Location of the Permeation Pathway in the Recombinant Type 1 Inositol 1,4,5-Trisphosphate Receptor

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Abstract

The inositol 1,4,5-trisphosphate receptor (InsP$_3$R) forms ligand-regulated intracellular Ca$^{2+}$ release channels in the endoplasmic reticulum of all mammalian cells. The InsP$_3$R has been suggested to have six transmembrane regions (TMRs) near its carboxyl terminus. A TMR-deletion mutation strategy was applied to define the location of the InsP$_3$R pore. Mutant InsP$_3$Rs were expressed in COS-1 cells and single channel function was defined in planar lipid bilayers. Mutants having the fifth and sixth TMR (and the interceding lumenal loop), but missing all other TMRs, formed channels with permeation properties similar to wild-type channels (gCs = 60 pS; P$_{Ca}$/P$_{Ca}^*$ = 60). These mutant channels bound InsP$_3$, but ligand occupancy did not regulate the constitutively open pore (P$_o$ > 0.80). We propose that a region of 191 amino acids (including the fifth and sixth TMR, residues 2398–2589) near the COOH terminus of the protein forms the InsP$_3$R pore. Further, we have produced a constitutively open InsP$_3$R pore mutant that is ideal for future site-directed mutagenesis studies of the structure-function relationships that define Ca$^{2+}$ permeation through the InsP$_3$R channel.

Key words: intracellular Ca$^{2+}$ release • Ca$^{2+}$ signaling • recombinant channel

Introduction

The inositol 1,4,5-trisphosphate receptor (InsP$_3$R) gene family encodes a highly homologous group of proteins localized to the endoplasmic reticulum (ER). The InsP$_3$R gene family has three members (types 1, 2, and 3) that are ubiquitously expressed in metazoans (Newton et al., 1994; Nakanishi et al., 1996; De Smedt et al., 1997). The InsP$_3$R proteins tetramerize to form ion channels that are responsible for the regulated release of Ca$^{2+}$ from intracellular Ca$^{2+}$ stores (reviewed in Bezprozvanny and Ehrlich, 1995; Joseph, 1996). The single channel properties of the three InsP$_3$R Ca$^{2+}$ channels (types 1, 2, and 3) have been defined by reconstituting the native receptor complex into planar lipid bilayers (Bezprozvanny et al., 1991; Hagar et al., 1998; Ramos-Franco et al., 1998a). Recently, the single channel properties of a recombinant type 1 InsP$_3$R channel and a splice variant have been defined (Kaznacheeva et al., 1998; Ramos-Franco et al., 1998b). The recombinant and native InsP$_3$R channels have nearly identical functional properties. The capacity to define single channel behavior of recombinant InsP$_3$Rs established a foundation from which new molecular/biophysical approaches can be used to define the structure-function properties of the InsP$_3$R channel family.

A first step towards understanding structure-function relationships in any protein is to locate the primary sequences that associated with its general features, such as ligand binding and/or transmembrane regions (TMRs). Analysis of the recombinant InsP$_3$R revealed that this protein is composed of three domains: the NH$_2$-terminal InsP$_3$-binding domain, the COOH-terminal channel domain, and the central coupling domain (Mignery and Südhof, 1990; Südhof et al., 1991). The originally proposed channel domain contains all the putative TMRs. This interpretation is consistent with several lines of evidence. For example, deletion of the channel-domain generates soluble monomeric InsP$_3$-binding proteins. The green fluorescent protein-tagged channel-domain, after deletion of the InsP$_3$-binding and coupling domains, oligomerizes and is localized to the ER (Sayers et al., 1997). Currently, it is thought that the InsP$_3$R’s channel domain has six TMRs. A six-TMR model is consistent with immunogold electron microscopy studies showing that the NH$_2$ and COOH termini are both localized in the cytoplasm (Mignery et al., 1989). It is also consistent with glycosylation data that demonstrates that the loop between the fifth and sixth TMR is lumenal (Michikawa et al., 1994). Moreover, the six-TMR model was experimentally confirmed by differential permeabilization combined with immunohistochemistry (Galvan, D., E. Borrego-Diaz, P.J. Perez, and G.A. Mignery, manuscript submitted for publication).

The InsP$_3$R protein is thought to tetramerize to form functional Ca$^{2+}$ release channels (Mignery et al., 1989). It is clear that important determinants of InsP$_3$R tet-
ramerization are associated with the TMRs (Mignery and Südhof, 1990; Galvan, D., E. Borrego-Diaz, P.J. Perez, and G.A. Mignery, manuscript submitted for publication). Galvan and co-workers constructed several TMR-deletion mutants from the full-length type 1 InsP$_3$R cDNA to define important determinants of InsP$_3$R tetramerization. At least two TMRs are required for the initiation of InsP$_3$R channel assembly (Galvan, D., E. Borrego-Diaz, P.J. Perez, and G.A. Mignery, manuscript submitted for publication). Tetramerization is also stabilized in the presence of additional TMRs and in the presence of the COOH terminus. Further, two studies have implicated the fifth and sixth TMRs as a particularly strong determinant of InsP$_3$R tetramerization (Joseph et al., 1997; Galvan, D., E. Borrego-Diaz, P.J. Perez, and G.A. Mignery, manuscript submitted for publication). This region of the InsP$_3$R has three notable attributes. First, the sixth TMR is a point of very high sequence homology with the RyR channel (Mignery et al., 1989). Second, the fifth and sixth TMRs contain a putative leucine zipper motif that could be important for stable tetramerization and/or pore formation (Galvan, D., E. Borrego-Diaz, P.J. Perez, and G.A. Mignery, manuscript submitted for publication). Third, the luminal 5–6 loop has been proposed to be analogous to the H-loop of voltage-activated Ca$^{2+}$, Na$^+$, and K$^+$ channels (Mignery and Südhof, 1993).

It is reasonable to hypothesize that the fifth and sixth TMR and the interceding loop are the most likely region of the InsP$_3$R to form the trans-ER ion permeation pathway. To experimentally test this hypothesis, single channel function of different type 1 InsP$_3$R TMR-deletion mutants was defined in planar bilayer studies. Our data indicate that the fifth and sixth TMRs and the interceding loop contain important structural determinants of the InsP$_3$R channel’s permeation pathway that govern its conduction and selectivity. Our data also suggest that the 1–4 TMR region of the pInsP$_3$R contains important sequence and/or structural elements that regulate gating of the pore.

**Materials and methods**

**Materials**

$[^{3}H]$InsP$_3$ (21 Ci/mmol) was obtained from Du Pont-New England Nuclear. Unlabeled InsP$_3$ was purchased from LC Laboratories Inc., and heparin was from Sigma Chemical Co. Ryanodine was purchased from Calbiochem Corp. Lipids, l-$\alpha$-phosphatidylcholine, l-$\alpha$-phosphatidylethanolamine, and l-$\alpha$-phosphatidylserine were obtained from Avanti Polar Lipids.

**Plasmid Construction and Expression**

The full-length type 1 plasmid (pInsP$_3$R-T1) was assembled from overlapping cDNA clones isolated from a rat brain library as previously described (Mignery et al., 1990). This plasmid is identical to the pInsP$_3$R plasmid we used previously (Ramos-Franco et al., 1998b). Two TMR-deletion plasmids were also used in this study. The expression plasmid pln$p^{*}$R1△4 encoded a protein missing residues 2211–2416. The expression plasmid pln$p^{*}$R△5-6 encoded a protein missing residues 2398–2589. Construction strategies of these two expression plasmids is described in detail elsewhere (Galvan, D., E. Borrego-Diaz, P.J. Perez, and G.A. Mignery, manuscript submitted for publication). Galvan and co-workers denoted the plasmids as TMR5-6+ and TMR1-4+ and pInsP$_3$R△1-4, and pInsP$_3$R△5-6 were transiently transfected into COS-1 cells. COS-1 cells were transfected with each plasmid or sheared salmon sperm (SS) DNA using the DEAE-dextran method as described by Gorman (1985). The sheared SS DNA was used to mock transflect COS-1 cells and served as a negative control. Cells were incubated at 37°C, 5% CO$_2$ for 48–72 h before 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.

**CHAPS Solubilization and Gradient Sedimentation**

COS-1 cells transfected with either pln$p^{*}$R-T1, pln$p^{*}$R△1-4 and pln$p^{*}$R△5-6, or the SS DNA were harvested 48–72 h after transfection, and microsomes were prepared as described previously (Mignery et al., 1990). COS-1 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), 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, 1.8% CHAPS β-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate on ice for 1 h. Insoluble fractions were eliminated by a 10-min centrifugation at 289,000 g, and the supernatant containing solubilized receptor was fractionated through 5–20% sucrose (wt/vol) gradients as previously described (Mignery et al., 1990). The sheared SS DNA was used to mock transflect COS-1 cells and served as a negative control. Cells were incubated at 37°C, 5% CO$_2$ for 48–72 h before 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.

**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 of membrane protein in 100 μl of 50 mM Tris-HCl, pH 8.3, 1 mM EDTA, 1 mM 2-mercaptoethanol, 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 then the radioactivity of the membrane pellets was determined by scintillation spectrometry. All assays were performed in quadruplicate and replicated three times.

**Single Channel Assay**

Planar lipid bilayers were formed across a 150-μm diameter aperture in the wall of a Delrin partition as described (Ramos-Franco et al., 1998b). 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 CsCl, 50 mM Na$_2$SO$_4$, 20 mM Hepes, pH 7.4, and 1 mM EGTA.
{[Ca\textsuperscript{2+}]\textsubscript{FREE} = 250 nM; Ca\textsuperscript{2+} added as Ca(CH\textsubscript{3}SO\textsubscript{4})\textsubscript{2}} The 
{[Ca\textsuperscript{2+}]\textsubscript{FREE} was verified using a Ca\textsuperscript{2+} electrode. The Ca\textsuperscript{2+} 
electrodes were comprised of the Ca ligand ETH 129 in a polyvinyl-
chloride membrane at the end of small (2 mm) polyethylene 
trodes were comprised of the Ca ligand ETH 129 in a polyvinyl-
odified under bionic conditions. The trans solution contained 30
mM Ca\textsuperscript{2+} 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 2
kHz. Ligands (1 μM InsP\textsubscript{3}, 50 μg/ml heparin, 10 μM ryanodine) were added symmetrically to reconstituted single channels. 
Open probability and unitary current amplitude was defined from 
Gaussian fitting of total amplitude histograms. Selectivity was de-
defined using a constant field equation:

\[
E_{\text{rev}} = \frac{RT}{2F} \ln \frac{4P_{\text{Ca}}[Ca]_{\text{cis}}}{P_{\text{Cs}}[Cs]_{\text{cis}}}
\]

where \textit{R}, \textit{T}, and \textit{F} have their usual meanings. Note that [\textit{Ca}]_{\text{trans}}/ 
[\textit{Ca}]_{\text{cis}} was equal to one.

**Results**

Expression and Isolation of Type 1 InsP\textsubscript{3}R 
TMR-deletion Mutants

The full-length (pInsP\textsubscript{3}R-T1) and TMR-deletion 
(pInsP\textsubscript{3}R-A5-6 and pInsP\textsubscript{3}R-A1-4) mutants were transfected 
into COS-1 cells using the DEAE-dextran method (Gorman, 1985). A schematic of the full-length 
deletion mutants used in this study is shown in Fig. 
1 A. The putative pore region of the InsP\textsubscript{3}R includes 
the 150 amino acids bounded by the fifth and sixth
TMRs. A sequence aligned between the type 1 InsP3R 
and ryanodine receptor 2 (RyR2) proteins over this 
region is illustrated in Fig. 1 B. The fifth and sixth InsP3R
TMRs are boxed, and identical residues in the InsP3R 
and RyR sequences are shaded. Residues marked by 
a star indicate those conserved between all three InsP3R 
isofoms and RyR2. These expression vectors were 
under the control of the cytomegalovirus promoter 
(Mignery et al., 1990), and these plasmids expressed 
immunoreactive InsP\textsubscript{3}R protein. Microsomes prepared 
from COS-1 cells transfected with sheared SS DNA re-
vealed no immunoreactive endogenous receptor protein 
(Fig. 1 C). Extended exposures of the SS DNA 
Western blots revealed only low levels of immunoreactive 
protein (data not shown). Microsomes (10 μg protein) 
from cells expressing the pInsP\textsubscript{3}R-T1, pInsP\textsubscript{3}R-A1-4, 
and pInsP\textsubscript{3}R-A5-6 plasmids were Western blotted with antibodies directed against the NH\textsubscript{2} and COOH termi-
ni of the receptor. Blots performed with the COOH-
terminus antibody are shown in Fig. 1 C. These data 
dicate that the expressed InsP\textsubscript{3}R proteins were of the

**Figure 1.** Construction and expression of the type 1 (SI \(-/\) SII+) 
InsP\textsubscript{3}R receptor membrane spanning domain deletion plasmids. 
(A) Schematic representation of the constructions used in this 
study. Membrane spanning region deletions pInsP\textsubscript{3}R-A5-6 and 
pInsP\textsubscript{3}R-A1-4 are illustrated below the full length receptor (InsP\textsubscript{3}R-T1). Deleted residues in pInsP\textsubscript{3}R-A5-6 and pInsP\textsubscript{3}R-A1-4 (residues 
2398-2589 and 2211-2416, respectively) are indicated as un-
shaded regions. Vertical bars represent the membrane spanning 
domains. (B) The 150 amino acids bounded by the fifth and sixth 
TMRs of the type 1 InsP\textsubscript{3}R are aligned with the RyR2 sequence. 
The fifth and sixth InsP\textsubscript{3}R TMRs are boxed. Identical residues are 
shaded. Marked residues indicate identity between all three InsP\textsubscript{3}R 
isofoms and RyR2. (C) Western immunoblot of microsomal protein 
(10 μg, all lanes) from COS-1 cells transiently transfected with 
control SS DNA, pInsP\textsubscript{3}R-A5-6, pInsP\textsubscript{3}R-A1-4, and the full-length 
type 1 receptor (InsP\textsubscript{3}R-T1). The Western blot was probed with 
a type 1 specific carboxyl-terminal antipeptide antibody (Ramos-
Franco et al., 1998b), and immunoreactive protein was detected 
using chemiluminescence reagents (Amersham Life Sciences, 
Inc.). Similar results were observed using a type 1 amino-terminal 
antibody (data not shown).
studies (Ramos-Franco et al., 1998b). Thus, proteoliposomes prepared from transfected COS-1 cells contain predominantly recombinant protein. These proteoliposomes can then be reconstituted into planar lipid bilayers to define the single channel properties of the mutant InsP3R channels. This strategy to define the function of recombinant InsP3R channels has been successfully applied by two laboratories (Kaznacheyeva et al., 1998; Ramos-Franco et al., 1998b).

**InsP3 Binding of the Type 1 InsP3R TMR-deletion Mutants**

Equilibrium InsP3 binding assays were performed using microsomal proteins from transfected COS-1 cells (Table I). The full-length recombinant receptor (pInsP3R-T1) and both TMR-deletion mutants (pInsP3RΔ1-4 and pInsP3RΔ5-6) bind significant amounts of InsP3. The SS DNA control microsomes did not bind InsP3 at significant levels above nonspecific background. The amount of InsP3 bound was normalized to the relative protein expression of each InsP3R construct by densitometry. 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 [3H]InsP3 (Mignery et al., 1990). In each case, the level of InsP3 binding was reduced in the presence of heparin or unlabeled InsP3. These data indicate that the expressed TMR-deletion mutant proteins are functional in terms of InsP3 binding.

**Single Channel Properties of the Type 1 InsP3R TMR-deletion Mutants**

The TMR-deletion mutant InsP3R receptor proteins were incorporated into proteoliposomes for fusion into planar lipid bilayer studies. Microsomes from COS-1 cells transfected with either pInsP3R-T1, pInsP3RΔ1-4, pInsP3RΔ5-6, or control SS DNA were solubilized in CHAPS detergent and sedimented over 5-20% sucrose density gradients. The tetrameric receptor complex (i.e., the channel complex) migrates to a position on the gradient beyond the majority of other proteins (Mignery and Südhof, 1990). Its position in the gradient was detected by Western immunoblotting and fractions containing the highest levels of recombinant receptor reconstituted into l-α-phosphatidylcholine and l-α-phosphatidylserine containing liposomes as described previously (Perez et al., 1997; Ramos-Franco et al., 1998b).

No detectable InsP3/heparin-sensitive Cs+ conducting channels were incorporated into the bilayer after fusion of proteoliposomes containing gradient receptor fractions from nontransfected COS-1 cells, control (SS DNA)-transfected cells, or pInsP3RΔ5-6-transfected cells. Incorporation of proteoliposomes containing the pInsP3RΔ1-4 construct resulted in the appearance of a high conductance (~300 pS) ion channel with high open probability (>0.80). Sample single channel activity from the pInsP3RΔ1-4 channel is shown in Fig. 2. The pInsP3RΔ1-4 channel was nearly always open with frequent and usually brief (~1 ms) flickers to the close state. Long closed events (>20 ms) were rare. Sample channel activity was observed in the presence (Fig. 2A) and absence (Fig. 2B) of InsP3. Single channel activity was also not impacted by the addition of 10 μM ryanodine or 50 μg/ml heparin (Fig. 2C). Corresponding total amplitude histograms under each experimental condition are also presented in Fig. 2. The channel was open most of the time with frequent but brief transitions to the closed state. Thus, these data suggest that the pore formed by the pInsP3RΔ1-4 protein was not modulated by agents (i.e., InsP3 and heparin) that modulate function of wild-type InsP3R channels. Additionally, the pInsP3RΔ1-4 pore was constitutively open (i.e., high P o, n = 6). Under optimal experimental conditions, the P o of full-length type 1 InsP3R channels is relatively low (Bezprozvanny et al., 1991; Ramos-Franco et al., 1998a,b). The high P o and absence of channel regulation by InsP3 and heparin indicates that the channel activity observed is not due to endogenous InsP3R channels. The absence of ryanodine action indicates that it is not due to endogenous RyR channels.

The permeation properties of the pInsP3RΔ1-4 pore were also defined. Stationary single channel activity was recorded for extended periods (~5 min) at several different membrane potentials. The unitary current amplitude (Cs+ charge carrier) was measured as a function of membrane potential. Sample single channel records at different membrane potential (0, 20, and 40 mV) are shown in Fig. 3 A. A sustained high P o was a
The unitary Cs\(^+\) current carried by the \(\text{pInsP}_3\text{R}\Delta 1-4\) pore was Ohmic with a slope conductance of 284 pS \((n = 9)\). Unitary \(\text{Ca}^{2+}\) current reversed at \(-22\) mV, indicating that the \(\text{pInsP}_3\text{R}\Delta 1-4\) pore was cation selective (Fig. 3 B). The unitary \(\text{Ca}^{2+}\) current was also Ohmic at relatively large negative membrane potentials, with a slope conductance of 60 pS \((n = 7)\). The selectivity of the channel was probed under bilionic conditions (Fig. 3 D). In brief, 30 mM \(\text{Ca}^{2+}\) was applied to one side of the channel and 30 mM \(\text{Cs}^{+}\) was applied to the other. The selectivity between \(\text{Ca}^{2+}\) and \(\text{Cs}^{+}\) can then be calculated from the reversal potential (see methods). The reversal potential was near +40 mV \((n = 10)\), indicating the \(\text{pInsP}_3\text{R}\Delta 1-4\) pore was \(\text{Ca}^{2+}\) selective \((P_{\text{Ca}}/P_{\text{Cs}} \approx 6.3)\). Thus, the \(\text{pInsP}_3\text{R}\Delta 1-4\) protein forms a high conductance and \(\text{Ca}^{2+}\) selective pore.

**discussion**

The principal functional attribute of the \(\text{InsP}_3\text{R}\) is its capacity to operate as an intracellular \(\text{Ca}^{2+}\) release channel. The permeation and \(\text{InsP}_3\) regulation of the native type 1 \(\text{InsP}_3\text{R}\) pore have been defined in bilayer studies (Bezprozvanny and Ehrlich, 1994). The permeation and regulatory properties of the native and recombinant \(\text{InsP}_3\text{R}\) channels are comparable (Kaznatcheyeva et al., 1998; Ramos-Franco et al., 1998a,b). The \(\text{InsP}_3\text{R}\) is a high conductance, poorly selective \(\text{Ca}^{2+}\) channel. It is activated by \(\text{InsP}_3\) (1 μM) and blocked by heparin (Bezprozvanny and Ehrlich, 1994; Hagar et al., 1998; Ramos-Franco et al., 1998a). In the presence of 1 μM \(\text{InsP}_3\) (250 nM \(\text{Ca}^{2+}\)), the native type 1 \(\text{InsP}_3\text{R}\) channel has a relatively low open probability (φ ~0.15; Bezprozvanny et al., 1991). The \(\text{InsP}_3\text{R}\) channels are permeable to a variety of monovalent (e.g., \(\text{K}^{+}\), \(\text{Na}^{+}\), and \(\text{Cs}^{+}\)) and divalent cations (e.g., \(\text{Ca}^{2+}\), \(\text{Ba}^{2+}\), and \(\text{Mg}^{2+}\)). The main conductance is near 300 pS for monovalent ions and ~60–80 pS for divalent cations (Hagar et al., 1998; Ramos-Franco et al., 1998a). The channel is also remarkable for its relatively poor selectivity. The estimated permeability ratio (divalent/monovalent) of the \(\text{InsP}_3\text{R}\) channel pore is near 6 (Bezprozvanny and Ehrlich, 1994). Surface membrane channels (e.g., L-type \(\text{Ca}^{2+}\) channel) typically have \(P_{\text{DIVALENT}}/P_{\text{MONOVALENT}} > 1,000\) (Lee and Tsien, 1982). The high conductance and poor selectivity of the \(\text{InsP}_3\text{R}\) channel is similar to that of the \(\text{RyR}\) \(\text{Ca}^{2+}\) release channel (Tinker and Williams, 1992; Bezprozvanny and Ehrlich, 1994). This is interesting because the transmembrane regions of the \(\text{InsP}_3\text{R}\) and \(\text{RyR}\) share significant (~40%) primary cDNA sequence homology (Mignery et al., 1989). Thus, the structural determinants defining the ion permeation pathway may be similar in the \(\text{InsP}_3\text{R}\) and \(\text{RyR}\) channels.

The original analysis of the \(\text{InsP}_3\text{R}\) cDNA suggested
the existence of a channel-forming domain near the COOH terminus of the protein (Mignery et al., 1989). This suggestion was based on hydropathy and sequence homology to the RyR protein. It is also clear that the InsP3R protein oligomerizes (i.e., tetramerizes) to form the functional Ca\textsuperscript{2+} release channel entity (Mignery et al., 1989; Sayers et al., 1997) and that the TMRs are involved in targeting and stabilizing the oligomer (Mignery and Südhof, 1990; Galvan, D., E. Borrego-Diaz, P.J. Perez, and G.A. Mignery, manuscript submitted for publication). Channel assembly is thought to be a multideterminant process involving interplay between the TMRs and the COOH terminus. Two studies have suggested that the fifth and sixth TMRs are key elements that stabilize the InsP3R tetramer (Joseph et al., 1997; Galvan, D., E. Borrego-Diaz, P.J. Perez, and G.A. Mignery, manuscript submitted for publication). The loop that links the fifth and sixth TMRs has been proposed to be analogous to the H loop of voltage-activated Ca\textsuperscript{2+}, Na\textsuperscript{+}, and K\textsuperscript{+} channels (Mignery and Südhof, 1993). A similar suggestion has been made for the corresponding sequence of the RyR protein (Balshaw et al., 1999). Balshaw et al. (1999) proposed that the region of the RyR protein bounded by its two most COOH-terminal TMRs contains a pore-forming segment analogous to the H loop. Point mutations in this region of RyR1 are known to modify channel function (Gao et al., 1999; Lynch et al., 1999). The fifth and sixth TMRs of the InsP3R may also contain a putative leucine zipper motif. The presence of a leucine zipper could confer a degree of structural rigidity that may be important in stabilizing a pore through coiled-coil interactions (Simmerman et al., 1996). Thus, it is reasonable to hypothesize that the fifth and sixth TMRs (and the intervening luminal loop) are the most likely region of the InsP3R to form the Ca\textsuperscript{2+}-selective pore.

Deletion of the sequence bounded by the fifth and sixth TMRs (i.e., the pInsP3RΔ5-6 mutant) did not form detectable Ca\textsuperscript{2+} channels. However, this mutant protein did occasionally (~15% of attempts) induce a very small, sustained nonspecific leak current. The leak current reversed at 0 mV and no clear opening or closing events were observed. Thus, the leak current was not attributed to the opening and closing of an ion channel. It is more likely that this leak current was due to destabilization of the bilayer after incorporation of integral non–channel-forming protein. A similar leak current was observed in a previous study with our pInsP3RΔT1ALT construct (Ramos-Franco et al., 1998b). This type 1 InsP3R construct codes a truncated protein...
missing the 310 amino-terminal amino acids of the InsP₃ binding domain. This is interesting because the plnSP₃RΔ1ALT construct contains all six TMRs. The implication is that the plnSP₃RΔ1ALT mutant formed a constitutively closed channel, while the plnSP₃RΔ5-6 mutant formed a constitutively open Ca²⁺ release channel.

Deletion of the first four TMRs (i.e., the plnSP₃RΔ1-4 mutant) did form high conductance fast gating ion channels. Control experiments with SS cDNA–transfected cells indicated that the appearance of these channels was not due to some endogenous COS-1 cell protein or factor. The activity of the plnSP₃RΔ1-4 channel was not modified by the addition of InsP₃ or heparin. This is interesting because the protein binds InsP₃, and this binding is blocked by heparin. In the absence of pharmacological tools, channel identity was thus confirmed by its permeation profile. The plnSP₃RΔ1-4 channel was permeable to both monovalent (i.e., Cs⁺) and divalent (Ca²⁺) cations. The Cs⁺ and Ca²⁺ conductances were ~280 and 60 pS, respectively. The channel was cation selective, with a Ca²⁺/Cs⁺ permeability ratio of 6.3. These values match those described for the wild-type InsP₃R channel (Bezprozvanny and Ehrlich, 1994). This suggests that amino acid residues 2398–2589 (i.e., fifth and sixth TMR and interceding loop) contains key determinants of the InsP₃R’s permeation pathway.

The absence of InsP₃ regulation despite InsP₃ binding suggests that the deleted sequence (TMRs 1–4, residues 2211–2416) may couple binding to channel gating. Alternatively, the missing sequence may annul ligand regulation by sterically limiting pore structure. For example, removing surrounding TMRs could energetically restrict molecular motions in pore structure needed for normal ligand regulation. Such restricted molecular motion could be manifested as a constitutively open pore.

In summary, this study has localized the InsP₃R pore to a region of 191 amino acids near the COOH terminus of the protein. This region includes the fifth and sixth TMR and interceding loop. We suggest that a putative leucine zipper may infer the structural integrity needed to form a stable pore. A sequence alignment between the RyR and the InsP₃R pore-forming region reveals potential “hot spots” for future mutagenesis studies. These hot spots include the valine/iso-leucine residues (i.e., the β-branched amino acids) in the fifth and sixth TMR and the cluster of conserved glycines in the fifth to sixth TMR loop.

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References

Baudet, S., L. Hove-Madsen, and D.M. Bers. 1994. How to make and use calcium-specific mini- and microelectrodes. Methods Cell Biol. 40:9–114.

Balshaw, D., L. Gao, and G. Meissner. 1999. Luminal loop of the ryanodine receptor: a pore-forming segment? Prog. Natl. Acad. Sci. USA. 96:3345–3347.

Bezprozvanny, I., J. Watras, and B.E. Ehrlich. 1991. Bell-shaped calcium-response curves of Ins(1,4,5)P₃ and calcium-gated channels from endoplasmic reticulum of cerebellum. Nature. 351:751–754.

Bezprozvanny, I., and B.E. Ehrlich. 1994. InsP₃ receptor: functional properties and regulation. In Handbook of Membrane Channels. C. Peracchia, editor. Academic Press, NY, NY. 511–526.

Bezprozvanny, I., and B.E. Ehrlich. 1995. The inositol 1,4,5-trisphosphate (InsP₃) receptor. J. Membr. Biol. 145:205–216.

De Smedt, H., L. Misiaen, J.B. Parys, R.H. Henning, I. Sienaert, S. Vanlingen, A. Gijsen, B. Himpens, and R. casteels. 1997. Isoform diversity of the inositol 1,4,5-trisphosphate receptor in cell types of mouse origin. Biochem. J. 322:575–583.

Gao, L., A. Tripathy, L. Xe, D. Paек, D. Balshaw, C. Xin, and G. Meissner. 1999. Mutation of charged amino acids in a putative luminal loop of the skeletal muscle Ca²⁺ release channel results in the loss of high affinity ryanodine binding. Biophys. J. 76:A303.

Gorman, C., 1985. DNA Cloning. Vol. II. D.M. Glover, editor. IRL Press, Oxford, UK. 143–190.

Hagar, R.E., A.D. Burgstahler, M.H. Nathanson, and B.E. Ehrlich. 1998. Type-III InsP₃ receptor channel stays open in the presence of increased calcium. Nature. 396:81–84.

Joseph, S.K. 1996. The inositol trisphosphate receptor family. Cell Signal. 8:1–7.

Joseph, S.K., D. Boehning, S. Pierson, and C.V. Nicchitta. 1997. Membrane insertion, glycosylation, and oligomerization of inositol trisphosphate receptors in a cell-free translation system. J. Biol. Chem. 272:1579–1588.

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

Lee, K.S., and R.W. Tisen. 1992. Reversal of current through Ca²⁺ channels in dialyzed single heart cells. Nature. 297:498–501.

Lynch, P.J., J. Tong, M. Lehane, A. Mallet, L. Giblin, J.J.A. Heffron, P. Vaughan, G. Zafra, D.H. MacLennan, and T.V. McCarthy. 1999. A mutation in the transmembrane/luminal domain of the ryanodine receptor is associated with abnormal Ca²⁺ release channel function and severe central core disease. Proc. Natl. Acad. Sci. USA. 96:4164–4169.

Michikawa, T., H. Hamanaka, H. Otusu, A. Yamamoto, A. Miyawaki, T. Furuichi, Y. Tashiro, and K. Micoshiba. 1994. Transmembrane topology and sites of glycosylation of the inositol trisphosphate receptor. J. Biol. Chem. 269:9184–9189.
Mignery, G.A., T.C. Südhof, K. Takei, and P. DeCamilli. 1989. Putative receptor for inositol 1,4,5-trisphosphate similar to ryanodine receptor. Nature. 342:192–195.

Mignery, G.A., and T.C. Südhof. 1990. The ligand-binding site and transduction mechanism in the inositol-1,4,5-trisphosphate receptor. EMBO (Eur. Mol. Biol. Organ.) J. 9:3893–3898.

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

Mignery, G.A., and T.C. Südhof. 1993. Molecular analysis of inositol 1,4,5-trisphosphate receptors. Methods Neurosci. 18:247–265.

Nakanishi, S., A. Fujii, S. Nakade, and K. Mikoshiba. 1996. Immunohistochemical localization of inositol 1,4,5-trisphosphate receptors in non-neural tissues, with special reference to epithelia, the reproductive system and muscular tissues. Cyt Tissue Res. 285:235–251.

Newton, C.L., G.A. Mignery, and T.C. Südhof. 1994. Co-expression in vertebrate tissues and cell lines of multiple inositol 1,4,5-trisphosphate (InsP₃) receptors with distinct affinities for InsP₃. J. Biol. Chem. 269:28613–28619.

Perez, P.J., J. Ramos-Franco, M. Fill, and G.A. Mignery. 1997. Identification and functional reconstitution of the type-2 InsP₃ receptor from ventricular cardiac myocytes. J. Biol. Chem. 272:23961–23969.

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

Ramos-Franco, J., M. Fill, and G.A. Mignery. 1998b. Single channel function of recombinant type-1 inositol 1,4,5-trisphosphate receptor ligand binding domain splice variants. Biophys. J. 75:2783–2793.

Sayers, L.G., A. Miyawaki, A. Muto, H. Takeshita, A. Yamamoto, T. Michikawa, T. Furuichi, and K. Mikoshiba. 1997. Intracellular targeting and homotetramer targeting of a truncated inositol 1,4,5-trisphosphate receptor-green fluorescent protein chimera in Xenopus laevis oocytes: evidence for the involvement of the membrane spanning domain in endoplasmic reticulum targeting and homotetramer complex formation. Biochem. J. 323:273–280.

Simmerman, H.K.B., Y.M. Kobayashi, J.M. Autry, and L.R. Jones. 1996. A leucine zipper stabilizes the pentameric membrane domain of phospholamban and forms a coiled-coil pore structure. J. Biol. Chem. 271:5941–5946.

Südhof, T.C., C.L. Newton, B.T. Archer, Y.A. Ushkaryov, and G.A. Mignery. 1991. The structure of a novel InsP₃ receptor. EMBO (Eur. Mol. Biol. Organ.) J. 10:3199–3206.

Tinker, A., and A.J. Williams. 1992. Divalent cation conduction in the ryanodine receptor of sheep cardiac muscle sarcoplasmic reticulum. J. Gen. Physiol. 100:479–493.