Single Channel Function of Recombinant Type-1 Inositol 1,4,5-Trisphosphate Receptor Ligand Binding Domain Splice Variants

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ABSTRACT In this study we describe the expression and function of the two rat type-1 inositol 1,4,5-trisphosphate receptor ($InsP_3R$) ligand binding domain splice variants (SI±/SII+). Receptor protein from COS-1 cells transfected with the type-1 $InsP_3R$ expression plasmids (p$InsP_3R$-T1, p$InsP_3R$-T1ALT) or control DNA were incorporated into planar lipid bilayers and the single channel properties of the recombinant receptors were defined. The unitary conductance of the two splice variants were ~290 pS with Cs^+ as charge carrier and ~65 pS with Ca^{2+} as charge carrier. Both $InsP_3R$ expression products consistently behaved like those of the native type-1 receptor isoform isolated from cerebellum in terms of their $InsP_3$, Ca^{2+}, and heparin sensitivity. An $InsP_3$ receptor ligand binding domain truncation lacking the 310 amino-terminal amino acids (p$InsP_3R$-ΔT1ALT) formed tetrameric complexes but failed to bind $InsP_3$ with high affinity, and did not form functional Ca^{2+} channels when reconstituted in lipid bilayers. These data suggest that 1) the ligand binding alternative splice site is functionally inert in terms of $InsP_3$ binding and single channel function, and 2) the single channel properties of the expressed recombinant type-1 channel are essentially identical to those of the native channel. This work establishes a foundation from which molecular/biophysical approaches can be used to define the structure-function properties of the $InsP_3$ receptor channel family.

INTRODUCTION

The inositol 1,4,5-trisphosphate receptor ($InsP_3R$) gene family encodes a highly homologous group of proteins localized to the endoplasmic reticulum, which is responsible for the regulated release of intracellular Ca^{2+} (reviewed in Bezprozvanny and Ehrlich, 1995; Joseph, 1996). The $InsP_3$ receptor family consists of three primary isoforms that appear to be ubiquitously expressed in metazoans. Despite their widespread distribution, the complement and abundance of the different isoforms can be highly variable between cell types (Newton et al., 1994; De Smedt et al., 1997; Nakanishi et al., 1996). In addition, type-1 receptor diversity may arise via two regions for alternative splicing events in the ligand binding and coupling domains (Mignery et al., 1990; Maeda et al., 1991; Nakagawa et al., 1991; Danoff et al., 1991). The role of the differentially spliced site located in the ligand binding domain is unclear. Newton et al. (1994) demonstrated that recombinant receptor with or without the spliced sequence bound $InsP_3$ with similar affinities, suggesting similar function. The spliced form of the receptor containing the insert predominates in neural tissues (Newton et al., 1994). The alternative splice site within the coupling domain is between two PKA phosphorylation sites and has been suggested to affect PKA phosphorylation (Danoff et al., 1991). The type-2 and type-3 receptors do not appear to be differentially processed.

In any event, it is clear that most cells contain at least two, if not all three, $InsP_3$ receptor isoforms. Expression of multiple isoforms of the $InsP_3$ receptor in a single cell implies that the different isoforms have different functions. This suggestion is consistent with the fact that intracellular Ca^{2+} release mediated by $InsP_3$ receptor Ca^{2+} channels has been implicated in numerous, very diverse cellular processes including the initiation/propagation of Ca^{2+} waves, cell growth, secretion, fertilization, and development (Berridge, 1993). Despite its broad physiological relevance, there are relatively few single channel studies of $InsP_3$ receptor function and regulation at the molecular level.

The single channel function of the type-1 $InsP_3$ receptor was first examined by Bezprozvanny et al. (1991). Recently, the single channel function of the native type-2 (Perez et al., 1997; Ramos-Franco et al., 1998) and native type-3 $InsP_3$ receptor (Hagar and Ehrlich, 1998) have been defined. These studies indicate that single channel function of the $InsP_3$ receptors is isoform-specific. The three different isoforms of the $InsP_3$ receptor apparently have unique and distinct functional properties in terms of Ca^{2+} and $InsP_3$ sensitivity. Interestingly, the permeation properties of all three receptor channels are very similar, suggesting that the pore-forming region of the proteins is well conserved between the channel isoforms. This is consistent with the high degree of homology in the channel domains of the three $InsP_3$ receptor isoforms. The functional heterogeneity in Ca^{2+} and $InsP_3$ sensitivity is consistent with the higher degree of heterogeneity in the regulatory and ligand binding domains of the three $InsP_3$ receptor isoforms. The well defined domains (Mignery and Sudhof, 1990) of the $InsP_3$ receptor channels (i.e., $InsP_3$ binding, regulatory/coupling,

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and channel domains) and the well defined functional differences/similarities among the three $InsP_3$ receptor isoforms establish the ideal foundation for a mutagenesis-based investigation of how the $InsP_3R$ structure defines its function.

The goal of this study was to find a way to define single channel function of exogenous $InsP_3$ cDNAs expressed in a cell line. To this end, two splice variants of the rat type-1 $InsP_3$ receptor protein and a type-1 $InsP_3$ receptor truncation mutant were expressed in transiently transfected COS cells. The recombinant receptors were localized to the endoplasmic reticulum and the full-length receptors bind $InsP_3$ at high levels, very similar as to those previously reported (Mignery et al., 1990). Fusion of proteoliposomes enriched in either of the two splice variants of the type-1 $InsP_3R$ into planar lipid bilayers resulted in the frequent appearance of robust single channel activity. No channel activity was observed after fusing proteoliposomes rich in an $InsP_3R$ truncation mutant missing the first 310 amino acids of the $InsP_3$ binding domain or from control COS-1 cells. Both type-1 $InsP_3$ receptor splice variants were activated by $InsP_3$ (EC_{50} ~130 nM), blocked by heparin, and had bell-shaped Ca^{2+} sensitivities. The unitary conductance values of the two splice variants were ~290 pS with Cs^+ as charge carrier and ~65 pS with Ca^{2+} as charge carrier. Channel gating was characterized by brief openings with few opening events lasting longer than 10 ms. Despite considerable efforts to reconstitute endogenous $InsP_3$ receptor channels from mock-transfected COS cells, there was no evidence that endogenous $InsP_3$ receptor channels were present in these studies. Thus, all single channel recordings here were attributed to exogenously expressed $InsP_3$ receptor channels.

These functions of the recombinant type-1 $InsP_3$ receptor channels are similar to those defined for the native type-1 $InsP_3$ receptor channel (Perez et al., 1997; Ramos-Franco et al., 1998; Bezprozvanny et al., 1991; Watras et al., 1991). Thus, these data show that overexpression of either of the type-1 $InsP_3$ receptor splice variants in COS cells produces functional type-1 $InsP_3$ receptor channels that can be reconstituted in planar lipid bilayers. They also show that the two splice variants have nearly identical functional attributes and that the $InsP_3$ binding domain may be required for the formation of a functional $InsP_3$ receptor channel. This study establishes a molecular approach that can be utilized to define the structure-function properties of the $InsP_3$ receptor family channels.

MATERIALS AND METHODS

Materials

[^{3}H]Inositol 1,4,5-trisphosphate (21 Ci/mmol) was obtained from Du Pont-New England Nuclear (Boston, MA). Unlabeled inositol 1,4,5-trisphosphate ($InsP_3$) was purchased from LC Laboratories Inc. (Woburn, MA) and heparin from Sigma (St. Louis, MO). Lipids, L-α-phosphatidylcholine, L-α-phosphatidylethanolamine, and L-α-phosphatidylserine were obtained from Avanti Polar Lipids (Alabaster, AL). The type-1 antipeptide antibody directed against the 19 carboxyl-terminal amino acids was previously described (Mignery et al., 1989).

Antibodies

The antibodies used in this study include amino- and carboxyl-terminal for all three isoforms of the $InsP_3R$ as well as one directed against the amino-terminal 15-amino-acid sequence of the differentially spliced type-1 isoform. The antipeptide antibodies correspond to the following sequences: T1NH = CLATGHYLAAEVDPDQDASR, T2NH = CPDYRDAQNEGKTVRDGELP, T3NH = CENPSYKGDVSDPKAAGPGA, T1C = CRIGLLGHPPHMNVNPQQPA, T2C = CNKQRLGFLGSNTPHENHHMPPH, T3C = CRRQRLGFVDVQNCMSR, and T1ALT15 = CVDPDFEEECLEFQPS. All peptide antibodies were affinity-purified using immunogenic peptide. The secondary antibody used in immunofluorescence staining was an FITC-goat anti-rabbit antibody (Organon Technica).

$InsP_3R$ Expression Plasmid Construction

The full-length type-1 receptor plasmid ($pInsP_3R$-T1) was assembled from overlapping cDNA clones isolated from a rat brain library as previously described (Mignery et al., 1990). An identical coding sequence has recently been used by Kaznacheyeva et al. (1998). The expression plasmid, $pInsP_3R$-T1ALT, encoding the full-length $InsP_3R$ with the amino-terminal alternatively spliced 15-amino-acid insert (residues 318–332, Mignery et al., 1990) was constructed by inserting the 1365 bp *Eco*RI-*Kpn*I fragment of pKCMVI-12 (type 1a, Newton et al., 1994) into a similarly digested $pInsP_3R$-T1 *Eco*RI-*Xba*I insert that had been ligated into *Eco*RI/*Xba*I sites of the pCMV5 vector. The alternatively spliced insert, containing (SI+), ligand binding domain deletion construct $pInsP_3R$-ΔT1ALT, lacking the amino-terminal 310 amino acids, was prepared using PCR. Briefly, an oligonucleotide primer pair GGAATTCCATGGGGCATTACTTGGCAGCAGAGG (nt. 1260–1280) and GGCAAAAGCTTCCTTGTCCTCC (nt. 1621–1601) were used along with pKCMVI-12 (type 1a, Newton et al., 1994) as template for the PCR reactions. The PCR product was digested with *Eco*RI/*Kpn*I and inserted into *Eco*RI/*Kpn*I digested $pInsP_3R$-T1ALT vector. The resulting plasmid ($pInsP_3R$-ΔT1ALT) encodes amino acids 311-2749 of the $InsP_3$ alternatively spliced (SI+) receptor isoform.

COS cell transfection

Equivalent numbers of COS-1 cells were transiently transfected with either $pInsP_3R$-T1, $pInsP_3R$-T1ALT and $pInsP_3R$-ΔT1ALT expression plasmid, or sheared salmon sperm (SS) DNA using the DEAE-dextran method as described by Gorman et al. (1985). The sheared salmon sperm DNA (SS DNA) was used to mock-transfect COS-1 cells and served as a negative control. Cells were incubated at 37°C, 5% CO_2 for 48–72 h prior to harvesting for biochemical and functional analysis. Typical transfection efficiencies were routinely 60% or greater as determined by indirect immunofluorescence or via green fluorescent reporter chimeras.

Immunocytochemical analysis of $InsP_3R$ expression

Transiently transfected COS cells were harvested by brief trypsinization followed by plating onto poly-D-lysine-coated glass coverslips. Following an attachment interval, the cells were fixed with 4% paraformaldehyde in 200 mM phosphate buffer, permeabilized with 0.3% Triton X-100 in phosphate buffered saline and blocked in the same buffer containing 10% goat serum. Expression of $pInsP_3R$-T1 was detected by incubation with an affinity-purified antipeptide antibody corresponding to the 19 carboxyl-terminal amino acids of the type-1 receptor (T1C). $pInsP_3R$-T1ALT and $pInsP_3R$-ΔT1ALT expression were assessed by using an affinity pure antibody generated against the 15 amino acids corresponding to the alternatively spliced insert (T1ALT15). All coverslips were then incubated with an FITC-goat anti-rabbit secondary antibody (Organon Technica). Coverslips were analyzed with a Nikon Diaphot 300 inverted microscope and photographed using Tmax 400 ASA film (Kodak).

Preparation of microsomes, CHAPS solubilization, and gradient sedimentation

COS-1 cells transfected with either $pInsP_3R$-T1, $pInsP_3R$-T1ALT, $pInsP_3R$-ΔT1ALT, or the SS DNA control were harvested 48–72 h post-transfection and microsomes were prepared as described previously (Mignery et al., 1990). COS cells were washed with PBS, harvested by scraping into 50 mM Tris-HCl pH 8.3, 1 mM EDTA, 1 mM 2-mercaptoethanol, 1 mM PMSF, and lysed by 40 passages through a 27-gauge needle. Membranes were pelleted by a 20-min centrifugation (289,000 × g_{av}), resuspended in buffer, and either used immediately or frozen at −80°C. Microsomal fractions were solubilized in 50 mM Tris-HCl pH 8.3, 1 mM EDTA, 1 mM 2-mercaptoethanol, 1 mM PMSF, and 1.8% CHAPS on ice for 1 h. Insoluble fractions were eliminated by a 10-min centrifugation at 289,000 × g_{av} and the supernatant containing solubilized receptor was fractionated through 5–20% sucrose (w/v) gradients as previously described (Mignery et al., 1989). Gradient fractions containing $InsP_3$ receptor protein were then identified by immunoblotting with type-1 receptor antibody and reconstituted into proteoliposomes as previously described (Mignery et al., 1992; Perez et al., 1997).

$InsP_3$ binding assays

[^{3}H]-$InsP_3$ ligand binding assays were performed as previously described (Mignery et al., 1990). Binding assays were performed using 50 μg membrane protein in 100 μl 50 mM Tris-HCl pH 8.3, 1 mM EDTA, 1 mM 2-mercaptoethanol, and 1 mM PMSF containing 9.52 nM [^{3}H]-$InsP_3$ ± 1 μM unlabeled $InsP_3$. Samples were incubated on ice for 10 min and the radioactivity of the membrane pellets was determined by scintillation spectrometry. All assays were performed in quadruplicate and replicated three times.

SDS-PAGE and immunoblotting

COS cell microsomes and sucrose gradient fractions were analyzed by 5% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (Mignery et al., 1990), followed by immunoblotting with $InsP_3R$ subtype-specific antibodies and detected using chemiluminescence reagents (Amersham Life Sciences Inc., Arlington Heights, IL).

Planar lipid/protein bilayer formation

Planar lipid bilayers were formed across a 220-μm diameter aperture in the wall of a Delrin partition as described (Perez et al., 1997). Lipid bilayer-forming solution contained a 7:3 mixture of phosphatidylethanolamine and phosphatidylcholine dissolved in decane (50 mg/ml). Proteoliposomes were added to the solution on one side of the bilayer (defined as the *cis*-chamber). The other side was defined as the *trans*-chamber (virtual ground). Standard solutions contained 220 mM $CsCH_3SO_3$ *cis* (20 mM *trans*), 20 mM HEPES (pH 7.4), and 1 mM EGTA ($[Ca^{2+}]_{Free}$ = 250 nM). The $[Ca^{2+}]_{Free}$ was verified using a Ca^{2+} electrode. The Ca^{2+} electrodes were comprised of the calcium ligand ETH 129 in a polyvinylchloride membrane at the end of a small (2 mm) polyethylene tube. These Ca^{2+} minielectrodes were made and used as described previously (Baudet et al., 1994). A custom current/voltage conversion amplifier was used to optimize single-channel recording. Acquisition software (pClamp: Axon Instruments, Foster City, CA), an IBM compatible 486 computer, and a 12-bit A/D-D/A converter (Axon Instruments) were used. Single channel data were digitized at 5–10 KHz and filtered at 1 KHz. Channel sidedness was determined by $InsP_3$ sensitivity. The orientation of the channels studied was such that the $InsP_3$ sensitive side (i.e., cytoplasmic side) was in the *cis* compartment.

RESULTS

Expression of the type-1 $InsP_3R$ ligand binding domain splice variants

The $InsP_3R$ was transfected into COS-1 cells using the DEAE-dextran method (Gorman, 1985). The $InsP_3R$ constructs expressed were the full-length type-1 $InsP_3R$ missing the 15-amino-acid alternatively spliced insert (SI-, $pInsP_3R$-T1), the full-length type-1 $InsP_3R$ containing the 15-amino-acid insert (SI+, $pInsP_3R$-T1ALT), or the amino-terminal truncation ($pInsP_3R$-ΔT1ALT). The expression vectors were under the control of the cytomegalovirus (CMV) promoter (Mignery et al., 1990) and these plasmids abundantly expressed immunoreactive receptor protein (Fig. 1, *A* and *B*). Microsomes prepared from an equivalent number of COS-1 cells transfected with sheared SS DNA revealed no immunoreactive endogenous receptor protein (Fig. 1 *A*) even when 20 μg microsomal protein was loaded. Extended exposures of the Western blots revealed only low levels of immunoreactive protein (data not shown). Microsomes (20 μg) from cells expressing $pInsP_3R$-T1ALT, $pInsP_3R$-ΔT1ALT, and control COS cells (SS DNA) were Western-blotted with an antibody directed against the alternatively spliced insert (T1ALT15) and revealed significant expression, yet no detectable endogenous receptor signal was observed (Fig. 1 *B*). In experiments to determine whether overexpression of transfected receptor constructs resulted in elevated expression of endogenous receptor, microsomes from $pInsP_3R$-T1ALT, $pInsP_3R$-ΔT1ALT, and SS DNA control were immunoblotted with the carboxyl-terminal (T1C) antibody (Fig. 1 *C*). If the endogenous type-1 receptor was up-regulated in cells overexpressing $pInsP_3R$-ΔT1ALT, it would be detected inasmuch as this recombinant protein is lacking the 310 amino-terminal amino acids and thus migrates on an SDS-PAGE to a position lower than full-length receptor. No differences in endogenous type-1 receptor expression were detected between the control COS cells and the truncation mutant (Fig. 1 *C*).

COS-1 cells, as well as all other cell lines or tissues, express $InsP_3$ receptors. Newton et al. (1994) demonstrated that, at the level of mRNA, both the type-1 and type-3 receptor isoforms are expressed in COS cells. No type-2 transcripts were detected in the PCR studies. We have investigated the endogenous receptor population in this study using amino- and carboxyl-terminal antibodies specific to each receptor subtype and found that the type-1 and type-3 receptors are indeed expressed (Fig. 2). The type-3 receptor is the most abundant isoform present as judged by signal intensity on Western blots (Fig. 2). However, it is difficult to assess the exact stoichiometry of the type-1 and -3 isoforms since different antibodies of similar titers often behave very differently (e.g., sensitivity to native or denatured protein, relative affinities, etc.). The type-2 isoform was not detected in control COS cells using either amino- or carboxyl-terminal antibodies (Fig. 2).

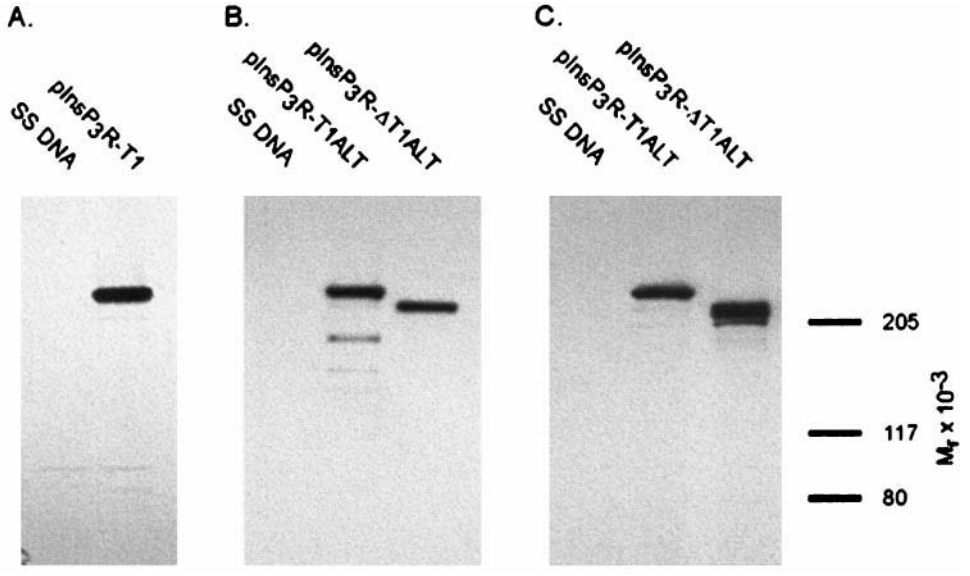

FIGURE 1 Analysis of $InsP_3R$ expression in transfected COS-1 cells by Western blotting. Microsomal protein (20 μg) from COS cells transfected with salmon sperm (SS) DNA, rat type-1 $InsP_3$ receptor minus the 15-amino-acid alternative spliced insert ($pInsP_3R$-T1), rat type-1 $InsP_3$ receptor containing the amino-terminal 15-amino-acid alternative spliced insert ($pInsP_3R$-T1ALT), and rat type-1 $InsP_3$ receptor containing the amino-terminal 15-amino-acid alternative spliced insert with the 310 amino-terminal amino acids deleted ($pInsP_3R$-ΔT1ALT), were resolved on 5% SDS-PAGE, transferred to nitrocellulose membranes, and Western-blotted with type-specific antibodies. (*A*) SS DNA and $pInsP_3R$-T1 microsomes immunoreacted with carboxyl-terminal type-1 antipeptide antibody (T1C); (*B*) SS DNA, $pInsP_3R$-T1ALT, and $pInsP_3R$-ΔT1ALT microsomes immunoreacted against T1ALT15 affinity pure antibody. (*C*) SS DNA, $pInsP_3R$-T1ALT, and $pInsP_3R$-ΔT1ALT immunoreacted with T1C antibody. Note the absence of detectable endogenous receptor bands above the $pInsP_3R$-ΔT1ALT expression products indicating that no significant induction of expression of endogenous receptors occurred.

In studies investigating the multiple membrane spanning regions of the $InsP_3R$ via mutational analysis we observed that some, not all, membrane spanning region truncations/deletions appeared to induce expression of endogenous receptors (D. Galvan and G. Mignery, unpublished observations). We hypothesized that this may possibly be a result of the massive overexpression of nonfunctional channel proteins titrating or recruiting the active endogenous receptors, thus inactivating them and necessitating de novo synthesis of receptor. In experiments designed to determine whether overexpression of $pInsP_3R$-T1 induced expression of endogenous receptors, we observed no apparent up-regulation of the type-3 isoform when microsomes from either control or overexpressing type-1 transfected COS cells were probed with two type-3 specific antibodies (Fig. 2). The type-2 receptor was not detectable in control (SS DNA) COS cell microsomes; however, in microsomes from cells overexpressing $pInsP_3R$-T1 a very faint signal was observed with both the amino- and carboxyl-terminal type-2 antibodies (Fig. 2). Whether this signal represents an induction of expression of the type-2 isoform in COS cells or reflects physical trapping of antibodies in the mass of overexpressed type-1 receptor is unclear. If this is indeed an induction of type-2 receptor, then the low levels of expression would probably not be detected in electrophysiological experiments because of the low rate of success in incorporating channels from vesicles or liposomes sparsely populated with receptor protein. In addition, if either of the endogenous type-2 or -3 isoforms was incorporated into the bilayer, they would be recognizable as such because of their unique regulation by Ca^{2+} and $InsP_3$ (Ramos-Franco et al., 1998; Hagar and Ehrlich, 1998).

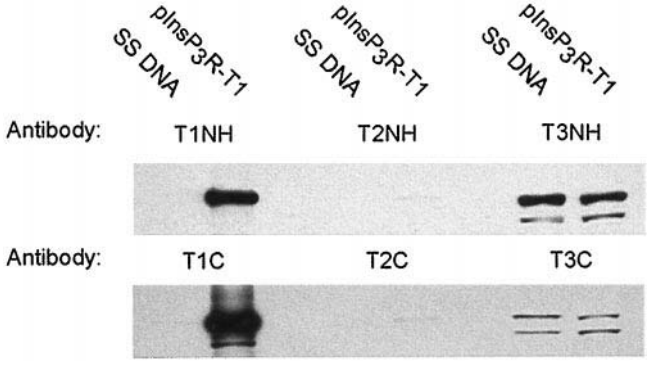

FIGURE 2 Expression of endogenous $InsP_3R$ isoforms in transfected COS-1 cells. Microsomal protein (25 μg) from either control (SS DNA) or $pInsP_3R$-T1 transfected COS cells were resolved on 5% SDS-PAGE and immunoblotted with $InsP_3R$ type-specific amino-terminal antibodies (*top panel*) and type-specific carboxyl-terminal antibodies (*bottom panel*).

$InsP_3$ binding

Equilibrium $InsP_3$ binding assays were performed using microsomal proteins from transfected COS cells (Table 1). Both the full-length ligand binding domain alternatively spliced forms of the receptor ($pInsP_3R$-T1, $pInsP_3R$-T1ALT) bind significant amounts of $InsP_3$. The amino-

TABLE 1 $InsP_3R$ binding of recombinant type-1 $InsP_3R$ splice variants

Sample	$InsP_3$ Bound (pmol/mg Protein)	
	Minus Competitor	Plus Competitor
$pInsP_3R$-T1	0.646 ± 0.069	0.109 ± 0.012
$pInsP_3R$-T1ALT	0.935 ± 0.110	0.111 ± 0.019
$pInsP_3R$-ΔT1ALT	0.114 ± 0.017	0.095 ± 0.011
SS Control	0.130 ± 0.016	0.099 ± 0.012

Data reported as means ± SD performed in quadruplicate and repeated three times.

terminal truncation expression product ($pInsP_3R$-ΔT1ALT) and SS DNA control microsomes did not bind $InsP_3$ at significant levels above nonspecific background. The lack of $InsP_3$ binding in the microsomes from the $pInsP_3R$-ΔT1ALT expression products is not surprising, since approximately one-half of the ligand binding domain was deleted. However, it should be noted that if the expression of endogenous isoforms were induced by overexpression of the recombinant plasmid we would anticipate increased [^{3}H]-$InsP_3$ binding. These results are consistent with previous studies in which microsomes of transfected COS-1 cells contained abundant amounts of immunoreactive receptor protein and bound significant amounts of [^{3}H]-$InsP_3$ (Mignery et al., 1990).

Immunolocalization

The expression and targeting of the recombinant receptor protein in COS-1 cells transfected with either $pInsP_3R$-T1, $pInsP_3R$-T1ALT, $pInsP_3R$-ΔT1ALT, or control DNA was analyzed by immunofluorescence microscopy using affinity-purified type-1 specific antipeptide antibodies T1C and T1ALT15. Examination of COS-1 cells transfected with $pInsP_3R$-T1 (Fig. 3, *panels 1* and *2*), $pInsP_3R$-T1ALT (Fig. 3, *panel 4*), and $pInsP_3R$-ΔT1ALT (Fig. 3, *panel 5*) reveals numerous brightly immunoreactive cells expressing the type-1 $InsP_3R$. The expressed receptor is targeted to the endoplasmic reticulum (ER) throughout the cell and into the apexes of extending processes. The cellular distribution of all expressed proteins was indistinguishable and the expressed proteins were previously shown to co-localize with an RER marker (BIP; Takei et al., 1994). COS-1 cells transfected with control SS DNA exhibit little, if any, specific immunoreactivity to T1C (Fig. 3, *panel 3*) or T1ALT15 (Fig. 3, *panel 6*) and reveal no clear ER staining.

Fractionation and functional reconstitution of the recombinant type-1 $InsP_3R$

In experiments designed to determine whether the recombinant type-1 receptor expressed in COS cells assembles into functional channels, we reconstituted partially purified receptor into proteoliposomes for planar lipid bilayer studies. This strategy was successfully used to examine the native type-1 receptor channel properties of cerebellum and the native type-2 isoform isolated from ventricular cardiac myocytes (Perez et al., 1997; Ramos-Franco et al., 1998). Microsomes from COS-1 cells transfected with either $pInsP_3R$-T1, $pInsP_3R$-T1ALT, $pInsP_3R$-ΔT1ALT, or control SS DNA were solubilized in 1.8% CHAPS and sedimented over 5–20% sucrose density gradients. The extremely large size of the receptor tetramer (calculated monomer MW = 313 kD) results in its sedimentation to a position on the gradient beyond the majority of the other proteins (Mignery and Sudhof, 1990). $InsP_3R$ protein was detected in gradient fractions by Western immunoblotting using the type-1 specific carboxyl-terminal (T1C) and T1ALT15 antibodies. Gradients containing detergent extracts from COS-1 cells transfected with the $pInsP_3R$-T1, $pInsP_3R$-T1ALT, and $pInsP_3R$-ΔT1ALT expression plasmids exhibited significant levels of receptor protein at positions consistent with those of tetramers (Fig. 4 *A, top panel*, and 4 *B*). No immunoreactive $InsP_3$ receptor was detected from COS-1 cells transfected with SS DNA using identical exposure times (Fig. 4 *A, middle panel*). An extended exposure interval (10×) revealed a weak signal from the control COS-1 cells and indicated that the endogenous receptor was present at very low levels (Fig. 4 *A, bottom panel*) and had similar sedimentation profiles to those of native or recombinant receptors. The protein species observed in fractions 4 and 6 of the 10× exposure of the SS DNA control gradient, illustrated in Fig. 4 *A* (*bottom panel*), may possibly be individual $InsP_3R$ subunits that were dissociated during the isolation and solublization, since monomeric receptor sediments to this position on 5–20% gradients (Mignery et al., 1990; Mignery and Sudhof, 1990). A more likely interpretation is that during the extended exposure times necessary to detect the endogenous $InsP_3R$, in Fig. 4 *A* (*bottom panel*), background signals due to nonspecific absorption of antibody were detected. These fractions, near the top of the gradient, have been shown to contain the bulk of the protein originally applied to gradient (Mignery et al., 1989; Mignery and Sudhof, 1990). The expression products from $pInsP_3R$-T1 have a broad distribution on the sucrose gradient. This is most likely due to the large amount of receptor protein initially applied to the gradient. Similar distributions of native type-1 receptor from cerebellum were observed (Mignery et al., 1992). The purpose of this gradient was as a preparative tool and not an analytical mechanism to assess sedimentation properties. Only the fractions rich in receptor, which sedimented to tetrameric positions on the gradient, were chosen for reconstitution into liposomes. Thus, potential artifacts resulting from reconstitution of protein aggregates are minimized. As is the case with most proteins, some aggregation is possible and we cannot rule out the chance that some of the receptor population that sediments to positions other than that of discrete tetramers is indeed aggregated. Gradient fractions containing the highest levels of receptor were recovered and reconstituted into L-α-phosphatidylcholine and L-α-phosphatidylserine containing liposomes as described previously (Perez et al., 1997).

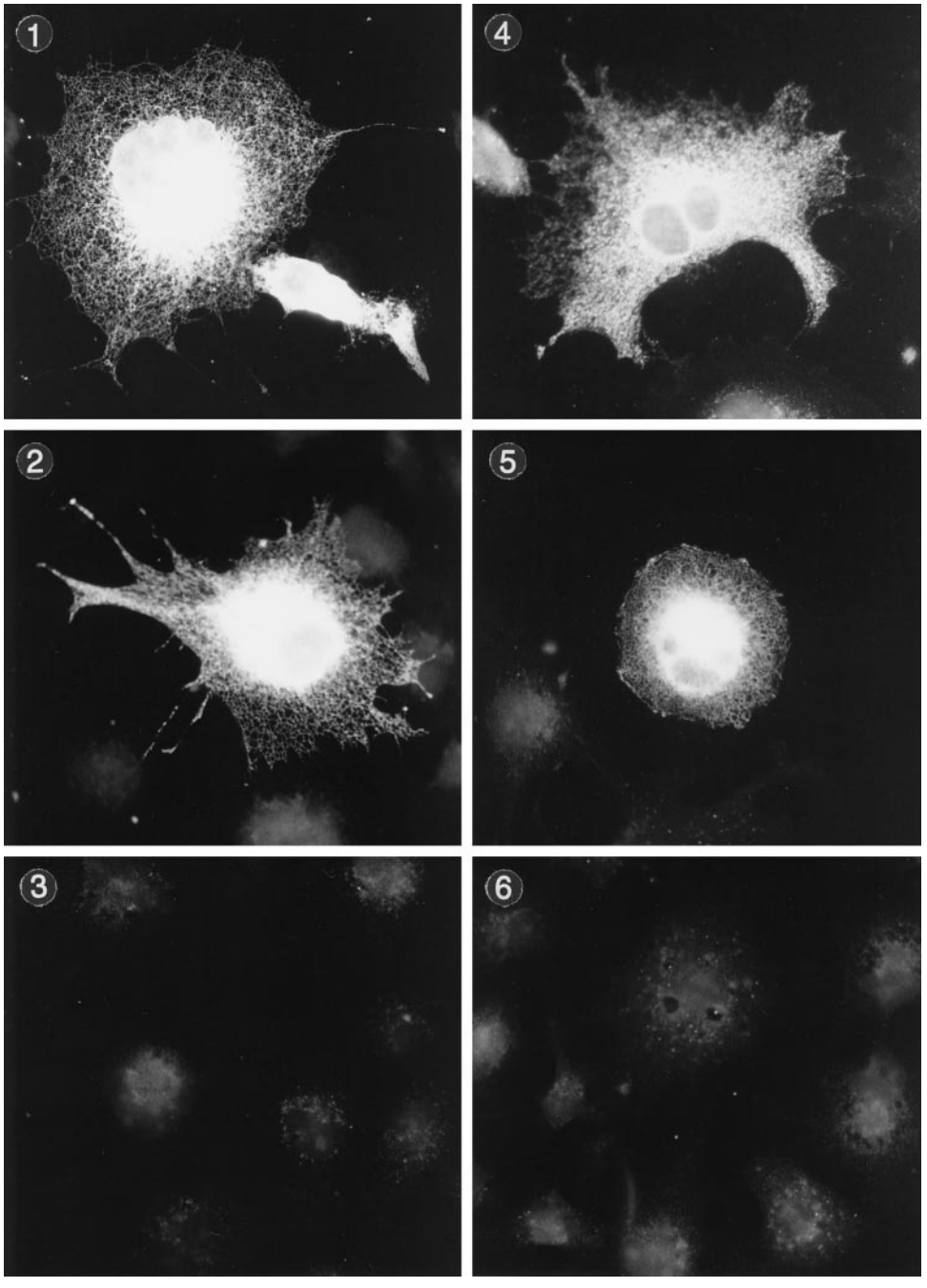

FIGURE 3 Analysis of $InsP_3$ receptor localization in transfected COS-1 cells. COS-1 cells transfected with either $pInsP_3R$-T1 (*panels 1* and *2*), control (SS) DNA (*panels 3* and *6*), $pInsP_3R$-T1ALT (*panel 4*) or $pInsP_3R$-ΔT1ALT (*panel 5*) were reacted with affinity purified antipeptide antibodies directed against the 19 carboxyl-terminal amino acids of the type-1 receptor (T1C) (*panels 1–3*) and the 15-amino-acid alternatively spliced insert (T1ALT15) (*panels 4–6*). The strong immunoreactivity of cells transfected with $pInsP_3R$-T1 (*panels 1* and *2*), $pInsP_3R$-T1ALT (*panel 4*), and $pInsP_3R$-ΔT1ALT (*panel 5*) is localized throughout the cell and is visible in filamentous networks, consistent with those of the endoplasmic reticulum. Analysis of control COS cells (*panels 3* and *6*), transfected with SS DNA, revealed only faint immunoreactivity observable in the thickest regions surrounding the nucleus with either antibody. No discrete ER staining could be detected with these low levels of expression. All coverslips were photographed with the same optics settings and similar exposure interval.

The functions of individual receptor channels were defined by fusion of the reconstituted proteoliposomes into planar lipid bilayers using Cs^+ as the cationic charge carrier. After incorporation of the proteoliposomes containing the recombinant $pInsP_3R$-T1 and $pInsP_3R$-T1ALT proteins, cationic channels appeared in the bilayer. In the absence of added $InsP_3$ these channels were quiescent with only rare opening events (Fig. 5, *top panel*). Upon addition of 100 nM $InsP_3$ to the *cis* buffer solution these channels activated, exhibiting frequent and rapid opening events (Fig. 5, *middle panel*) that increased the P_o to 0.078 ± 0.007 ($n = 4$). The mean open time was 2.2 ms and the mean closed time was 51.5 ms. These values are in close agreement with those obtained for the native type-1 receptor isolated from cerebellum (Ramos-Franco et al., 1998). Addition of 0.5 mg/ml heparin to the *cis* buffer solution significantly inhibited single channel activity (Fig. 5, *bottom panel*). Infrequent channel openings were occasionally observed in the presence of heparin; however, the P_o was markedly reduced (~0.002).

No detectable $InsP_3$/heparin-sensitive Cs^+ conducting channels were incorporated into the bilayer after fusion of proteoliposomes containing gradient receptor fractions from the control (SS DNA) transfected or nontransfected COS-1 cells (see Table 2). These experiments were done over a range of $InsP_3$ concentrations (0.1–5 μM). These results

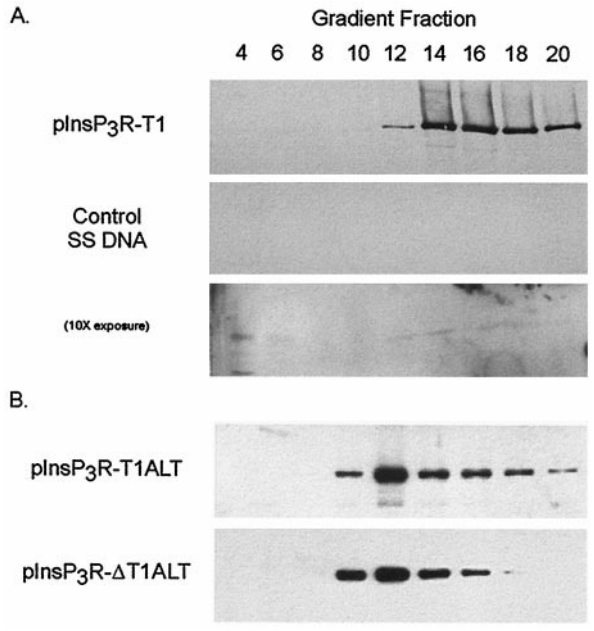

FIGURE 4 Partial purification of $InsP_3R$ proteins by sucrose density sedimentation. (*A*) Microsomes from an equivalent number of COS-1 cells (900E3) transfected with either the full-length type-1 $InsP_3$ receptor ($pInsP_3R$-T1) or control (SS) DNA were solubilized in 1.8% CHAPS and fractionated over 5–20% sucrose gradients. Fractions were analyzed by SDS-PAGE and immunoblotted with the type-1 receptor antibody (T1C) to identify the receptor containing fractions (*A*, *all panels*). Gradients containing $pInsP_3R$-T1 COS cell expression product exhibited a strong immunoreactive signal in fractions consistent with those of $InsP_3R$ tetramers (*A, top panel*). The gradient fractions containing membrane extracts from SS DNA control transfected COS cells revealed no detectable immunoreactivity at equivalent exposure times (*A, middle panel*). Extended exposure intervals (10× those in *A, middle panel*) reveal a low level of endogenous $InsP_3R$ in the control COS cell gradient fractions (*A, bottom panel*). (*B*) Partial purification of $pInsP_3R$-T1ALT (*top panel*) and $pInsP_3R$-ΔALT (*bottom panel*) on sucrose gradients Western-blotted with T1ALT15 affinity pure antibody. The slight heterogeneity between the two panels in (*A*) and (*B*) represents small differences in run times of the gradients.

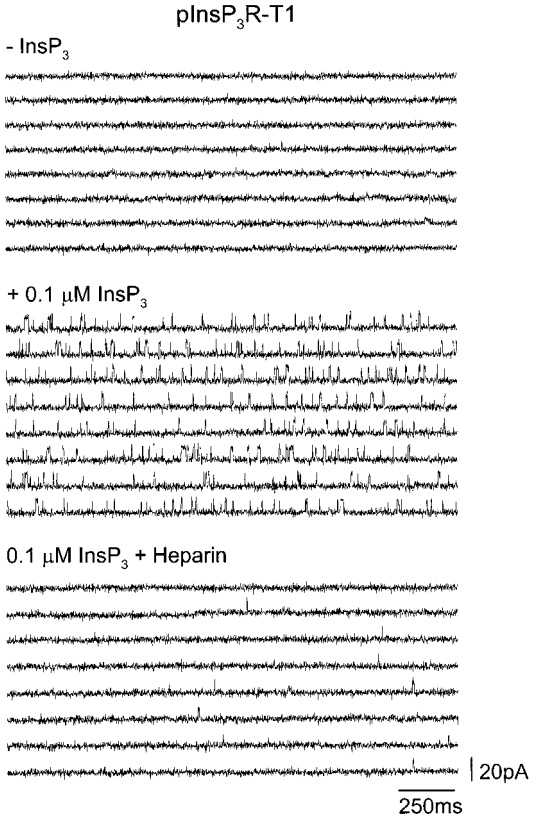

FIGURE 5 Reconstitution of single $pInsP_3R$-T1 recombinant channels in planar lipid bilayers. Proteoliposomes containing gradient-enriched recombinant $InsP_3R$ protein were fused to planar lipid bilayers for electrophysiological analysis. Solutions contained 220/20 mM $CsCH_3SO_3$ (*cis/trans*), 1 mM EGTA, 250 nM $[Ca^{2+}]_{Free}$, and 20 mM HEPES (pH 7.4). Open events are shown as upward deflections from the zero current level. Control records (*top panel*) were obtained before the addition of $InsP_3$ ($P_o < 0.001$). The addition of 100 nM $InsP_3$ to the *cis* chamber of the bilayer apparatus evoked channel activity ($P_o = 0.08$; *middle panel*). After several minutes of single channel recording, heparin (0.5 mg/ml) was added to the *cis* chamber ($P_o = 0.002$; *bottom panel*).

reflect the low level abundance of the endogenous receptor compared to the expression of the recombinant receptor as illustrated in Figs. 1–3. It should be noted, however, that the endogenous receptors in COS cells are sufficient to sustain the intracellular calcium signaling in this cell line. The overexpression of recombinant receptor in COS-1 cells essentially inundates the background levels of endogenous receptors.

The electrophysiological properties of the recombinant type-1 receptor isoform closely resemble those measured for the native type-1 channels under identical experimental conditions (Perez et al., 1997; Ramos-Franco et al., 1998). The current-voltage relationships for the recombinant ($pInsP_3R$-T1) channels conducting Cs^+ or Ca^{2+} are illustrated in Fig. 6. Expressed $pInsP_3R$-T1 channels conducting Cs^+ (*circles*; 220–20 mM gradient) exhibited a reversal potential of −23.6 mV, indicating that this channel was cation-selective (Fig. 6). The Cs^+ conductance (290 pS) of recombinant (*solid line*) and the native channels (*dashed line*) were nearly identical. Expressed $pInsP_3R$-T1 channels conducting Ca^{2+} (*triangles*) exhibited a reversal potential of +20 mV and a Ca^{2+} conductance of 62 pS (*solid line*). Again, the Ca^{2+} permeation attributes of native (*dashed*

TABLE 2 Frequency of $InsP_3R$ channel incorporation into planar lipid bilayers

Sample	Number of Incorporation Attempts	Number of Channels Incorporated	Success Rate
Wild-type T1	702	147	21%
$pInsP_3R$-T1	238	69	29%
$pInsP_3R$-T1ALT	224	54	24%
$pInsP_3R$-ΔT1ALT	318	0	0
SS Control	561	0	0
Nontransfected	872	0	0

An "incorporation attempt" refers to new bilayer formation and application of channel protein. Number of "channels incorporated" refers to single and multiple channel incorporations. Success rate was calculated as "incorporation attempts" divided by "channels incorporated."

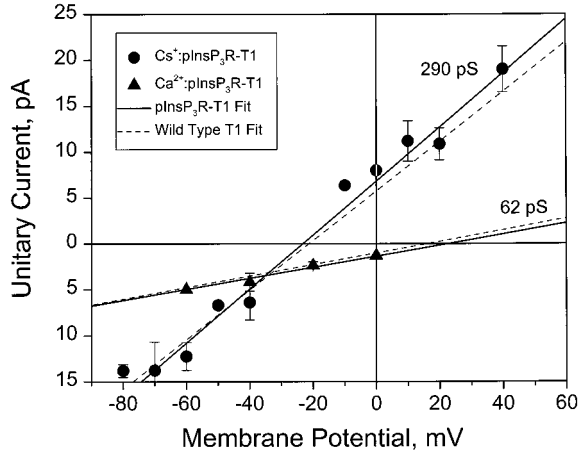

FIGURE 6 Permeation properties of $pInsP_3R$-T1 channels. The current-voltage relationships for $pInsP_3R$-T1 channels with Cs^+ (*circles*) or Ca^{2+} (*triangles*) as the charge carrier are shown. The data sets are well fit by a linear regression ($r = 0.99$, *solid line*) with a slope of 290 pS or 62 pS, respectively. These conductance values are very similar to those reported for the native type-1 $InsP_3R$ measured under identical conditions (*dashed lines*) (Ramos-Franco et al., 1998). All points represent mean ($\pm$SEM; $n \geq 4$). For the monovalent currents, the solutions contained 220/20 mM $CsCH_3SO_3$ (*cis/trans*), 1 mM EGTA, 250 nM $[Ca^{2+}]_{Free}$, and 20 mM HEPES (pH 7.4). For the divalent currents, the solutions contained 100 mM HEPES-Tris (pH 7.4), 1 mM EGTA, 250 nM $[Ca^{2+}]_{Free}$ (*cis*), and 50 mM calcium methanesulfonate (*trans*).

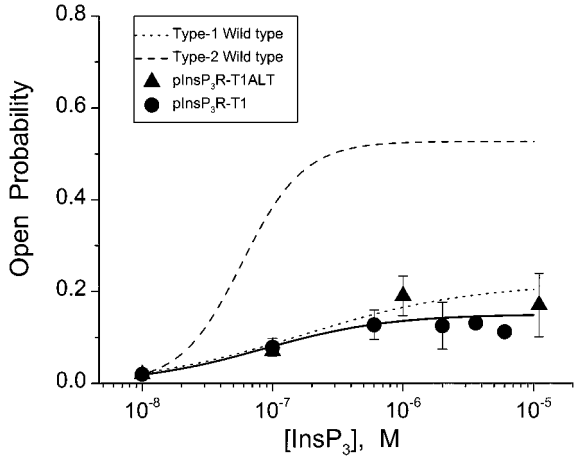

FIGURE 7 $InsP_3$ sensitivity of expressed type-1 $InsP_3R$ channels. The $InsP_3$ sensitivities of single $pInsP_3R$-T1 (*circles*) and $pInsP_3R$-T1ALT (*triangles*) channels were defined in planar lipid bilayer studies. Each point represents mean ($\pm$SEM) of four to six determinations from different channels. The solid line represents a Hill fit of the $pInsP_3R$-T1 data points. The long dashed line represents the $InsP_3$ sensitivity reported for native type-2 $InsP_3R$ channels (Ramos-Franco et al., 1998). The short dashed line represents the $InsP_3$ sensitivity reported for native type-1 $InsP_3R$ channels (Ramos-Franco et al., 1998). The apparent EC_{50} values of the $pInsP_3R$-T1 and $pInsP_3R$-T1ALT channel are listed in Table 3. Solutions contained 220/20 mM $CsCH_3SO_3$ (*cis/trans*), 1 mM EGTA, 250 nM $[Ca^{2+}]_{Free}$, and 20 mM HEPES (pH 7.4).

line) and the recombinant channels were nearly identical. The unitary Cs^+ and Ca^{2+} conductances of the native and recombinant channels ($pInsP_3R$-T1 and $pInsP_3R$-T1ALT) are listed in Table 3. The permeation properties of the native cerebellar type-1 channel represent those reported previously by our lab group (Ramos-Franco et al., 1998). These permeation properties are also quite similar to those reported for the type-2 and type-3 receptors (Perez et al., 1997; Ramos-Franco et al., 1998; Hagar and Ehrlich, 1998).

The $InsP_3$ sensitivities of the $pInsP_3R$-T1 and $pInsP_3R$-T1ALT channels to $InsP_3$ are displayed in Fig. 7. The open probability (P_o) of both channels was dependent on $InsP_3$ concentration. The $InsP_3$ sensitivities were fit by the Hill equation. The dose of $InsP_3$ required for half-maximal activation (EC_{50}) of the $pInsP_3R$-T1 channel was 97 nM and 135 nM for the $pInsP_3R$-T1ALT channel. Native cerebellum type-1 channels under the same experimental conditions had an EC_{50} of 194 nM (Ramos-Franco et al., 1998). The mean ($\pm$SD) EC_{50} values for native and the recombinant channels are listed in Table 3. There was no statistically significant difference in the $InsP_3$ sensitivity of the native and recombinant channels. The EC_{50}, Hill coefficient ($\sim$1), and extent of channel activation (P_o) reported here for the recombinant type-1 channel are very similar to those reported by Watras et al. (1991) for the native cerebellum type-1 $InsP_3R$ channel. The similar $InsP_3$ sensitivity of the two splice variants suggests that differential splicing does not have an impact on the $InsP_3$ regulation of the channel.

TABLE 3 Comparison of native and recombinant type-1 $InsP_3R$ splice variants

	Unitary Conductance		Ligand Sensitivity (EC_{50})	
Sample	Ca^{2+}	Cs^+	Ca^{2+}	$InsP_3$
Wild-type T1	64 $\pm$ 3 pS	270 $\pm$ 13 pS	309 $\pm$ 86 nM	194 $\pm$ 55 nM
$pInsP_3R$-T1	65 $\pm$ 3 pS	293 $\pm$ 25 pS	267 $\pm$ 62 nM	97 $\pm$ 66 nM
$pInsP_3R$-T1ALT	65 $\pm$ 4 pS	276 $\pm$ 23 pS	278 $\pm$ 102 nM	135 $\pm$ 72 nM

Data reported as means $\pm$ SD obtained from at least three experiments. No statistically significant differences (*t*-test, $p > 0.05$) between the three $InsP_3R$ variants tested.

The ability of the $pInsP_3R$-ΔT1ALT construct to form an ion channel was also evaluated. The $pInsP_3R$-ΔT1ALT is an $InsP_3R$ truncation missing 310 amino-terminal amino acids of the $InsP_3$ binding domain. Our $InsP_3$ binding studies confirmed that this receptor does not bind $InsP_3$ (see Table 1). Several attempts were made to reconstitute ion channels from $pInsP_3R$-ΔT1ALT-rich liposomes (see Table 2). No evidence of single channel activity was observed in 318 attempts, although a nonspecific leak current was observed occasionally ($\sim$10% of attempts). The nonspecific nature of the leak current suggests that it does not arise from the well characterized Ca^{2+}-selective $InsP_3R$ pore. Thus, these data indicate that the $pInsP_3R$-ΔT1ALT construct does not form functional ion channels. This implies that the presence of the $InsP_3$ binding domain is required for the $InsP_3R$ channel to open, and thus its absence appears to lock the channel into a state that does not gate (open and close).

The Ca^{2+} sensitivity of the native type-1 $InsP_3R$ has been defined (Bezprozvanny et al., 1991; Ramos-Franco et al., 1998). The Ca^{2+} sensitivity of the $pInsP_3R$-T1 (*circles*) and

the $pInsP_3R$-T1ALT (*triangles*) recombinant receptors is illustrated in Fig. 8. The Ca^{2+} sensitivity of the native $InsP_3R$ is illustrated by the solid line (Ramos-Franco et al., 1998). The recombinant $InsP_3Rs$ have a bell-shaped Ca^{2+} sensitivity that is nearly identical to each other and to that of the native receptor. These data, therefore, show that bell-shaped Ca^{2+} sensitivity of the type-1 $InsP_3R$ is conserved in the two recombinant forms of the channels. It also suggests that the alternate splicing events that create the amino-terminal splice variants do not have an impact on the Ca^{2+} regulation of the channel.

DISCUSSION

This study demonstrates that the recombinant type-1 $InsP_3$ receptor ligand binding domain splice variants form functional ion channels when transiently expressed in COS-1 cells. All of the $InsP_3$ receptor expression plasmids ($pInsP_3R$-T1, $pInsP_3R$-T1ALT, and $pInsP_3R$-ΔT1ALT) were expressed at high levels and localized to the endoplasmic reticulum in the transfected cells (Fig. 3). Isolation of the purified receptors on sucrose density gradients (Fig. 4) followed by reconstitution of the protein into liposomes enabled electrophysiological characterization of $InsP_3$ receptor constructs. The full-length $InsP_3$ receptor constructs formed functional Ca^{2+} channels. The truncation mutant ($pInsP_3R$-ΔT1ALT) did not form a functional Ca^{2+} channel. The recombinant Ca^{2+} channels were activated by $InsP_3$ and blocked by heparin. Their unitary conductance (Cs^+ or Ca^{2+}) was very similar to that reported for the native type-1 $InsP_3R$ channel (Table 3). The two full-length $InsP_3$ receptor splice variants ($pInsP_3R$-T1 and $pInsP_3R$-T1ALT) had nearly identical $InsP_3$ and Ca^{2+} sensitivities and are compared to the native type-1 receptor from cerebellum (Table 3).

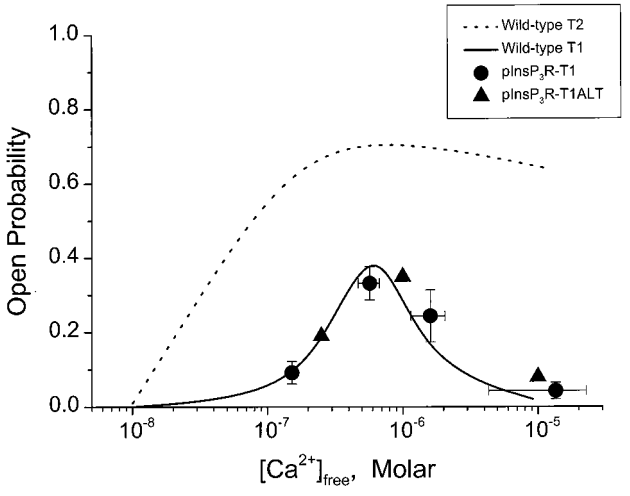

FIGURE 8 Calcium sensitivity of expressed $InsP_3R$ channels. The Ca^{2+} sensitivities of single $pInsP_3R$-T1 (*circles*) and $pInsP_3R$-T1ALT (*triangles*) channels were defined in planar lipid bilayer studies. The data point represents means ($\pm$SEM) of two to six determinations. The solid line represents the Ca^{2+} sensitivity reported for native type-1 $InsP_3R$ channels (Ramos-Franco et al., 1998). The dashed line represents the Ca^{2+} sensitivity reported for native type-2 $InsP_3R$ channels (Ramos-Franco et al., 1998). The apparent EC_{50} values of the $pInsP_3R$-T1 and $pInsP_3R$-T1ALT channels are listed in Table 1. Solutions contained 220/20 mM $CsCH_3SO_3$ (*cis/trans*), 1 mM EGTA, 250 nM $[Ca^{2+}]_{Free}$, and 20 mM HEPES (pH 7.4).

This study demonstrates that overexpression of either of the type-1 $InsP_3$ receptor splice variants in COS cells produces functional type-1 $InsP_3$ receptor channels with nearly identical functional attributes. The data suggest that the amino terminal truncation mutant did not form functional Ca^{2+} channels, and thus the $InsP_3$ binding domain may be required for normal protein function. Perhaps the most notable contribution of this study is that it demonstrates that it is possible to apply a mutagenesis-based experimental approach to define the structure-function properties of the $InsP_3$ receptor channel. This is exciting because this approach has not yet been successfully applied to the $InsP_3$ receptor.

What about the endogenous $InsP_3$ receptors?

Most cells contain one or more endogenous $InsP_3$ receptors. The COS cell is no exception. The COS cell contains endogenous $InsP_3$ receptors and the impact of endogenous receptors must be addressed.

All cell lines that we have tested (HEK 293, SF9, CHO, COS, PC12, 3T3) contain endogenous $InsP_3$ receptors (Newton et al., 1994). Application of a strategy to transiently or permanently knock out one (two or three) $InsP_3R$ isoform(s) of course is possible. Before undertaking this potentially difficult task, we elected to use an existing cell line (i.e., COS-1) and simply overexpress the recombinant $InsP_3$ receptor to the extent that the endogenous receptors represent an almost negligible fraction of the total $InsP_3$ receptor protein pool. Three lines of experimental evidence suggest that this was indeed the case. First, Western blots of type-1 $InsP_3R$ in control (SS-DNA transfected) and test ($pInsP_3R$-T1, $pInsP_3R$-T1ALT, and $pInsP_3R$-ΔT1ALT transfected) revealed that very little endogenous receptor was present compared to recombinant receptor. No significant induction of expression of endogenous isoforms was observed by Western immunoblotting using a total of seven receptor type-specific affinity pure antibodies (Figs. 1 *A* and 2). Second, immunofluorescence illustrated that SS-DNA transfected cells had very little immunoreactivity to type-1 receptor antibodies (T1C and T1ALT15) compared to $pInsP_3R$-T1, $pInsP_3R$-T1ALT, and $pInsP_3R$-ΔT1ALT transfected cells (Fig. 3). Third, no detectable $InsP_3$ receptor channels were found when proteoliposomes prepared from SS-DNA or the ligand binding truncation, $pInsP_3R$-ΔT1ALT, transfected cells were fused into planar lipid bilayers. In contrast, frequent and robust single channel activity was observed when proteoliposomes prepared from $pInsP_3R$-T1 or $pInsP_3R$-T1ALT transfected cells were fused into planar bilayers.

It is possible that the unique functional attributes of the type-2 and -3 $InsP_3R$ may make endogenous copies of these

channels difficult to detect in our study. Our single channel studies, however, explored channel function over wide ranges of $InsP_3$ and Ca^{2+} concentrations. Under these conditions all three types of $InsP_3R$ should be detectable. The functional profile of all detected channels in our study matched that of the type-1 $InsP_3R$. Thus, there was no evidence that endogenous type-2 or -3 $InsP_3R$ were incorporated into the planar lipid bilayer in this study.

It is important to note that these data (as is the case for testing any negative) do not definitively show that endogenous $InsP_3$ receptors were absent in our channel preparations. These data simply show that recombinant $InsP_3$ receptor populations dramatically out numbered endogenous receptors in the pInsPR-T1 and pInsP$_3$R-T1ALT transfected cells and that it is reasonable to conclude that the single channel properties defined here represent the properties of those proteins.

Significance

The sequence of the cDNA that encodes the type-1 $InsP_3$ receptor has been known for several years (Mignery et al., 1990; Furuichi et al., 1989) and has been expressed in several different types of cells. The type-1 $InsP_3$ receptor is alternately spliced at two different sites (Mignery and Sudhof, 1993; Furuichi and Mikoshiba, 1995). Thus, there may be at least six different type-1 $InsP_3$ receptor forms, two in the ligand binding and four in the coupling domain. The cerebellum contains the type-1 $InsP_3$ receptor that correlates to the expression product of the pInsP$_3$R-T1 vector. Other cells contain the type-1 $InsP_3$ receptor splice variant that correlates to the expression product of the pInsP$_3$R-T1ALT vector. This splice variant is a small 15-amino-acid insertion in the $InsP_3$ binding domain. The functional implications of this particular splice variation has not been extensively explored. Here, we show that the function of the single Ca^{2+} channels formed by the pInsP$_3$R-T1 and pInsP$_3$R-T1ALT expression products were nearly identical and that they had similar efficacies to $InsP_3$.

The $InsP_3$ receptor consists of three domains ($InsP_3$ binding, coupling/regulatory, and channel domains) (Mignery and Sudhof, 1990). The channel domain clearly contributes its ion permeation properties. The $InsP_3$ binding and coupling/regulatory domains contain the $InsP_3$ binding site and numerous motifs that regulate the channel. How the different domains interact to establish the function of the protein is not yet well understood. In this study we expressed an $InsP_3$ receptor truncation mutant that was missing 310 amino-terminal amino acids of the $InsP_3$ binding domain. This $InsP_3$ receptor construct did not appear to make functional channels. This suggests that the truncation of most of the $InsP_3$ binding domain does not leave the channel in an open state. Instead, it implies that the presence of the $InsP_3$ binding domain is fundamental to channel operation.

Most importantly, this manuscript establishes a strategy that allows the single channel properties of recombinant $InsP_3$ receptors to be defined. It shows that, by the simple strategy of overexpressing a recombinant $InsP_3$ receptor in COS cells, single channel function of the recombinant receptor can be examined. Defining the function of single recombinant $InsP_3$ receptors represents a critical first step in the dissection of the molecular aspects of the $InsP_3$ receptor's structure-function.

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