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

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ABSTRACT Inositol 1,4,5-trisphosphate ($InsP_3$) receptors ($InsP_3Rs$) are intracellular Ca^{2+} channels gated by the second messenger $InsP_3$. Here we describe a novel approach for recording single-channel currents through recombinant $InsP_3Rs$ 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 $InsP_3R$. Single channels that were activated by $InsP_3$ and inhibited by heparin were observed in 45% of patches from nuclei prepared from transfected cells overexpressing recombinant $InsP_3Rs$. In contrast, nuclei from cells transfected with the vector alone had $InsP_3$-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 Ca^{2+} over K^+, and their open probabilities were biphasically regulated by cytoplasmic [Ca^{2+}]. This approach provides a powerful new methodology to study the permeation and gating properties of recombinant mammalian $InsP_3Rs$ in a native mammalian membrane environment at the single-channel level.

INTRODUCTION

The second messengers inositol 1,4,5-trisposphate ($InsP_3$) and diacylglycerol are generated in response to agonists that activate phospholipase C (Berridge and Irvine, 1989). $InsP_3$ diffuses through the cytoplasm and binds to its receptor ($InsP_3R$), which is an intracellular Ca^{2+} channel (Patel et al., 1999). Binding of $InsP_3$ to the $InsP_3R$ gates the channel open. The resulting rapid release of stored Ca^{2+} from the endoplasmic reticulum generates elevations of the concentration of free Ca^{2+} in the cytoplasm ($[Ca^{2+}]_i$), which is transduced into the modulation of a diverse array of cellular processes (Marks, 1997).

Structurally, the $InsP_3R$ is a tetrameric channel composed of subunits derived from three separate genes (types 1, 2, and 3 $InsP_3Rs$) (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 $[Ca^{2+}]_i$ 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 $InsP_3R$ 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 $InsP_3R$ channels specifically. We previously showed that $^{45}Ca^{2+}$ fluxes from microsomal vesicles prepared from COS-7 cells engineered to overexpress recombinant $InsP_3Rs$ and sarcoplasmic- and endoplasmic-reticulum Ca^{2+}-ATPase type 2b (SERCA-2b) could be attributed specifically to the recombinant $InsP_3Rs$ (Boehning and Joseph, 2000). However, this approach cannot provide detailed information about gating or ion permeation through $InsP_3R$ channels. We therefore sought to develop a method to measure the activities of recombinant $InsP_3Rs$ in mammalian endoplasmic reticulum membranes at the single-channel level.

The intracellular location of $InsP_3Rs$ 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 $InsP_3Rs$ (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 $InsP_3R$ 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 $InsP_3R$ 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 $InsP_3R$ isoforms expressed in cultured mammalian cells. We have recorded single recombinant $InsP_3R$ channels by patch-clamping nuclei isolated from COS-7 cells engineered to transiently overexpress recombinant rat $InsP_3Rs$. COS-7 cells were chosen for these studies because recombinant $InsP_3Rs$ overexpressed in these cells do not associate with the endogenous $InsP_3R$ 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 $InsP_3R$ in native mammalian membranes. Both channels required $InsP_3$ for gating and they had similar permeation properties and ionic selectivities. Examination of the $[Ca^{2+}]_i$ 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 $InsP_3R$ channels in native mammalian membranes. The simplicity and reproducibility of expressing and detecting recombinant $InsP_3R$ channels on COS-7 cell nuclei should significantly advance efforts to elucidate the structure-function relationships in this important intracellular Ca^{2+} channel.

EXPERIMENTAL PROCEDURES

Expression constructs

The neuronal rat type 1 $INSP_3R$ 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 $InsP_3R$ cDNA has been described in detail elsewhere (Boehning and Joseph, 2000). Briefly, 75-cm^2 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× 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 $InsP_3$-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 μl 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 $InsP_3R$ 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 $InsP_3R$ 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 μM BAPTA, 0.001% trypan blue, and 250 nM $[Ca^{2+}]_{free}$ (~220 μM added Ca^{2+}) adjusted to pH 7.1 with KOH. Bath trypan blue concentrations >0.002% reduced $InsP_3R$ single-channel P_o (data not shown), and were therefore avoided. Pipette solutions contained 140 mM KCl, 10 mM HEPES, 100 μM BAPTA, 0.5 mM NaATP, 10 μM $InsP_3$, 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 $InsP_3R$ 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 Affolter, 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^+ (osmolarity not adjusted) pipette solution with 14 mM KCl, 10 mM HEPES, 100 μM BAPTA, 0.5 mM NaATP, 10 μM IP_3 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 $InsP_3Rs$ on isolated nuclei

We have shown previously that $InsP_3R$ isoforms expressed in COS-7 cells form tetramers, bind $InsP_3$, 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 $InsP_3Rs$ (Joseph et al., 2000; Boehning and Joseph, 2000). Recombinant $InsP_3Rs$ 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 $InsP_3R$ 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 $InsP_3Rs$ can be similarly recorded, using batches of oocytes that express low levels of native $InsP_3Rs$ (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 $InsP_3Rs$ (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 $InsP_3R$ 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 $InsP_3Rs$ 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 μm in diameter (Fig. 1 *A*). Nuclei suitable for patching were identified as those that

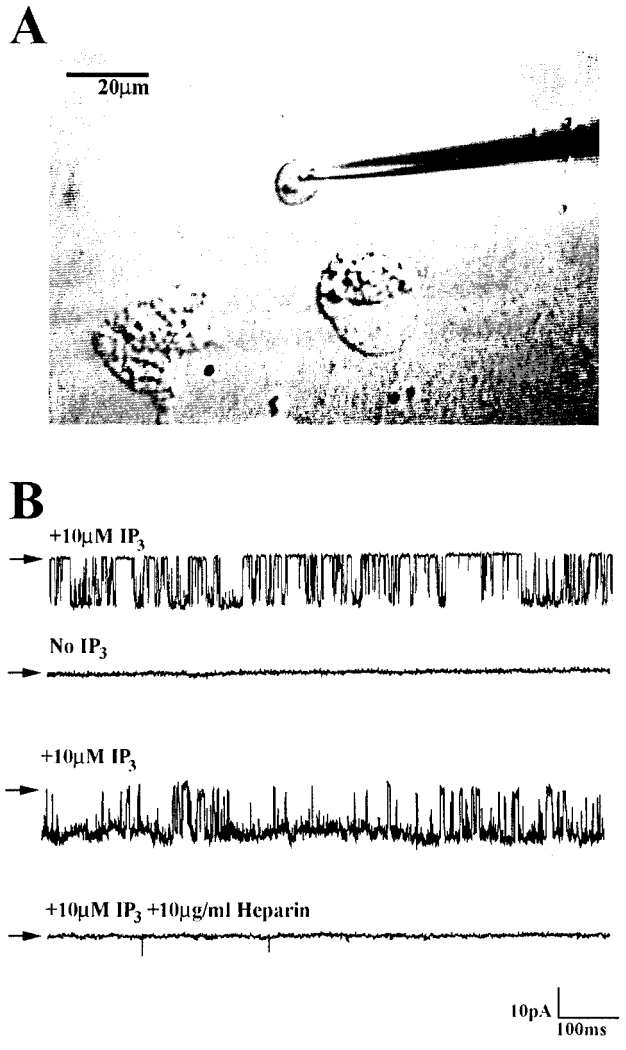

FIGURE 1 Detection of recombinant $InsP_3R$ channels. (*A*) Isolated nuclei free of cellular debris were visually identified for patch clamp electrophysiology. An intact cell is visible directly below the isolated nucleus being patched, and a damaged cell is present in bottom left. (*B*) The $InsP_3$ dependence of gating was confirmed by repeatedly patching onto the same nucleus with pipettes alternatively containing 10 μM $InsP_3$, no $InsP_3$, or $InsP_3$ plus heparin (10 μg/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 $InsP_3R$ channel activities, a saturating concentration of $InsP_3$ (10 μM) was included in the pipette solution, because the ligand-binding region of the expressed $InsP_3R$ is located on the cytoplasmic aspect of the channel, which faced into the pipette. In nuclei from COS-7 cells transiently transfected with $InsP_3R$ type 1 cDNA, large conductance channels were detected in 45% of nuclei examined with $InsP_3$ in the pipette solution (Table 1; Fig. 1 *B*). This rate of detecting channel activity is consistent with the 40- 60% transfection efficiency of $InsP_3R$ cDNA into COS-7 cells (Joseph et al., 2000). Multiple channel levels were detected in ~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 $InsP_3Rs$.

To confirm that the observed channel activities were contributed by the $InsP_3R$, the $InsP_3$ dependence of channel activity was investigated by repeated patching of the same nucleus with pipettes alternately containing either 10 μM $InsP_3$, no $InsP_3$, or $InsP_3$ plus the $InsP_3R$ competitive antagonist heparin (10 μg/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 $InsP_3$ 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 $InsP_3R$.

Ionic conductance and selectivity of alternatively spliced rat $InsP_3R$-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 ± 0.5 mV; the SII(−) splice variant displayed a similar reversal potential (+44.8 ± 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 $InsP_3R$ (Mak and Foskett, 1994). To determine the relative permeabilities of Ca^{2+} 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 Ca^{2+} 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 Ca^{2+} to K^+ (P_{Ca2+}:P_{K+}) was determined to be ~4. Thus, both

TABLE 1 Detection of $InsP_3$-sensitive channels on transfected COS-7 cell nuclei

Condition	$InsP_3R$	PD	>1 channel (%)	Inactivation (%)*
Recombinant type 1 $InsP_3R$ + $InsP_3$	317 (708)	45.0%	27%	9.3% ($Ca^{2+} \leq 1$ μM) 77.0% ($Ca^{2+} \geq 10$ μM)
Recombinant type $InsP_3R$ − $InsP_3$	0 (11)	0.0%	0%	N/D
Recombinant type $InsP_3R$ + heparin	0 (20)	0.0%	0%	N/D
Recombinant type $InsP_3R$ + heparin + $InsP_3$	0 (19)	0.0%	0%	N/D
pcDNA3.1 + $InsP_3$	2 (126)	1.5%	0%	N/D

$InsP_3R$, number of $InsP_3$-gated channels detected, with total attempts in parenthesis; PD, probability of detecting an $InsP_3$-gated channel; N/D, not determined.

Sensitivities to heparin (10 μg/ml) or the absence of $InsP_3$ were determined using nuclei previously demonstrating $InsP_3$-sensitive recombinant channel activity (PD > 95% when membrane patches were obtained from $InsP_3$-transfected nuclei previously demonstrating $InsP_3$-sensitive channel activity).

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

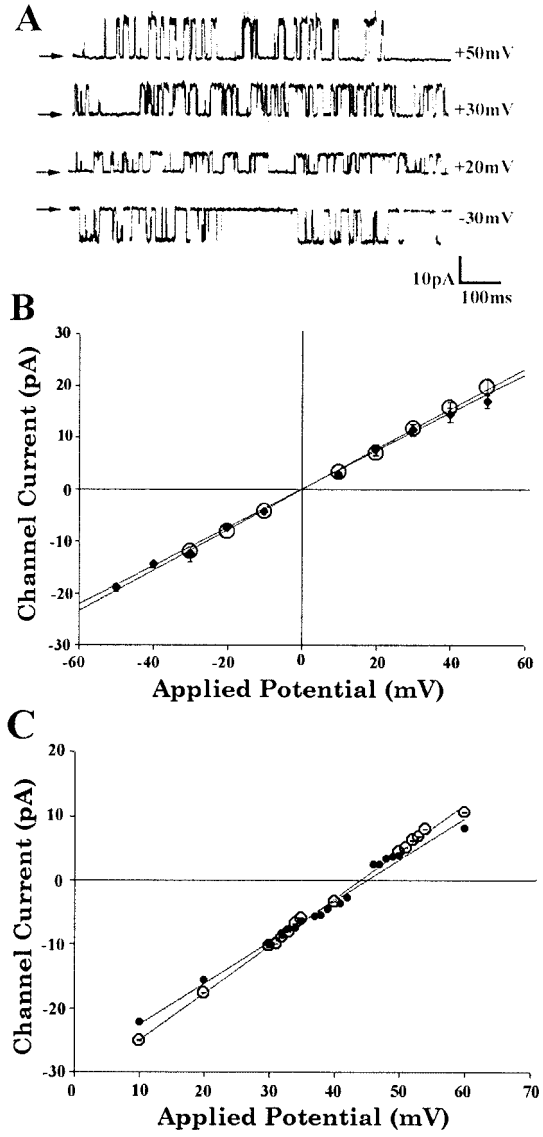

FIGURE 2 Conductances and ion selectivities of alternatively spliced $InsP_3Rs$. (*A*) Typical current traces at various holding potentials of recombinant $InsP_3R$ activities in symmetrical 140 mM KCl solutions. The traces are a continuous recording of the type 1 SII(−) splice variant channel. The SII(+) 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 ± 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.

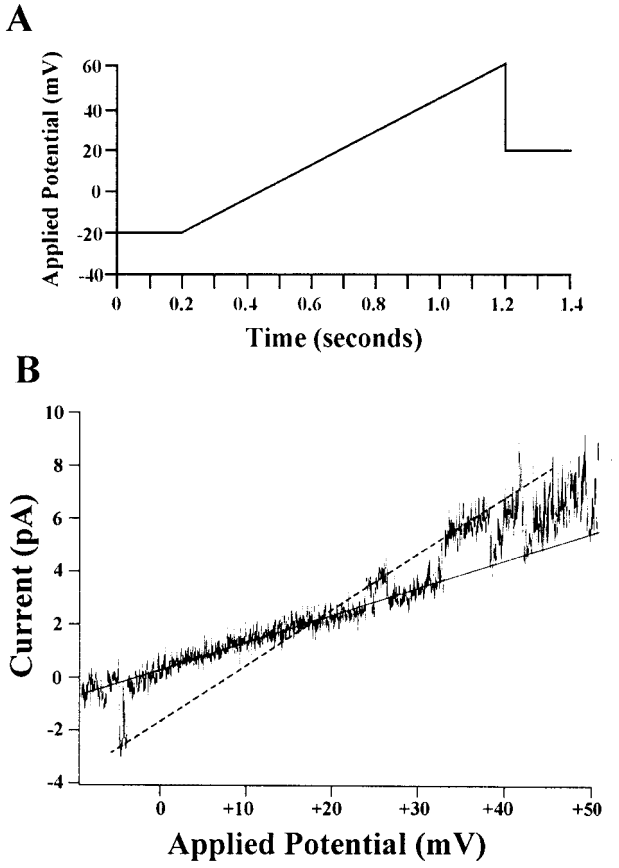

FIGURE 3 Divalent cation selectivities of recombinant $InsP_3R$-I splice variants. (*A*) Voltage ramp protocol used to determine the reversal potential in the presence of 140 mM KCl in pipette and high Ca^{2+} in the bath (see Patch-clamp solutions). A ramp protocol was employed to minimize Ca^{2+} flux 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).

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 Foskett, 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* $InsP_3R$-1 (Mak and Foskett, 1994, 1997), was observed (Fig. 4 *A*), the $InsP_3R$-1 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.

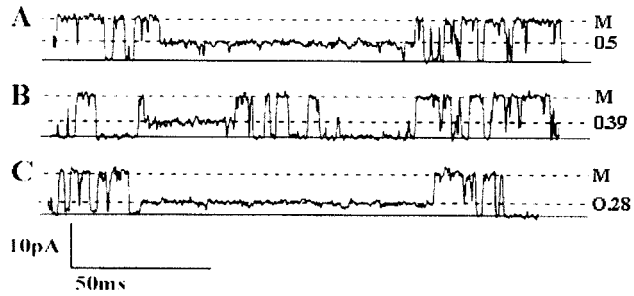

FIGURE 4 Recombinant $InsP_3R$ channels have multiple subconductance states. Brief transitions to subconductance states were occasionally observed, including a half (H) subconductance state (0.5; *A*), and others, including one at 39% (*B*) and 28% (*C*) of the main state, were also observed. *Dashed 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 μM Ca^{2+}. Similar sub-conductance states were observed in the SII(−) splice variant (not shown).

Dependence of channel open probability on $[Ca^{2+}]_i$

Gating of the $InsP_3R$ is sensitive to $[Ca^{2+}]_i$ as well as $[InsP_3]$. Low $[Ca^{2+}]_i$ stimulate $InsP_3$-liganded channels, whereas higher $[Ca^{2+}]_i$ are inhibitory (Patel et al., 1999; Taylor, 1998; Thomas et al., 1996). The biphasic effects of $[Ca^{2+}]_i$ on $InsP_3$-mediated Ca^{2+} release are believed to underlie oscillations, waves, and transitions from localized to global cellular responses. Although it is generally agreed that the type 1 isoform is inhibited by high $[Ca^{2+}]_i$, it has been suggested that the types 2 (Ramos-Franco et al., 1998b, 2000) and 3 (Hagar et al., 1998) isoforms are not. Gating of both the *Xenopus* $InsP_3R$-1 (Mak et al., 1998) and recombinant rat $InsP_3R$-3 (Mak et al., 2001) channels in the oocyte nuclear envelope membrane is regulated biphasically by $[Ca^{2+}]_i$. Because of the central role of $[Ca^{2+}]_i$ in regulating the channel, we investigated the effects of $[Ca^{2+}]_i$ on the gating of the recombinant rat SII(+) $InsP_3R$-1 in COS-7 cell nuclear membranes.

To examine specifically the effects of $[Ca^{2+}]_i$ on $InsP_3R$-1 channel gating, a functionally saturating $[InsP_3]$ of 10 μM was applied to the cytoplasmic (pipette) side of the channel to stimulate it fully at all experimental $[Ca^{2+}]_i$. At $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ was raised from 100 nM to 1 μM (Fig. 5). Between $[Ca^{2+}]_i$ of 1 μM and 25 μM, P_o remained high (~0.8). As $[Ca^{2+}]_i$ was increased beyond 25 μM, P_o dropped precipitously (Fig. 5). This biphasic $[Ca^{2+}]_i$ dependence of the recombinant rat SII(+) type 1 channel is remarkably similar to that of the *Xenopus* type 1 $InsP_3R$ 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).

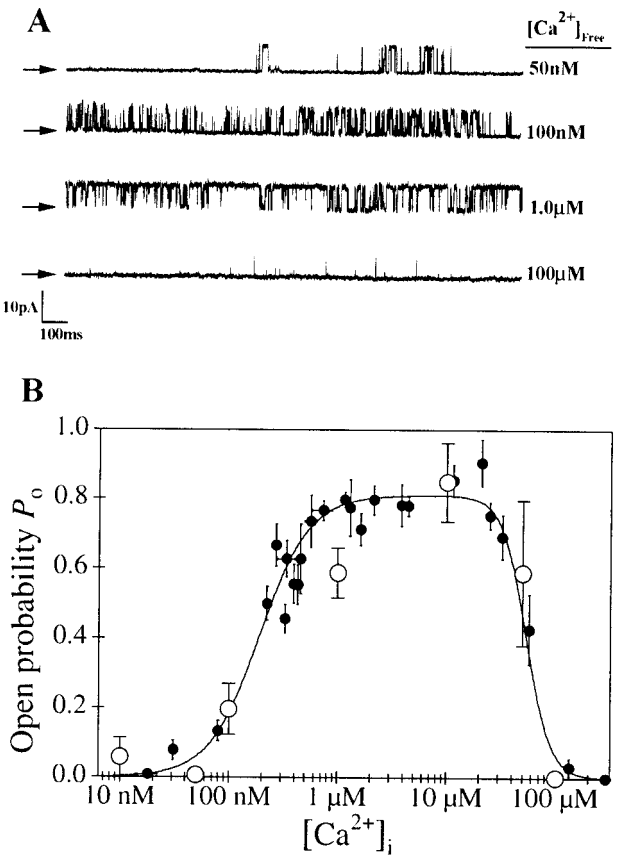

FIGURE 5 $[Ca^{2+}]_i$ dependence of recombinant $InsP_3R$ channel open probability. (*A*) Typical current traces of the recombinant SII(+) channel at various free Ca^{2+} concentrations. *Arrow*, zero current level. Recordings performed in symmetrical 140 mM KCl at holding potential of +20 mV. (*B*) Dependence of $InsP_3R$ channel open probability on free Ca^{2+} concentration. Data points represent mean ± S.E.M. of at least three separate determinations. $[Ca^{2+}]_i$ dependence of native *Xenopus* $InsP_3R$ 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 $[Ca^{2+}]_i$ (≤1 μM), durations of channel activities were longer, with only ~9% of channels inactivating within 2 min, whereas at $[Ca^{2+}]_i$ >10 μM, >75% of channels inactivated within 2 min (Table 1). These observations suggest that the loss of $InsP_3R$ activity observed during patch-clamp recording may be due to an $InsP_3$ and Ca^{2+}-dependent inactivation process.

DISCUSSION

In this report we have described a new methodology for studying recombinant $InsP_3R$ Ca^{2+} 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 $InsP_3$-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 $InsP_3R$-1 cDNA. The rate of detection of recombinant $InsP_3R$ channel activity in the present study (~45%) is somewhat greater than the probability of detecting recombinant $InsP_3Rs$ 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 $InsP_3R$ 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 $InsP_3R$ channels is the consistently low rate of detection of the endogenous $InsP_3R$ 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 $InsP_3R$-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 $InsP_3R$ 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 $InsP_3R$ 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 $InsP_3Rs$ recorded in nuclei from transfected COS-7 cells may more faithfully reflect native $InsP_3R$ channel function in vivo.

In this regard, it is notable that the recombinant rat $InsP_3R$-1 channel activities recorded in the present study share many of the properties of the endogenous *Xenopus* $InsP_3R$-1 recorded in its native oocyte nuclear membrane. In addition to displaying the basic properties of activation by $InsP_3$ and inhibition by heparin, the recombinant channels displayed rare sub-conductance states, they were similarly Ca^{2+} selective, and their gating was regulated with a biphasic $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ sensitivity (Ramos-Franco et al., 1998a). A characteristic of both native type 1 and recombinant type 3 $InsP_3Rs$ recorded in *Xenopus* oocyte nuclei is that channel activity inactivates within a few minutes in the continued presence of $InsP_3$ (Mak and Foskett, 1997; Mak et al., 2000). Loss of channel activity is an $InsP_3$-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 $InsP_3R$-1 channels measured on COS-7 cell nuclei, although channel activity could sometimes be recorded for many minutes. The rate of $InsP_3$-induced channel inactivation appeared to be $[Ca^{2+}]_i$ dependent, although further studies will be necessary to elucidate the mechanisms of $InsP_3R$ inactivation.

A different $[Ca^{2+}]_i$ dependence of the recombinant type 1 channel activity was determined previously using a $^{45}Ca^{2+}$ flux assay (Boehning and Joseph, 2000). The results from those measurements indicated a more bell-shaped $[Ca^{2+}]_i$ dependence, with the $[Ca^{2+}]_i$ for both the peak activity and inhibition of the channel activity displaced to much lower concentrations than observed here. The discrepancy between the $[Ca^{2+}]_i$ sensitivities determined in nuclear patching compared with those derived from measurements of $^{45}Ca^{2+}$ fluxes from microsomal vesicles suggest that uncontrolled variables can effect the apparent $[Ca^{2+}]_i$ dependence of channel activity. The $[Ca^{2+}]_i$ dependence measured in global Ca^{2+} release assays may be influenced by Ca^{2+} fluxes through the channel that act upon cytoplasmic sites, and/or by the luminal Ca^{2+} concentration, variables which are highly controlled in the patch clamp experiments. It may also be possible that outer nuclear membrane-localized $InsP_3Rs$ may have unique sensitivities to $[Ca^{2+}]_i$, 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 $InsP_3Rs$. 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 $InsP_3R$ population. Utilization of this technique should significantly advance efforts to elucidate the structure-function relationships in $InsP_3R$ Ca^{2+} channels.

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REFERENCES

Berridge, M. J., and R. F. Irvine. 1989. Inositol phosphates and cell signalling. *Nature.* 341:197–205.

Blondel, O., J. Takeda, H. Janssen, S. Seino, and G. Bell. 1993. Sequence and functional characterization of a third inositol trisphosphate receptor

subtype, IP3R-3, expressed in pancreatic islets, kidney, gastrointestinal tract, and other tissues. *J. Biol. Chem.* 268:11356–11363.

Boehning, D., and S. K. Joseph. 2000. Functional properties of recombinant type I and type III inositol 1,4,5-trisphosphate receptor isoforms expressed in COS-7 cells. *J. Biol. Chem.* 275:21492–21499.

Danoff, S. K., C. D. Ferris, C. Donath, G. Fischer, S. Munemitsu, S. Ullrich, S. H. Snyder, and C. A. Ross. 1991. Inositol 1,4,5-trisphosphate receptors: distinct neuronal and non-neuronal forms derived by alternative splicing differ in phosphorylation. *Proc. Natl. Acad. Sci. U.S.A.* 88:2951–2955.

Hagar, R. E., A. D. Burgstahler, M. H. Nathanson, and B. E. Ehrlich. 1998. Type III $InsP_3$ receptor channel stays open in the presence of increased calcium. *Nature.* 396:81–84.

Hille, B. 1992. Ionic Channels of Excitable Membranes. Sinauer Associates Inc. Sunderland, MA.

Joseph, S. K., D. Boehning, and C. Lin. 1999. Inositol 1,4,5-trisphosphate receptors: molecular aspects. *In* Calcium Signaling. Putney, J.W., editor. CRC Press, New York. 203–226.

Joseph, S. K., S. Bokkala, D. Boehning, and S. Zeigler. 2000. Factors determining the composition of inositol trisphosphate receptor hetero-oligomers expressed in COS cells. *J. Biol. Chem.* 275:16084–16090.

Joseph, S. K., C. Lin, S. Pierson, A. P. Thomas, and A. R. Maranto. 1995. Heteroligomers of type-I and type-III inositol trisphosphate receptors in WB rat liver epithelial cells. *J. Biol. Chem.* 270:23310–23316.

Kaznacheyeva, E., V. D. Lupu, and I. Bezprozvanny. 1998. Single-channel properties of inositol (1, 4, 5)-trisphosphate receptor heterologously expressed in HEK-293 cells. *J. Gen. Physiol.* 111:847–856.

Lin, C., J. Widjaja, and S. K. Joseph. 2000. The interaction of calmodulin with alternatively spliced isoforms of the type-I inositol trisphosphate receptor. *J. Biol. Chem.* 275:2305–2311.

Mak, D. D., and J. K. Foskett. 1994. Single channel inositol 1,4,5-trisphosphate receptor currents revealed by patch clamp of isolated *Xenopus* oocyte nuclei. *J. Biol. Chem.* 269:29375–29378.

Mak, D. D., and J. K. Foskett. 1997. Single-channel kinetics, inactivation and spatial distribution of inositol trisphosphate receptors in *Xenopus* oocyte nuclei. *J. Gen. Physiol.* 109:571–587.

Mak, D. D., and J. K. Foskett. 1998. Effects of divalent cations on single-channel conduction properties of *Xenopus* IP_3 receptor. *Am. J. Physiol.* 275:C179–C188.

Mak, D. D., S. McBride, and J. K. Foskett. 1998. Inositol 1,4,5-trisphosphate activation of inositol trisphosphate receptor Ca^{2+} channel by ligand tuning of Ca^{2+} inhibition. *Proc. Natl. Acad. Sci. U.S.A.* 95:15821–15825.

Mak, D. D., S. McBride, and J. K. Foskett. 1999. ATP regulation of type I inositol 1,4,5-trisphosphate receptor channel gating by allosteric tuning of Ca^{2+} activation. *J. Biol. Chem.* 274:22231–22237.

Mak, D. O. D., S. McBride, and J. K. Foskett. 2001. Regulation by Ca^{2+} and inositol 1,4,5-trisphosphate ($InsP_3$) of single recombinant type 3 $InsP_3$ receptor channels. Ca^{2+} activation uniquely distinguishes types 1 and 3 $InsP_3$ receptors. *J. Gen. Physiol.* 117:435–466.

Mak, D. D., S. McBride, V. Raghuram, Y. Yue, S. K. Joseph, and J. K. Foskett. 2000. Single-channel properties in endoplasmic reticulum membrane of recombinant type 3 inositol trisphosphate receptor. *J. Gen. Physiol.* 115:241–255.

Marks, A. R. 1997. Intracellular calcium-release channels: regulators of cell life and death. *Am. J. Physiol.* 272:H597–H605.

Mignery, G. A., Newton, C. L., Archer, B. T., I. I. I. and Südhof, T. C. 1990. Structure and expression of the rat inositol 1,4,5-trisphosphate receptor. *J. Biol. Chem.* 265:12679–12685.

Miyakawa, T., A. Maeda, T. Yamazawa, K. Hirose, T. Kurosaki, and M. Iino. 1999. Encoding of Ca^{2+} signals by differential expression of IP_3 receptor subtypes. *EMBO J.* 18:1303–1308.

Monkawa, T., Miyawaki, A., Sigiyama, T., et al., 1995.Heterotetrameric complex formation of inositol 1,4,5-trisphosphate receptor subunits. *J. Biol. Chem.* 270:14700–14704.

Neher, E. 1995. Voltage offsets in patch-clamp experiments. *In* Single-Channel Recording. Sakmann, B. and Neher, E., editors. Plenum Press, New York. 147–153.

Newton, C. L., G. A. Mignery, and T. C. Südhof. 1994. Coexpression in vertebrate tissues and cell lines of multiple inositol 1,4,5-trisphosphate receptors with distinct affinities for $InsP_3$. *J. Biol. Chem.* 269: 28613–28619.

Nucifora, F. C., A. H. Sharp, S. L. Milgram, and C. A. Ross. 1996. Inositol 1,4,5-trisphosphate receptors in endocrine cells: localization and association in hetero and homotetramers. *Mol. Biol. Cell.* 7:949–960.

Patel, S., S. K. Joseph, and A. P. Thomas. 1999. Molecular properties of inositol 1,4,5 trisphosphate receptors. *Cell Calcium.* 25:247–264.

Ramos-Franco, J., D. Bare, S. Caenepeel, A. Nani, M. Fill, and G. Mignery. 2000. Single-channel function of recombinant type 2 inositol 1,4,5-trisphosphate receptor. *Biophys. J.* 79:1388–1399.

Ramos-Franco, J., S. Caenepeel, M. Fill, and F. Mignery. 1998a. Single channel function of recombinant type-I inositol 1,4,5-trisphosphate receptor ligand binding domain splice variants. *Biophys. J.* 75:2783–2793.

Ramos-Franco, J., M. Fill, and G. A. Mignery. 1998b. Isoform-specific function of single inositol 1,4,5-trisphosphate receptor channels. *Biophys. J.* 75:834–839.

Sigel, E., and H. Affolter. 1987. Preparation and utilization of an ion-specific calcium minielectrode. *In* Methods in Enzymology. Academic Press. 25–36.

Südhof, T. C., Newton, C. L., Archer, I. I. I., B. T., Ushkaryov, Y. A., and Mignery, G. A. 206. 1991. Structure of a novel $InsP_3$ receptor. *EMBO J.* 10:3199–3.

Taylor, C. W. 1998. Inositol trisphosphate receptors: Ca^{2+}-modulated intracellular Ca^{2+} channels. *Biochim. Biophys. Acta.* 1436:19–33.

Taylor, C. W., A. A. Genazzani, and S. A. Morris. 1999. Expression of inositol trisphosphate receptors. *Cell Calcium.* 26:237–251.

Thomas, A. P., G. J. Bird, G. Hajnoczky, L. D. Robb-Gaspers, and J. W. Putney. 1996. Spatial and temporal aspects of cellular calcium signaling. *FASEB J.* 10:1505–1517.

Watras, J., I. Bezprozvanny, and B. E. Ehrlich. 1991. Inositol 1,4,5-trisphosphate-gated channels in cerebellum: presence of multiple conductance states. *J. Neurosci.* 11:3239–3245.

Wojcikiewicz, R. J. H. 1995. Type I, II and III inositol 1,4,5-trisphosphate receptors are unequally susceptible to down-regulation and are expressed in markedly different proportions in different cell types. *J. Biol. Chem.* 270:11678–11683.

Wojcikiewicz, R. J. H., and Y. He. 1995. Type I, II and III inositol 1,4,5-trisphosphate receptor co-immunoprecipitation as evidence for the existence heterotetrameric receptor complexes. *Biochem. Biophys. Res. Commun.* 213:334–341.