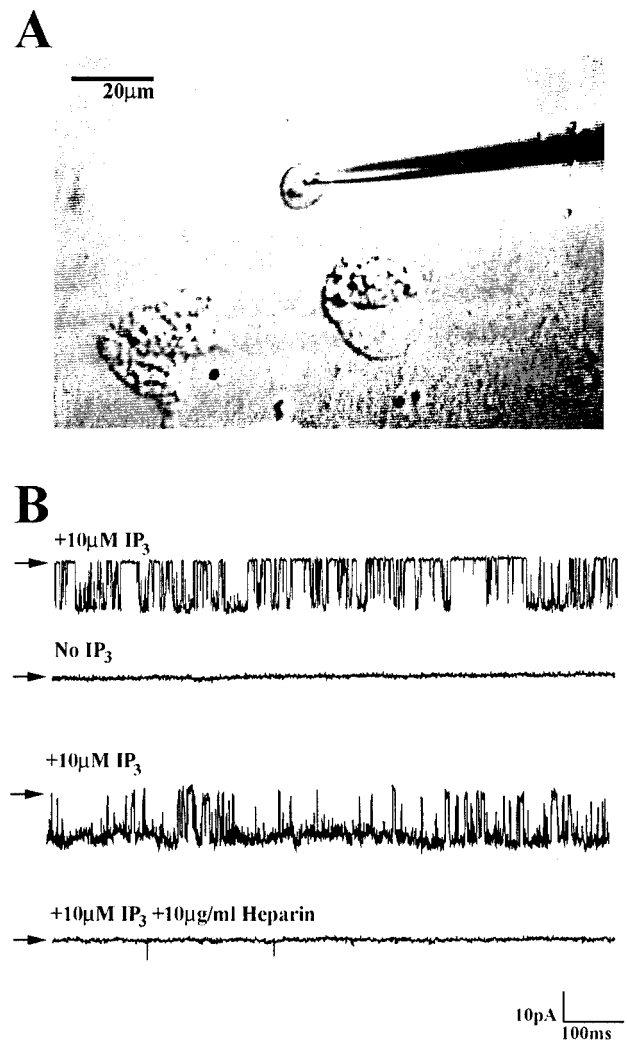


FIGURE 1 Detection of recombinant InsP₃R channels. (A) Isolated nuclei free of cellular debris were visually identified for patch clamp electrophysiology. An intact cell is visible directly below the isolated nucleus being patched, and a damaged cell is present in bottom left. (B) The InsP₃ dependence of gating was confirmed by repeatedly patching onto the same nucleus with pipettes alternatively containing 10 μ M InsP₃, no InsP₃, or InsP₃ plus heparin (10 μ g/ml). Pipette solutions contained low K^+ buffer, and the bath contained 140 mM KCl (see Patch-clamp solutions). Arrow, zero current level. Traces from type 1 SII(+) splice variant recorded at 0 mV holding potential. Variability in P_o among patches is typical.

displayed a smooth aspect over 50–100% of their surface area, and we attempted to form seals on those smooth areas. It was possible to routinely obtain seals with resistances >5 gigohms on such nuclei. To record InsP₃R channel activities, a saturating concentration of InsP₃ (10 μ M) was included in the pipette solution, because the ligand-binding region of the expressed InsP₃R is located on the cytoplasmic aspect of the channel, which faced into the pipette. In nuclei from COS-7 cells transiently transfected with InsP₃R type 1 cDNA, large conductance channels were detected in 45% of nuclei examined with InsP₃ in the pipette solution (Table 1; Fig. 1 B). This rate of detecting channel activity is consistent with the 40–60% transfection efficiency of InsP₃R cDNA into COS-7 cells (Joseph et al., 2000). Multiple channel levels were detected in $\sim 30\%$ of the patches (Table 1). The probability of detecting channel activity increased to $>95\%$ when membrane patches were obtained from the same nucleus that had previously shown channel activity. In similar experiments using nuclei isolated from cells transfected with the vector alone, channels were observed in only 2 of 126 seals (1.5%; Table 1). Therefore, the probability of detecting channels was dependent upon the expression of recombinant InsP₃Rs.

To confirm that the observed channel activities were contributed by the InsP₃R, the InsP₃ dependence of channel activity was investigated by repeated patching of the same nucleus with pipettes alternately containing either 10 μ M InsP₃, no InsP₃, or InsP₃ plus the InsP₃R competitive antagonist heparin (10 μ g/ml; Sigma Chemical Co., St. Louis, MO; low molecular weight). Membrane patches could be obtained successively from the same nucleus, as demonstrated in the series of traces in Fig. 1 B, and this sequence could be repeated for as long as the nucleus remained undamaged and attached to the surface of the coverslip. Channel activity was observed when InsP₃ was present in the pipette solution, but not when it was absent or present together with heparin. (Fig. 1 B). We therefore conclude that the channels we observed were the expressed InsP₃R.

Ionic conductance and selectivity of alternatively spliced rat InsP₃R-1

In symmetric K⁺ solutions, the SII(+) splice variant channel had a linear current-voltage relation, with a slope conductance of 369 ± 6 picosiemens (pS). The SII(−) splice variant channel had a similar slope conductance of 389 ± 5 pS (Figs. 2 A and 2 B). These values are similar to those determined for the types 1 and 3 channels measured under similar recording conditions on *Xenopus* oocyte nuclei (320–360 pS; Mak and Foskett, 1998; Mak et al., 2000). To determine the ion selectivities of the recombinant channels, reversal potentials were measured in the presence of asymmetrical KCl solutions. With a low K⁺ buffer in the pipette and 140 mM KCl in the bath (see Patch-clamp solutions), current through the SII(+) channel reversed at $+44.9 \pm 0.5$ mV; the SII(−) splice variant displayed a similar reversal potential ($+44.8 \pm 0.2$ mV) (Fig. 2 C). Using the Goldman-Hodgkin-Katz equations (Hille, 1992), the relative permeabilities of the channel to K⁺ and Cl[−] ($P_{K^+} : P_{Cl^-}$) was determined to be 15. Thus, the channel is cation selective. This value is in good agreement with the relative K⁺:Cl[−] permeability determined for the *Xenopus* type 1 InsP₃R (Mak and Foskett, 1994). To determine the relative permeabilities of Ca²⁺ and K⁺, channels were first detected in symmetrical 140-mM KCl solutions, and then the patch was excised and the bath was replaced with a high Ca²⁺ solution (see Patch-clamp solutions). Voltage ramps from -20 mV to $+60$ mV were employed to determine the reversal potential (Fig. 3 A). Under these conditions, both SII(+) and SII(−) splice variant channels had reversal potentials of $+18$ mV when corrected for the liquid junction potential (Fig. 3 B). The value for the reversal potential is similar to those determined for the *Xenopus* type 1 (Mak and Foskett, 1994) and rat type 3 (Mak et al., 2000) channels in native membranes. Using the Goldman-Hodgkin-Katz equations and the value of $P_{K^+} : P_{Cl^-}$, the relative permeability of Ca²⁺ to K⁺ ($P_{Ca^{2+}} : P_{K^+}$) was determined to be ~ 4 . Thus, both

TABLE 1 Detection of InsP₃-sensitive channels on transfected COS-7 cell nuclei

Condition	InsP ₃ R	PD	>1 channel (%)	Inactivation (%)*
Recombinant type 1 InsP ₃ R + InsP ₃	317 (708)	45.0%	27%	9.3% (Ca ²⁺ ≤ 1 μ M) 77.0% (Ca ²⁺ ≥ 10 μ M)
Recombinant type InsP ₃ R − InsP ₃	0 (11)	0.0%	0%	N/D
Recombinant type InsP ₃ R + heparin	0 (20)	0.0%	0%	N/D
Recombinant type InsP ₃ R + heparin + InsP ₃	0 (19)	0.0%	0%	N/D
pcDNA3.1 + InsP ₃	2 (126)	1.5%	0%	N/D

InsP₃R, number of InsP₃-gated channels detected, with total attempts in parenthesis; PD, probability of detecting an InsP₃-gated channel; N/D, not determined.

Sensitivities to heparin (10 μ g/ml) or the absence of InsP₃ were determined using nuclei previously demonstrating InsP₃-sensitive recombinant channel activity (PD $> 95\%$ when membrane patches were obtained from InsP₃-transfected nuclei previously demonstrating InsP₃-sensitive channel activity).

*Inactivation defined as channel activity lasting fewer than two minutes from the start of recording.

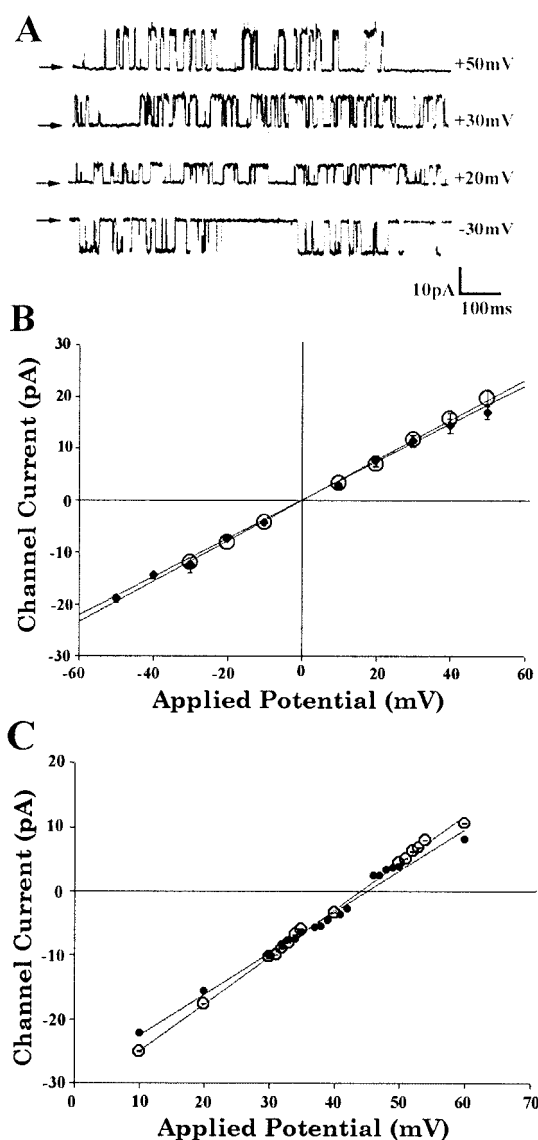


FIGURE 2 Conductances and ion selectivities of alternatively spliced InsP_3Rs . (A) Typical current traces at various holding potentials of recombinant InsP_3R activities in symmetrical 140 mM KCl solutions. The traces are a continuous recording of the type 1 SII(-) splice variant channel. The SII(+) splice variant demonstrated similar gating behavior (not shown). (B) Current-voltage relationship of the SII(-) splice variant (open circles) and the SII(+) splice variant (closed circles). Data points represent mean \pm S.E.M. of three separate determinations. In most cases error bars are smaller than the symbols. Solid lines, linear regressions through the data points. The SII(-) channel had a slope conductance of 389 ± 5 pS, and the SII(+) channel had a slope conductance of 369 ± 6 pS. (C) Current-voltage relationship of the SII(-) (open circles) and the SII(+) (closed circles) splice variants with low K^+ in pipette and 140 mM KCl in bath (see Patch-clamp solutions). Current amplitudes determined at holding potentials between +10 mV and +60 mV. Solid lines, linear regressions through the data points.

recombinant splice variant of the rat type 1 InsP_3R are Ca^{2+} -selective cation channels.

A feature of InsP_3R channel gating is the presence of sub-conductance states (Watras et al., 1991; Mak and Fos-

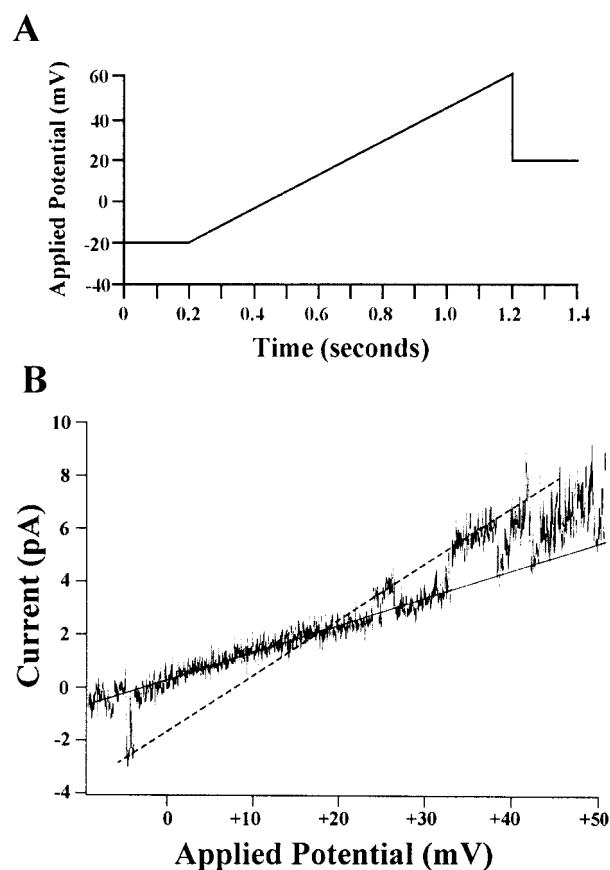


FIGURE 3 Divalent cation selectivities of recombinant $\text{InsP}_3\text{R-I}$ splice variants. (A) Voltage ramp protocol used to determine the reversal potential in the presence of 140 mM KCl in pipette and high Ca^{2+} in the bath (see Patch-clamp solutions). A ramp protocol was employed to minimize Ca^{2+} flux through the channel toward the cytoplasmic face, to minimize possible Ca^{2+} inhibition of channel activity. (B) Single-channel openings during voltage ramp. Zero current level, indicated by solid line, was not leak subtracted. Dashed line was fitted to channel openings. This recording of the SII(-) splice variant is representative of at least three separate determinations. The SII(+) splice variant was not significantly different (not shown).

kett, 1994, 1997; Mak et al., 2000). The recombinant InsP_3Rs observed in the present study occasionally opened to clearly defined sub-conductance levels for short periods (~ 10 to 100 ms). However, these events were rare, observed in $<0.01\%$ of all channel openings. Whereas a half (H) conductance state, reminiscent of an H state in the *Xenopus* $\text{InsP}_3\text{R-I}$ (Mak and Foskett, 1994, 1997), was observed (Fig. 4 A), the $\text{InsP}_3\text{R-I}$ channels recorded in COS-7 cell nuclei also exhibited other sub-conductance states that have not been previously reported (Fig. 4 B and C). The flicker kinetic mode and the double (D) substates, which have been observed in both the rat type 3 (Mak et al., 2000) and the native *Xenopus* type 1 (Mak and Foskett, 1997) InsP_3Rs recorded in the oocyte nuclear envelope, were not observed in the InsP_3R channels recorded in COS-7 cells.

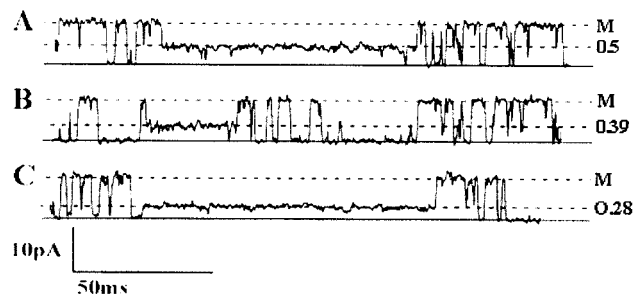


FIGURE 4 Recombinant InsP₃R channels have multiple subconductance states. Brief transitions to subconductance states were occasionally observed, including a half (H) subconductance state (0.5; *A*), and others, including one at 39% (*B*) and 28% (*C*) of the main state, were also observed. *Dashed lines*, open levels; *solid line*, zero current level; *M*, the main open state. All traces from type 1 SII(+) channels in symmetrical 140 mM KCl at +20 mV in 1.0 μ M Ca²⁺. Similar sub-conductance states were observed in the SII(-) splice variant (not shown).

Dependence of channel open probability on [Ca²⁺]_i

Gating of the InsP₃R is sensitive to [Ca²⁺]_i as well as [InsP₃]. Low [Ca²⁺]_i stimulate InsP₃-liganded channels, whereas higher [Ca²⁺]_i are inhibitory (Patel et al., 1999; Taylor, 1998; Thomas et al., 1996). The biphasic effects of [Ca²⁺]_i on InsP₃-mediated Ca²⁺ release are believed to underlie oscillations, waves, and transitions from localized to global cellular responses. Although it is generally agreed that the type 1 isoform is inhibited by high [Ca²⁺]_i, it has been suggested that the types 2 (Ramos-Franco et al., 1998b, 2000) and 3 (Hagar et al., 1998) isoforms are not. Gating of both the *Xenopus* InsP₃R-1 (Mak et al., 1998) and recombinant rat InsP₃R-3 (Mak et al., 2001) channels in the oocyte nuclear envelope membrane is regulated biphasically by [Ca²⁺]_i. Because of the central role of [Ca²⁺]_i in regulating the channel, we investigated the effects of [Ca²⁺]_i on the gating of the recombinant rat SII(+) InsP₃R-1 in COS-7 cell nuclear membranes.

To examine specifically the effects of [Ca²⁺]_i on InsP₃R-1 channel gating, a functionally saturating [InsP₃] of 10 μ M was applied to the cytoplasmic (pipette) side of the channel to stimulate it fully at all experimental [Ca²⁺]_i. At [Ca²⁺]_i corresponding to resting levels in cells (10–100 nM), the open probability (*P*_o) of the channel was low (<0.1, Fig. 5). The *P*_o increased to 0.6–0.8 when [Ca²⁺]_i was raised from 100 nM to 1 μ M (Fig. 5). Between [Ca²⁺]_i of 1 μ M and 25 μ M, *P*_o remained high (~0.8). As [Ca²⁺]_i was increased beyond 25 μ M, *P*_o dropped precipitously (Fig. 5). This biphasic [Ca²⁺]_i dependence of the recombinant rat SII(+) type 1 channel is remarkably similar to that of the *Xenopus* type 1 InsP₃R recorded in the oocyte nuclear membrane system (Fig. 5 *B*; Mak et al., 1998). Similar results were obtained in recordings of the SII(-) type 1 channel (data not shown).

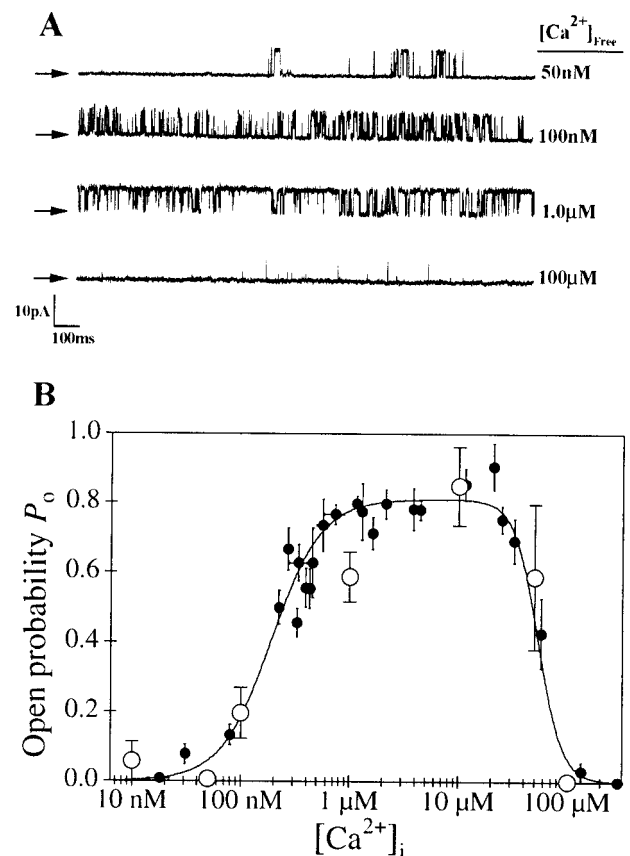


FIGURE 5 [Ca²⁺]_i dependence of recombinant InsP₃R channel open probability. (*A*) Typical current traces of the recombinant SII(+) channel at various free Ca²⁺ concentrations. *Arrow*, zero current level. Recordings performed in symmetrical 140 mM KCl at holding potential of +20 mV. (*B*) Dependence of InsP₃R channel open probability on free Ca²⁺ concentration. Data points represent mean \pm S.E.M. of at least three separate determinations. [Ca²⁺]_i dependence of native *Xenopus* InsP₃R open probability, measured by patch clamping isolated oocyte nuclei (Mak et al., 1998) is plotted for comparison (*filled circles*). *Solid lines*, biphasic Hill equation fit to the *Xenopus* data (Mak et al., 1998).

Inactivation of the channels was consistently observed (Table 1), although channel activities sometimes lasted for >20 min (data not shown). At low [Ca²⁺]_i (≤ 1 μ M), durations of channel activities were longer, with only ~9% of channels inactivating within 2 min, whereas at [Ca²⁺]_i >10 μ M, >75% of channels inactivated within 2 min (Table 1). These observations suggest that the loss of InsP₃R activity observed during patch-clamp recording may be due to an InsP₃ and Ca²⁺-dependent inactivation process.

DISCUSSION

In this report we have described a new methodology for studying recombinant InsP₃R Ca²⁺ channels at the single-channel level involving patch clamp electrophysiology of nuclei isolated from transfected COS-7 cells. Because gigohm electrical seals were readily obtained on isolated

COS-7 cell nuclei, the limiting factor in recording channel activities was the level of recombinant channel expression. The probability of detecting InsP₃-gated channel activities was very low in nuclei isolated from mock-transfected cells (1.5%). In contrast, it was dramatically enhanced by 40-fold in nuclei isolated from cells transfected with InsP₃R-1 cDNA. The rate of detection of recombinant InsP₃R channel activity in the present study (~45%) is somewhat greater than the probability of detecting recombinant InsP₃Rs expressed in COS cells and incorporated into planar lipid bilayers (20–24%; Ramos-Franco et al., 1998a, 2000). It is, however, similar to the probability of detecting recombinant InsP₃R channels expressed in *Xenopus* oocyte nuclei (50%) (Mak et al., 2000). A notable advantage of the COS-7 cell system for patch-clamp electrophysiology of recombinant InsP₃R channels is the consistently low rate of detection of the endogenous InsP₃R channels. In contrast, use of the oocyte expression system requires identification of particular batches of oocytes that functionally express very low levels of the endogenous InsP₃R-1 channel (Mak et al., 2000). In theory, a significant advantage of the nuclear patch-clamping approach for studying permeation, gating and regulatory properties of InsP₃R channels is that it enables recording of channels in their native mammalian endoplasmic reticulum membrane. This approach obviates the requirement for the extensive purification and reconstitution procedures necessary for recording recombinant InsP₃R channels in planar lipid bilayers. Thus, regulatory factors that might be associated with the endoplasmic reticulum membrane or within its lumen are more likely to be retained with this technique, by comparison with reconstitution approaches, and the channels are exposed to their natural complement of membrane lipids. Therefore, the single-channel properties observed for recombinant InsP₃Rs recorded in nuclei from transfected COS-7 cells may more faithfully reflect native InsP₃R channel function in vivo.

In this regard, it is notable that the recombinant rat InsP₃R-1 channel activities recorded in the present study share many of the properties of the endogenous *Xenopus* InsP₃R-1 recorded in its native oocyte nuclear membrane. In addition to displaying the basic properties of activation by InsP₃ and inhibition by heparin, the recombinant channels displayed rare sub-conductance states, they were similarly Ca²⁺ selective, and their gating was regulated with a biphasic [Ca²⁺]_i dependence that was remarkably similar to that of the *Xenopus* type 1 channel. No differences in these properties between the SII(+) and SII(−) splice variants were observed. Likewise, a previous study of SI(±) splice variants recorded in bilayers indicate that they shared similar conduction properties and [Ca²⁺]_i sensitivity (Ramos-Franco et al., 1998a). A characteristic of both native type 1 and recombinant type 3 InsP₃Rs recorded in *Xenopus* oocyte nuclei is that channel activity inactivates within a few minutes in the continued presence of InsP₃ (Mak and Foskett, 1997; Mak et al., 2000). Loss of channel activity is an

InsP₃-induced process, although the underlying mechanisms involved are not known (Mak and Foskett, 1997). A similar inactivation phenomenon was observed in the present study for rat InsP₃R-1 channels measured on COS-7 cell nuclei, although channel activity could sometimes be recorded for many minutes. The rate of InsP₃-induced channel inactivation appeared to be [Ca²⁺]_i dependent, although further studies will be necessary to elucidate the mechanisms of InsP₃R inactivation.

A different [Ca²⁺]_i dependence of the recombinant type 1 channel activity was determined previously using a ⁴⁵Ca²⁺ flux assay (Boehning and Joseph, 2000). The results from those measurements indicated a more bell-shaped [Ca²⁺]_i dependence, with the [Ca²⁺]_i for both the peak activity and inhibition of the channel activity displaced to much lower concentrations than observed here. The discrepancy between the [Ca²⁺]_i sensitivities determined in nuclear patching compared with those derived from measurements of ⁴⁵Ca²⁺ fluxes from microsomal vesicles suggest that uncontrolled variables can effect the apparent [Ca²⁺]_i dependence of channel activity. The [Ca²⁺]_i dependence measured in global Ca²⁺ release assays may be influenced by Ca²⁺ fluxes through the channel that act upon cytoplasmic sites, and/or by the luminal Ca²⁺ concentration, variables which are highly controlled in the patch clamp experiments. It may also be possible that outer nuclear membrane-localized InsP₃Rs may have unique sensitivities to [Ca²⁺]_i, or that important regulatory mechanisms are different in the two approaches. Further experiments will be necessary to resolve the quantitative differences obtained between the two approaches.

In conclusion, we have developed a new method for the measurement of the single-channel activities of recombinant InsP₃Rs. The advantages of this nuclear patch clamp approach include the ability to record mammalian recombinant channel activity in native mammalian endoplasmic reticulum membrane, and the ability to record these activities in isolation from those of the endogenous InsP₃R population. Utilization of this technique should significantly advance efforts to elucidate the structure-function relationships in InsP₃R Ca²⁺ channels.

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