Critical Regions for Activation Gating of
Inositol 1,4,5-Trisphosphate Receptor

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Abbreviations used:

IP$_3$, inositol 1,4,5-trisphosphate; IP$_3$R, inositol 1,4,5-trisphosphate receptor; Kd, equilibrium dissociation constant; EC$_{50}$, half-maximally effective concentration; Ca$^{2+}$, calcium; IICR, IP$_3$-induced Ca$^{2+}$ Release

Running title:

Critical regions for IP$_3$R gating
Summary

To understand the molecular mechanism of ligand-induced gating of the inositol 1,4,5-trisphosphate (IP₃) receptor (IP₃R)/Ca²⁺ release channel, we analyzed the channel properties of deletion mutants that retained both the IP₃-binding and channel-forming domains of type 1 IP₃R (IP₃R1). Using intrinsically IP₃R-deficient cells as the host cells for the receptor expression, we determined that six of the mutants, those lacking amino acid residues 1-223, 651-1130, 1267-2110, 1845-2042, 1845-2216, or 2610-2748, did not exhibit any measurable Ca²⁺-release activity, whereas the mutants lacking residues 1131-1379 or 2736-2749 retained the activity. Limited trypsin digestion showed that not only the IP₃-gated Ca²⁺-permeable mutants lacking amino acid residues 1131-1379 or 2736-2749, but two non-functional mutants lacking residues 1-223 or 651-1130 retain the normal folding structure of at least the C-terminal channel-forming domain. These results indicate that two regions of type 1 IP₃R, namely, amino acid residues 1-223 and 651-1130, are critical for the IP₃-induced gating. We also identified a highly conserved cysteine residue at position 2613, which is located within the C-terminal tail, as being essential for the channel opening. Based on these results, we propose a novel five-domain structure model in which both N-terminal and internal coupling domains transduce ligand binding signals to the C-terminal tail, which acts as a gatekeeper that triggers opening of the activation gate of IP₃R1 following IP₃ binding.
Introduction

The inositol 1,4,5-trisphosphate (IP₃) is a second messenger that is produced by hydrolysis of phosphatidyl-inositol 4,5-bisphosphate in response to activation by extracellular stimuli of the G protein- or tyrosine-kinase-coupled receptors on the plasma membrane in various cell types (1). IP₃ mediates the release of Ca²⁺ from intracellular storage sites, such as the endoplasmic reticulum, by binding to the IP₃ receptor (IP₃R)/Ca²⁺ release channel. IP₃-induced Ca²⁺ release (IICR) regulates numerous physiological processes, including fertilization, cellular proliferation, development, muscle contraction, secretion, learning and memory. In this signal transduction pathway, the IP₃R works as a switch that converts the information carried by extracellular stimuli into intracellular Ca²⁺ signals.

IP₃-gated intracellular Ca²⁺ release channels are composed of four IP₃R subunits (2). There are at least three types of IP₃Rs (IP₃R1, IP₃R2, and IP₃R3) (3), and they exist as both homo- and heterotetramers (4). The structure of IP₃Rs has traditionally been divided into three functional domains (3,5): the N-terminal ligand-binding domain, the modulatory/coupling domain, and the C-terminal transmembrane/channel-forming domain which contains six putative membrane-spanning regions. The transmembrane region is required for the intermolecular interaction in the formation of a tetrameric complex (6-9), and it is likely that the C-terminal cytoplasmic region just following the putative membrane-spanning regions has a supportive role in the association among the subunits (6,9). An ion conduction pore has been proposed to be located in the hydrophobic segment between the fifth and sixth transmembrane regions (10,11). The primary sequence of the transmembrane domain adjacent to the pore-forming segment is highly homologous with that of the ryanodine receptor (RyR), another type of intracellular Ca²⁺ release channel, suggesting that these two channels might share a common structure for the conduction of Ca²⁺ ions.

Each IP₃R subunit has a single high-affinity IP₃-binding site (2). The IP₃-binding core, a minimum essential region for specific IP₃-binding (12), resides among residues 226-578 of
mouse IP₃R₁ (2749 amino acids) (13), and it contains eleven essential basic amino acids for IP₃ binding (14). The N-terminal 225 amino acid residues, which are close to the IP₃-binding core, have been thought to function as a suppressor for IP₃-binding, because deletion of the N-terminal 225 amino acids from the N-terminal 734 amino acid region (T734) results in significant enhancement of IP₃ binding activity (12,15). IIICR is a positively cooperative process (16–18), that is, the binding of at least two IP₃ molecules to a single tetrameric IP₃R channel is required for channel opening. IP₃ binding elicits a large conformational change in the N-terminal cytoplasmic portion of IP₃R (19). Furthermore, the C-terminal cytoplasmic region following the transmembrane domain is thought to be involved in the IP₃-induced gating of the receptor, because the monoclonal antibody (mAb) 18A10, whose epitope is located in the C-terminal portion of mouse IP₃R₁ (13,20,21), has an inhibitory effect on IIICR, without causing any decrease in the affinity of the receptor for IP₃ (21). Controlled trypsinization induced fragmentation of the mouse IP₃R₁ into five major fragments, and all of the four N-terminal cytoplasmic fragments, which contain the IP₃-binding core, were associated directly or indirectly with the other remaining C-terminal fragment which contains the channel domain (22). The trypsinized IP₃R retained significant IIICR activity, indicating that intramolecular interaction within a subunit and/or intermolecular interaction between neighbouring subunits could effect functional coupling between IP₃ binding and channel opening (22). The sites of the interfaces between the cytoplasmic fragments and the channel domain and the molecular mechanism of their coupling, however, remain to be elucidated.

IIICR has been shown to occur in a quantal manner in permeabilized cells and isolated ER membranes (23,24). Addition of submaximal concentrations of IP₃ in the presence of Ca²⁺-pump inhibitors leads to the partial release of sequestered Ca²⁺, and the amount of released Ca²⁺ varies with the concentration of IP₃ (24). Although the Ca²⁺ release terminates abruptly, since it can be reinitiated by an additional increment in IP₃ concentration (24), the rapid termination of Ca²⁺ release is not due to ordinary inactivation or desensitization of the receptor. Purified IP₃Rs reconstituted into lipid vesicles revealed a
quantal Ca\textsuperscript{2+} flux (17,25), indicating that the quantal release of Ca\textsuperscript{2+} is an intrinsic property of the IP\textsubscript{3}R. Similar behavior was observed for RyR which mediates Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release from intracellular Ca\textsuperscript{2+} stores (26), but has not been observed for other ligand-gated ion channels on the plasma membrane, suggesting that the quantal release is a fundamental and unique property of the intracellular Ca\textsuperscript{2+} release channels.

To understand the molecular basis of the ligand-induced gating of IP\textsubscript{3}R, we analyzed a series of internal deletion mutants and site-directed mutants of mouse IP\textsubscript{3}R1 expressed in intrinsically IP\textsubscript{3}R-deficient R23·11 cells (27). We found that at least two regions and a cysteine residue are essential for IP\textsubscript{3}-dependent gating of IP\textsubscript{3}R1. These findings provide us with new insight into the gating mechanism of IP\textsubscript{3}R.
Experimental Procedures

Plasmid constructions

For transfection of wild-type mouse IP₃R1 cDNA, pBact-STneoB-C1 (28) was used. Seven deletion-mutant cDNAs of mouse IP₃R1, D651-1130, D1131-1379, D1267-2110, D1692-1731, D1845-2042, D1845-2216, and D2610-2748 (6), were subcloned into pAneo (27) at SalI sites. To construct D1-223, a XhoI site was introduced at nucleotide 998 of mIP₃R1 by PCR using GFP-IP₃R-D223 (Tateishi et al., unpublished data) as template DNA. A XhoI-KpnI fragment isolated from GFP-IP₃R-D223 was ligated to a SalI-KpnI fragment of pBact-STneoB-C1. The resultant plasmid pBact-STneoB-D1-223 uses nucleotides 998-1000, which correspond to an intrinsic methionine residue at position 224, as a start codon (ATG) for transcription. To construct D2736-2749, a KpnI-XhoI fragment isolated from EGFP-IP₃R/Δ18A10 (29) was ligated to a KpnI-SalI fragment of pBact-STneoB-C1. To substitute serine for cysteine residues at positions 1976, 2610 and 2613, or both 2610 and 2613, of mouse IP₃R1, site-directed mutagenesis was performed with the MutanK kit (Takara) using primers containing the appropriate substitutions (5’-GTGGTTTTCAGACAGCAGCTG-3’ for the nucleotides 6244-6264, 5’-CAGATGAAGCTCGTGGTTTTT-3’ for the nucleotides 8146-8167, 5’-TTCCAAGCCGGAGATGAAGCA-3’ for the nucleotides 8156-8176, and 5’-AAGCCGGAGATGAAGCTCGTGGT-3’ for the nucleotides 8150-8172). The EcoRI fragment from the EcoRI site (6646-nucleotide position) internal to the 3’ end of the mouse IP₃R1 isolated from pBactS-C1 (13) was subcloned into pBluescript SK (+) and used as template DNA. After the mutated EcoRI fragments were put back into pBactS-C1, the mutated cDNAs were subcloned into pBact-STneoB (28) at the SalI sites. All PCR products and mutations were confirmed by DNA sequencing.

Generation of wild-type and deletion-mutant-expressing cell lines

R23·11 cells (27) were cultured in RPMI 1640 supplemented with 10% fetal calf
serum, 1% chicken serum, 50 µM 2-mercaptoethanol, 4 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin at 39.5°C in 5% CO₂. Expression plasmids were linearized and transfected into R23-11 cells by electroporation, as previously described (27), or lipofection (Effectene, Qiagen) (Miyauchi et al. manuscript in preparation). Several stable clones were selected in medium containing 2 mg/ml G418 (Sigma), about 7-10 days after the transfection. Expressions of the IP₃R and its mutants were confirmed by immunoblotting with the mAbs 4C11 and/or 18A10, using cell lysates boiled in SDS-PAGE sample buffer (5 mM EDTA, 50 mM Tris·HCl, pH 6.8, 100 mM DTT, 2% SDS, 10% glycerol). The immunoblot analysis was performed as described previously (22).

**Preparation of membrane fractions from stable cells expressing mouse IP₃R1 and their mutants**

Membrane fractions were prepared in accordance with the protocol for mouse cerebella described by Michikawa T. et al (18), with minor modification. Cells were collected by centrifugation, washed twice with cold PBS, and homogenized in ice-cold homogenizing buffer (5 mM NaN₃, 0.1 mM EGTA, 1 mM 2-mercaptoethanol, and 20 mM, HEPES·NaOH, pH 7.4) containing protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 10 µM leupeptin, 10 µM pepstatin A and 10 µM E64) by 40 strokes in a chilled glass-Teflon Potter homogenizer at 1,000 rpm. The homogenate was centrifuged at 1,100 x g for 10 min at 2°C. The supernatant was centrifuged at 100,000 x g in a Beckman TLA100.3 rotor for 30 min at 2°C. The pellet was resuspended in an appropriate volume of the wash buffer (600 mM KCl, 5 mM NaN₃, 20 mM Na₄P₂O₅, 1 mM 2-mercaptoethanol, 10 mM HEPES·HCl, pH 7.2) containing protease inhibitors. The suspension was centrifuged at 1,100 x g for 10 min, and the supernatant was centrifuged at 63,000 x g for 30 min at 2°C. The pellet was finally suspended in an appropriate volume of Ca²⁺ release buffer (110 mM KCl, 10mM NaCl, 5 mM KH₂PO₄, 1 mM 2-mercaptoethanol, 50 mM HEPES·KOH, pH 7.2), containing protease inhibitors to obtain a final concentration of about 15 mg/ml protein. The Ca²⁺ release buffer was passed over CHELEX100 (Bio-Rad) to eliminate any extra free Ca²⁺ before use. The
membrane fractions were either used immediately, or frozen in liquid nitrogen and stored at -80°C until use.

\[ ^{[3]} \text{H} \]IP$_3$ binding assay using the membrane fractions

The IP$_3$ binding assay was performed as described previously (6). The membrane fractions (50 – 200 µg/tube) were incubated with 9.6 nM \[^{3}H\]IP$_3$ (Perkin Elmer) in 100 µl of binding buffer (50 mM Tris·HCl, pH 8.0, 1 mM EGTA, 1 mM 2-mercaptoethanol) for 10 min at 4 °C. After centrifugation, the pellets were dissolved in Solvable (Perkin Elmer) and the radioactivities were measured with a scintillation counter (Beckman LS6500). Nonspecific binding was measured in the presence of 10 µM unlabelled IP$_3$ (Dojindo).

IICR assay for the membrane fractions

The membrane fractions were suspended in the Ca$^{2+}$ release buffer supplemented with 1 µg/ml oligomycin (Sigma), 2 mM MgCl$_2$, 25 µg/ml creatine kinase (Roche), 10 mM creatine phosphate (Sigma) and 2 µM Fura-2 (Molecular Probes) and used at the concentration of 200-300 µg protein/ml. Fluorescence was recorded at 510 nm with alternate excitation of 340 and 380 nm (F340 and F380, respectively). Using the spectrofluorometer CAF-110 (Japan Spectroscopic Co.), signals were recorded every 0.01 second with MacLab (ver. 3.6, ADInstruments) at 30°C. When the Ca$^{2+}$ uptake induced by the addition of 1 mM ATP reached a steady level, 2 µM thapsigargin was added to eliminate active Ca$^{2+}$ uptake through intrinsic Ca$^{2+}$ pumps. The rate of leakage from the membrane fractions following the addition of thapsigargin was almost linear. When the ratio of fluorescence intensity (F340/F380) reached 1.2, corresponding to approximately 170 nM of free Ca$^{2+}$, various concentrations of IP$_3$ were added. At the end of each experiment, 2 mM CaCl$_2$ and 10 mM EGTA were added successively for normalization and calibration (30).

Limited trypsin digestion of mutant receptors

Limited trypsin digestion was performed as described previously (22). Microsomal
fractions (0.25–5 mg/ml) of wild-type and mutant IP₃R1-expressing cells were incubated with 0.01 – 10 µg/ml trypsin in trypsinizing buffer (120 mM KCl, 1 mM EDTA, 1 mM 2-mercaptoethanol, 20 mM Tris·HCl, pH 8.0) at 35°C for 10 min. The reaction was terminated by the addition of 50 µg/ml soybean trypsin inhibitor (Sigma) and 0.1 mM PMSF. After addition of an equal volume of SDS-PAGE sample buffer, reaction mixtures were incubated at 55°C for 30 min. The digested proteins were separated on 8% SDS-PAGE and then analyzed by Western blot with anti-IP₃R1 antibodies N1, 10A6, anti(1718-31), 1ML1, and 18A10 (Fig. 1A) (22).
Results

Expression of deletion mutants of IP$_3$R1 in intrinsically IP$_3$R-deficient R23·11 cells

As previously reported, we constructed 17 internal deletion mutants of the mouse type1 IP$_3$R (6). Among these mutants, we selected 7 (Fig. 1B) that contained both the IP$_3$·binding region (amino acid residues 226-578) and the putative transmembrane domain (amino acid residues 2276-2589), in order to investigate the critical regions for the coupling between ligand binding and channel opening. In addition, we constructed two mutants lacking amino acid residues 1-223 and 2736-2749, respectively (Fig. 1B). To express these mutant receptors, we introduced the mutant cDNAs into R23·11 cells, and established stable cell lines by selection with 2 mg/ml of G418. Fig. 2A illustrates the Western blot analysis of the membrane fractions prepared from these stable cell lines using the anti-IP$_3$R1 polyclonal antibody 1ML1, whose epitope lies within amino acid residues 2504-2523 of the IP$_3$R1 (Fig. 1A) (10). All mutant receptors except D2610·2748 were detected with an appropriate molecular weight (Fig. 2A). Since D2610·2748 was not well recognized by the antibody 1ML1, we confirmed the expression of D2610·2748 by Western blot analysis using anti-IP$_3$R1 mAb 4C11 (20). As shown in Fig. 2B, an additional signal with a low molecular weight was detected (open triangle), indicating that degradation (or truncation) of D2610·2748 occurs in R23·11 cells.

$[^3]$H IP$_3$·binding activities of the deletion-mutant IP$_3$Rs

The IP$_3$·binding activities of the internal deletion mutant receptors expressed in R23·11 cells were measured by the equilibrium $[^3]$HIP$_3$·binding analysis as described previously (6). There was no significant IP$_3$·binding activity in the membrane fraction obtained from the R23·11 cells (data not shown). Therefore we measured the ligand·binding activity of the exogenously expressed IP$_3$R using membrane fractions obtained from the stable cell lines. The IP$_3$·binding properties of wild-type and deletion-mutant IP$_3$Rs are summarized in Table 1. Wild-type IP$_3$R1 expressed in R23·11 cells showed a single,
high-affinity IP$_3$-binding site with a dissociation constant of 20 ± 5 (n=3) nM. This value is close to that of the native IP$_3$R1 expressed in the mouse cerebellum (31), and the cDNA-derived IP$_3$R1 expressed in L cells (28), NG108-15 cells (6) and Sf9 cells (32). The mutant receptors D1131-1379, D1692-1731, D2610-2748, and D2736-2749 exhibited binding affinity similar to that of wild-type IP$_3$R1 (Table 1). IP$_3$-binding affinity of mutant receptors D1267-2110, D1845-2042, and D1845-2216 was 2-3 fold lower (Table 1), and mutant D651-1130 had 7.5-fold lower affinity for IP$_3$ (Table 1). Mutant D1-223 exhibited, however, significantly higher affinity for IP$_3$ (Table 1), consistent with the previous report showing that amino acid residues 1-223 act as a suppressor for IP$_3$-binding (12). It has been reported that IP$_3$ binding to IP$_3$R is not cooperative (31,33), and that the same property holds for wild-type and all of the mutant receptors except D1-223 expressed in R23-11 cells (Table 1). Both the Western blot (Fig. 2) and IP$_3$-binding analyses (Table 1) showed that the amount of the IP$_3$R protein expressed in each cell line was different. The amounts of the mutant IP$_3$Rs expressed were in the range of 1.5 – 9.2 pmol/mg protein (Table 1), and therefore we used two stable cell lines (KMN13 and KMN107; Miyauchi et al., manuscript in preparation) expressing different amounts of wild-type IP$_3$R1 as controls in the following experiments. The $B_{\text{max}}$ values of KMN13 and KMN107 were 13 ± 9 and 0.77 ± 0.2 pmol/mg protein, respectively (Table 1).

IICR activity of the wild-type and the deletion-mutant IP$_3$Rs

To investigate the Ca$^{2+}$ release activity of the mutant IP$_3$Rs, IICR from the membrane fractions prepared from each stable cell line was measured in the presence of the Ca$^{2+}$-pump inhibitor thapsigargin. No Ca$^{2+}$ release was observed from membrane fractions prepared from R23-11 cells even after the addition of 10 µM IP$_3$ (Fig. 3A), indicating that by using R23-11 cells as a host cell for transfection allows evaluation of definite Ca$^{2+}$ release activity by exogenously expressed IP$_3$Rs. Fig. 3B shows the time-course of the Ca$^{2+}$ release mediated by recombinant wild-type IP$_3$R1 after addition of various concentrations of IP$_3$. Both the rate and the amplitude of Ca$^{2+}$ release depended on the concentration of IP$_3$ added,
indicating that the IP₃R1 expressed in R23·11 cells exhibits the quantal Ca²⁺ release that is known to be an intrinsic property of native IP₃R1 (17,25). As previously reported (11), D1692-1731, which corresponds to the SII alternative splicing variant of IP₃R1 observed in peripheral tissues (34,35) exhibits Ca²⁺ release after addition of 10 µM IP₃ (Fig. 3A). Among the eight artificial mutants (D1-223, D651-1130, D1131-1379, D1267-2110, D1845-2042, D1845-2216, D2610-2748, and D2736-2749), only D1131-1379 and D2736-2749 possessed measurable Ca²⁺ release activity (Fig. 3A). Under the conditions employed, we could detect IICR from membrane fractions prepared from the low-amount-IP₃R1-expressing KMN 107 cells (Fig. 3A), that contained 0.77 ± 0.2 pmol of IP₃ binding sites per mg protein. The expression levels of all the internal deletion mutants in each stable cell line were higher than the expression level of wild-type IP₃R1 in KMN 107 cells (Table 1). These findings suggest that none of the mutants except D1131-1379 and D2736-2749 act as IP₃-gated Ca²⁺ release channels.

**Limited trypsin digestion of the mutant IP₃Rs**

Mouse cerebellar IP₃R1 is trypsinized into five major fragments (I-V) (Fig. 1A; (22)). Limited proteolysis provides direct evidence of protein folding (36). To probe the tertiary structure of the mutated IP₃Rs, we analyzed trypsinized fragments of the recombinant IP₃R1. Since R23·11 cells contain an intrinsic 4C11-reactive protein whose molecular weight is similar to that of tryptic fragment II of IP₃R1 (data not shown), we analyzed four tryptic fragments (I, III, IV, and V) of the recombinant receptors. As shown in Fig. 4A, the wild-type IP₃R1 expressed in R23·11 cells was digested into the same four fragments, indicating that the recombinant IP₃R1 retains native structure. We found that trypsin digestion of the Ca²⁺-releasing mutant D2736-2749 generated the same four trypsinized fragments (Fig. 4B), indicating that D2736-2749 folds in the same manner as the wild-type IP₃R1. Trypsinization of the other functional mutant, D1131-1379, also generated fragments IV and V (Fig. 4C). However, D1131-1379, which exhibited markedly decreased Ca²⁺ release activity (Fig. 3A), was digested with a much lower concentrations of trypsin (Fig. 4C). This difference in
trypsin sensitivity suggests that the deletion of amino acids 1131-1379 influences the structure of the C-terminal channel domain. Tryptic fragments of the three functionless mutants, D1-223, D651-1130, and D2610-2748, are shown in Fig. 4D, E, and F, respectively. Fragment III, IV, and V were generated by trypsin digestion of D1-223 (Fig. 4D). Trypsinization of D651-1130, which lacks a cleavage site between fragments II and III (22), generated fragments IV and V (Fig. 4E). Both D1-223 and D651-1130 exhibited trypsin sensitivity similar to that of wild-type IP₃R₁, suggesting that at least the C-terminal channel domains of these mutants fold correctly. By contrast, only fragments I and III were generated by trypsin digestion of D2610-2748 (Fig. 4F), and fragments IV (40 kD) and V (91 kD) were not detected (outlined arrows in Fig. 4F). These results indicate that deletion of amino acid residues 2610-2748 induced a significant distortion in the folding structure of the C-terminal channel domain of IP₃R₁.

Identification of the cysteine residue essential for IP₃-induced gating of the IP₃R₁

The C-terminal cytoplasmic region following the transmembrane domain has been found to be highly conserved in both the IP₃R and RyR families (13), indicating that this region is involved in the formation of a critical structure that is required for some common functions of the intracellular Ca²⁺ release channels. One of the remarkable features of this region is that it contains two cysteine residues which are conserved in all the intracellular Ca²⁺ release channels. Both of IP₃R and RyR channels are known to be modified by sulphydryl reagents (37-39), and therefore, we analyzed whether or not the two conserved cysteine residues, cysteine 2610 and cysteine 2613, are essential for the gating function in the mouse IP₃R₁. We generated three mutant receptors (C2610S, C2613S and C2610/2613S) in which cysteine 2610 and/or cysteine 2613 was replaced by serine (Fig. 1B). We also constructed a mutant receptor (C1976S) in which cysteine 1976 was replaced by serine (Fig. 1B). All of the mutated cDNAs were introduced into R23-11 cells, and stable cell lines expressing mutant receptors were established. Equilibrium [³H]IP₃ binding assay showed that all of the cysteine mutants bound IP₃ with similar affinities to that of wild-type IP₃R₁.
(Table 2). The expression levels of the mutant receptors were in the range of 1-10 pmol/mg protein (Table 2), and the amounts of all the mutant receptors expressed in each established cell line were higher than the expression level of wild-type IP₃R1 in KMN 107 cells.

We then examined the CA²⁺ channel activities of the mutant receptors by measuring IICR from the membrane fractions prepared from the stable cell lines. We found that the substitution of serine for cysteine 2610 and/or 2613 completely abolished the CA²⁺ release activity, whereas no such effect was apparent with the substitution of serine for cysteine 1976 (Fig. 6). Limited trypsin digestion of C1976S, C2613S, and C2610/2613S generated fragments I, III, IV, and V (solid arrows in Fig. 7A, C, and D), indicating that these mutants retain a normal structure. By contrast, trypsin digestion of C2610S generated fragments I, III, and IV (solid arrows in Fig. 7B), but not fragment V (open arrow in Fig. 7B), indicating that the single amino acid substitution at cysteine 2610 induced a significant structural alteration of the C-terminal channel domain of IP₃R1.
Discussion

Most cells, including mammalian cultured cells, express two, or all the three types of IP₃R (4,40). Thus, measurement of the actual channel activities of recombinant IP₃Rs in most cells is difficult because of the background activities of the endogenous IP₃Rs in these cells. In this study, we used R23·11 cells that intrinsically lack all the three IP₃Rs (27) as the host cells, in order to exclude the background effects of the endogenous IP₃Rs. Under the conditions used neither IP₃ binding activity nor IP₃-elicited Ca²⁺ release activity was detected in membrane fractions prepared from R23·11 cells (Fig. 3A). The wild-type IP₃R1 expressed in KMN13 cells revealed affinity for IP₃ (Kₐ = 20 ± 5 nM) (Table 1) similar to that of native (31) and recombinant (6,28,32) mouse IP₃R1, and it mediated Ca²⁺ release from microsomal vesicles in an-IP₃-dependent manner (Fig. 3B). The wild-type IP₃R1 expressed in KMN13 cells revealed quantal Ca²⁺ release (Fig. 3B), which is thought to be an intrinsic property of native IP₃Rs (17,25), suggesting that the recombinant IP₃R1 in R23·11 cells functions properly. By using this system, we determined that six of the mutants investigated in this study, lacking amino acid residues 1-223, 651-1130, 1267-2110, 1845-2042, 1845-2216 or 2610-2748, did not exhibit any measurable Ca²⁺ release activity, while the mutants lacking residues 1131-1379 or 2736-2749 retained the activity. [³H]IP₃ binding analysis showed that all of these non-functional mutants except D1-223 possessed lower IP₃ binding affinity (Table 1). However, 98.5% of the receptors of even the lowest affinity mutant, D651-1130, whose Kₐ is 150 nM (Table 1), would have been occupied when 10 µM IP₃ was applied. Therefore, the decrease in IP₃-binding affinity is unlikely to be the primary cause of the loss of function of these mutants. The limited trypsin digestion of the crude membrane fractions prepared from the mutant-expressing cells showed that not only the IP₃-gated Ca²⁺-permeable mutants, D1131-1379 and D2736-2749, but two non-functional mutants, D1-223 and D651-1130, generated fragments IV and V (Fig. 4). All of these mutants except D1131-1379 exhibited trypsin sensitivity similar to that of the wild-type IP₃R1, indicating that these mutants retain a normal folding structure in at least the C-terminal...
channel-forming domain. Immunocytochemical staining suggested that all the mutants were localized on the Ca\textsuperscript{2+} stores of the cells (data not shown). Hence, we concluded that at least two regions, namely, 1-223 and 651-1130, are required for the IP\textsubscript{3}\textsuperscript{-}dependent gating of IP\textsubscript{3}R1.

The essential region 1-223, which has been known as a suppressor of IP\textsubscript{3} binding

Deletion of the amino acid residues of the N-terminal side of the IP\textsubscript{3} binding core had a complicated effect on the IP\textsubscript{3} binding activity (12). A short deletion of the N-terminal 31 amino acid residues from T734 resulted in a significant reduction in binding activity even though the mutant included the entire IP\textsubscript{3}\textsuperscript{-}binding core sequence. Such effects were also found in serial N-terminal deletions up to 215 residues. However, binding activity recovered when deleted up to the first 220, 223, or 225 amino acids. The mutant lacking the first 223 amino acids showed more than 10-fold higher affinity for IP\textsubscript{3} than the parental T734. Based on these results, Yoshikawa et al. proposed that the first ~225 N-terminal amino acids function as a suppressor of IP\textsubscript{3}\textsuperscript{-}binding (41). In the present study, we found that D1-223 has 10-fold higher affinity for IP\textsubscript{3} than wild-type IP\textsubscript{3}R1 (Table 1). In addition, we found that the IP\textsubscript{3}\textsuperscript{-}binding of D1-223 is positively cooperative (Table 1), indicating that the intersubunit interaction may be elicited (or modified) in the tetrameric complex composed of D1-223. Limited trypsin digestion showed that the mutant is likely to retain the normal folding structure of the C-terminal channel-forming domain (Fig. 4D). Surprisingly, the mutant D1-223 did not exhibit any measurable Ca\textsuperscript{2+}-release activity (Fig. 3A). These data, therefore, clearly indicate that amino acid residues 1-223 are required for the functional coupling between IP\textsubscript{3}\textsuperscript{-}binding and channel opening.

Homer (42) and calmodulin (CaM) (43) binding sites are localized in the 1-223 region of IP\textsubscript{3}R1. Homer forms an adaptor complex that couples between group 1 metabotropic glutamate receptors and IP\textsubscript{3}Rs, and it has recently been reported to be capable of associating with type 1 RyR and upregulating its Ca\textsuperscript{2+} release activity (44). The Homer binding motif (PPXXFR) is present in amino acid residues 49-54 (PPKKFR) of mouse IP\textsubscript{3}R1 (42). CaM
interacts with amino acid residues 49-81 and 106-128 in a Ca\textsuperscript{2+}-dependent and Ca\textsuperscript{2+}-independent manner (43). These binding proteins within the critical region 1-223 may modulate the gating of IP\textsubscript{3}R1.

The essential region 651-1130 which is close to the IP\textsubscript{3} binding core

In the region 651-1130 of IP\textsubscript{3}R1, the alternative splicing site (amino acid position between 917 and 918) referred to as SIII is present (45), but the functional significance of the SIII segment (9 amino acid residues) has not yet been elucidated. There are three possible Ca\textsuperscript{2+} binding sites within amino acid regions 660-745, 741-849 and 994-1059 (46), suggesting that the region 651-1130 is involved in the Ca\textsuperscript{2+}-dependent regulation of the IP\textsubscript{3}R function. Recently, Bosanac et al. unveiled the three-dimensional structure of the IP\textsubscript{3}-binding domain (224-604) that covers the IP\textsubscript{3}-binding core, amino acid residues 226-578 of IP\textsubscript{3}R1 in the presence of IP\textsubscript{3} (14). The IP\textsubscript{3}-binding domain forms an asymmetric boomerang-like structure that consists of an N-terminal \(\beta\)-trefoil domain (224-436) and a C-terminal \(\alpha\)-helical domain (437-604) containing three armadillo repeat-like folds. IP\textsubscript{3} fits into a cleft formed by these two arms of the boomerang. Our data described here suggest that region 651-1130, which immediately follows the IP\textsubscript{3}-binding core, is essential for IP\textsubscript{3}-induced gating of the channel. What kind of roles does this region have? It is known that other proteins containing armadillo repeats, such as \(\beta\)-catenin (47) and importins (48), have more than ten repeats. Based on analysis of the amino acid sequence of the IP\textsubscript{3}R1, Bosanac et al. suggested that in IP\textsubscript{3}R1, the armadillo repeat-like folds extend to the C-terminal region of the IP\textsubscript{3}-binding core. It is predicted that many \(\alpha\)-helical domains are formed over the entire 651-1130 region (14), and deletion of 1131-1379 did not abolish the IP\textsubscript{3}-dependent Ca\textsuperscript{2+} release activity (Fig. 3A). We therefore speculate that the armadillo repeat-like fold-containing \(\alpha\)-helical domain, which is essential for the IP\textsubscript{3}-induced gating, is formed within the residues 440-1130. This region might constitute a part of the bridge that connects the IP\textsubscript{3}-dependent conformational change of the IP\textsubscript{3}R with channel opening.

Recently, Hamada et al. showed that purified IP\textsubscript{3}R from the mouse cerebellum
contains two distinctive structures: a windmill-like structure and a square-shaped structure (49). Ca\(^{2+}\) reversibly promotes transition from the square- to the windmill-shaped structure, with relocation of the four peripheral IP\(_{3}\)-binding domains. This observation predicts the presence of a hinge region which changes its conformation drastically following Ca\(^{2+}\) binding to the receptor. By limited protease digestion analysis, Hamada et al. examined the Ca\(^{2+}\)-dependent structural change of the purified IP\(_{3}\)R. The results showed that a 38-kDa fragment detected with using anti-IP\(_{3}\)R1 mAb 4C11 was specifically generated by cleavage in a solution containing CaCl\(_{2}\), but not in an EDTA-containing solution. The epitope of the antibody 4C11 was mapped within residues 679-727 in IP\(_{3}\)R1 (13), and therefore the 651-1130 region found in this study is a strong candidate for the hinge region. The presence of Ca\(^{2+}\)-binding sites within this region (46) also supports this hypothesis.

**Non-functional mutants D1267-2110, D1845-2042 and D1845-2216**

Since the non-functional mutants, D1267-2110, D1845-2042, and D1845-2216 do not have cleavage sites between tryptic fragments IV and V (arginine 1931, arginine 1923, or lysine 1924) (22), we could not evaluate the folding structure of the C-terminal channel-forming domain of these mutants. Therefore we did not determine whether the deleted regions include critical regions for activation gating or the deletions simply distort the structure of the receptor. Notably, the deleted regions in these mutants include (or are close to) the Ca\(^{2+}\) sensor region found in IP\(_{3}\)R1 (50). Cytoplasmic Ca\(^{2+}\) is a coagonist for the IP\(_{3}\)R (51), and thus the IP\(_{3}\)-binding signal and Ca\(^{2+}\)-binding signal must be combined on the IP\(_{3}\)R. More detailed analysis of the regions deleted in these mutants may help to better understand the molecular mechanism of gating, in particular, the Ca\(^{2+}\)-dependent processes during channel opening.

**The region 2610-2748 may be required for the correct folding of the IP\(_{3}\)R1**

The C-terminal region following the sixth transmembrane region may play some part in channel gating of the IP\(_{3}\)R, because mAbs that recognize this region have been reported to
either inhibit (21) or enhance (52) IICR. In addition, it has been suggested that the C-terminus is involved in subunit assembly of the IP$_3$R channel complex (6,9). It has been shown that while a truncation-mutant IP$_3$R1 which lacks all of the transmembrane regions and the succeeding C-terminus (amino acids 2218-2749) is present as a monomer, a deletion mutant which lacks only the transmembrane regions (amino acids 2112-2605) forms dimers (6), suggesting that the C-terminal 144 amino acids (2606-2749) are involved in the intersubunit interaction of IP$_3$R1. Facilitation of multimer formation of mutant IP$_3$Rs having two or more transmembrane regions was observed if the mutants were fused to the C-terminal 145 amino acid residues (9); however, recombinant IP$_3$R1 lacking the C-terminal 145 amino acid residues formed tetramers (9), suggesting that this C-terminal region is not essential for the formation of the tetrameric channel complex. In the present study, we found that the deletion of amino acids 2610-2748 completely abolished the activity of the IP$_3$R channel. D2610-2748 was not well recognized by the antibody 1ML1 (Fig. 2A). Limited trypsin digestion of mutant D2610-2748 did not generate tryptic fragment IV and V (Fig. 4F), suggesting that deletion of amino acid residues 2610-2748 affects the folding structure around the cleavage sites between tryptic fragments IV and V.

The essential cysteine residue in the C-terminal tail

We found that the site-directed mutants C2610S, C2613S, and C2610/2613S did not exhibit any measurable Ca$^{2+}$ release activity (Fig. 6). As shown in Fig. 7, limited trypsin digestion of C2613S and C2610/2613S generated all four tryptic fragments (I, III, IV, and V), whereas trypsinization of C2610S generated only three tryptic fragments (I, III, and IV). These results indicate that cysteine 2613 is required for the functional coupling between IP$_3$ binding and channel opening. The results of trypsinization of C2610S suggest that substitution of cysteine 2610 disrupts the correct folding in at least the C-terminal channel-forming domain of IP$_3$R. This is puzzling, however, because C2610/2613S generated all four tryptic fragments. The effect of a single amino acid substitution at cysteine 2610 could be explained if the cysteine at position 2613 in the mutant C2610S elicits an artificial
modification, such as disulfide formation, S-nitrosylation, or palmitoylation, which induces a serious distortion in the folding structure of the C-terminal channel domain. These modifications might be prevented in the presence of cysteine 2610. This explanation suggests a direct or indirect structural interaction between cysteine residues 2610 and 2613 in wild-type IP₃R1.

These cysteine residues are also conserved in the RyR family (Fig. 5). Involvement of the cysteine residues in intracellular Ca²⁺ channel gating has been postulated on the basis of evidence that thiol reagents, such as oxidized glutathione or thimerosal, enhance the Ca²⁺-mobilizing activity of both IP₃R and RyR channels (1). There are nine cysteine residues conserved in both families (13). As shown in Fig. 6, substitution for the conserved cysteine residue at position 1976 did not affect the activity of the IP₃R1, while substitution for cysteine 2610 or 2613 caused loss of function of the IP₃R1. It is possible that thiol reagents directly attack the cysteine residues at positions 2610 and/or 2613 and enhance channel activity. Further studies are required to elucidate the exact target sites of thiol reagents and the mechanism of the enhancement of channel gating induced by these reagents.

Cysteine residues located in the C-terminal region following the transmembrane domain are known to be involved in the gating of some ion channels on the plasma membrane, such as cyclic nucleotide-gated channels (53-55), and voltage-dependent and inward rectifier K⁺ channels (56,57). In the presence of oxidants, a certain C-terminal cysteine residue in both these plasma membrane channels reacts with a cysteine residue located in the N-terminal region on the same or different subunit. This reaction depends on the states of the channels, and the formation of disulfide bonds results in channel potentiation. In the N-terminal side of the transmembrane domain, IP₃R possesses 18 cysteine residues that are conserved in the IP₃R family. Thus, examining the interaction between the N-terminus and C-terminus via the formation of disulfide bonds in the presence of some oxidants according to the gating states would be useful in understanding the conformational changes that occur during IP₃R gating.
A novel five-domain structure model for the IP$_3$R

We found that two regions, namely, 1-223 and 651-1130, and cysteine 2613 are crucial for the IP$_3$-induced gating of IP$_3$R1. How do they contribute to the activation gating of the IP$_3$R1? Recently, the open pore conformation of K$^+$ channels (MthK) was resolved at a resolution of 3.3 Å (58,59). Structural comparison between KcsA and MthK, closed and open K$^+$ channels, revealed that pore-lining inner helices form the channel gate, and bending of the inner helices causes channel opening. In the bent configuration, the inner helices form a wide (12 Å) entryway. The gating hinge is a glycine residue located in the middle of the inner helices. The glycine residue is highly conserved in voltage- or ligand-gated channels with two- or six-membrane spanning segments per subunit, suggesting that the bending of the inner helices is a common mechanism for channel opening. The IP$_3$R has been proposed to have the same structural arrangement of the pore-forming domain to that of the voltage-gated K$^+$ channels (10); therefore the IP$_3$R pore may also be equipped with the same gating mechanism. Pore-lining inner helices that contain the gating hinge in the MthK channel correspond to the sixth membrane-spanning segments of the IP$_3$R. It is of interest to note that a glycine residue is also present within the sixth membrane-spanning segment of all the three types of IP$_3$R. This structural similarity suggests that the channel gate is formed by the sixth membrane-spanning helices of the IP$_3$R. One of the striking differences between the IP$_3$R and other ligand-gated channels with six membrane-spanning segments, such as cyclic nucleotide-gated channels, is in the location of the ligand-binding site. Both the IP$_3$-binding site and the Ca$^{2+}$-binding sites are positioned on the N-terminal side of the transmembrane domain of IP$_3$R, while in other ligand-gated channels, the ligand-binding sites are located on the C-terminal cytoplasmic region that is close to the pore-lining inner helices. In these ligand-gated channels, the ligand-binding signals may be transferred directly to the pore domain and cause bending of the inner helices to open the channels. Ligand-binding signals of IP$_3$R may be transferred to the pore domain in a different manner. Based on the results presented here, we propose a novel five-domain structure model in which the C-terminal tail works as a gatekeeper for activation-induced gating of the IP$_3$R.
(Fig. 8). In this model, conformational changes in the IP$_3$-binding domain caused by IP$_3$ binding are transmitted through both the N-terminal coupling and internal coupling domains to the C-terminal tail, which then triggers the channel opening. Cysteine 2613 in the C-terminal tail may be critical for receiving the IP$_3$-binding signal and/or for triggering the channel opening. Further studies on the structure and function of IP$_3$R using the described experimental approach may provide us with an exact answer for the long-asked question, “How does the binding of IP$_3$ at the N-terminus gate the C-terminal Ca$^{2+}$ permeation pore?”
Acknowledgments

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Figures Legends

Figure 1. Deletion- and site-directed mutants of IP_3R1. (A) Schematic diagram of the primary structure of the mouse IP_3R1. SI, SII, and SIII represent the regions spliced out in alternative splicing variants. The hatched box represents the IP_3-binding core (amino acid residues 226-578). The black boxes M1 to M6 represent putative membrane spanning regions. The regions containing epitopes for the antibodies N1, 4C11, 10A6, anti(1718-31), 1ML1, and 18A10 are indicated by thick horizontal lines. Five tryptic fragments (I, II, III, IV, and V) (22) are indicated by thin horizontal lines. (B) Structures of the deletion- and point mutants. Open boxes represent deleted regions. Open circles denote the sites of single amino acid substitution of serine for cysteine.

Figure 2. Western blot analysis of wild-type and mutant IP_3R1 molecules expressed in R23-11 cells. (A) Membrane proteins prepared from stable cell lines were analyzed by Western blot analysis using antibody 1ML1. Lane 1: R23-11 cells (10 µg); Lane 2: high-dose wild-type IP_3R1-expressing cells (KMN 13) (1 µg); Lane 3: low-dose wild-type IP_3R1-expressing cells (KMN 107) (10 µg); Lane 4 to 11: cells expressing D1-223, D651-1130, D1131-1379, D1692-1731, D1845-2042, D1845-2216, D1267-2110, and D2736-2749, respectively (10 µg each). Molecular size markers are shown on the left (x 10^{-3}). (B) Western blot analysis with mAb 4C11. Lane 1: R23-11 cells (1 µg); Lane 2: high-dose wild-type IP_3R1-expressing cells (KMN 13) (0.1 µg); Lane 3: cells expressing D2610-2748 (1 µg). Molecular size markers are shown on the left (x 10^{-3}).

Figure 3. IP_3-dependent Ca^{2+} release activity of wild-type and mutants of IP_3R1. (A) Representative time-courses of the Ca^{2+} release from the microsomal fractions containing wild-type and mutants of IP_3R1. Ca^{2+} release was monitored in the presence of 2 µM thapsigargin. IP_3 (10 µM) was added at the times indicated by the arrowheads. Constant leakage (Experimental Procedures) was subtracted from each trace. Ca^{2+}-release activity of
the mutated IP₃R1 was measured in at least three independent experiments. (B) Time courses of Ca²⁺ release through wild-type IP₃R1 following the addition of different IP₃ concentrations. IP₃ was added at time 0.

Figure 4. Fragmentation of wild-type and mutated IP₃R1 by limited trypsin digestion. Crude microsomal fractions (0.25–5 mg protein/ml) were treated with various concentrations of trypsin. Tryptic fragments I, III, IV, and V (22) were detected by Western blot using the antibodies N1, 10A6, anti(1718-31), and 1ML1, respectively. Each major tryptic fragment is indicated by a solid arrow. (A) wild-type IP₃R1, (B) D2736-2749, (C) D1131-1379, (D) D1-223, (E) D651-1130, (F) D2610-2748. Trypsin concentrations are indicated on each lane (µg/ml). Molecular size markers are shown on the left (x 10⁵).

Figure 5. Conserved cysteine residues in the C-terminal tail. The C-terminal 48-amino acid sequences next to the transmembrane regions in all the three types of IP₃Rs and RyRs are compared. Asterisks and double dots indicate identical and similar residues, respectively. Cysteine residues 2610 and 2613 in mouse IP₃R1 are pointed with arrowheads. These amino acid sequences were aligned using the ClustalW algorithm. Abbreviations (Genbank accession number): mIP₃R1, mouse type 1 IP₃R (X15373); rIP₃R1, rat type 1 IP₃R (J05510); hIP₃R1, human type 1 IP₃R (D26070); XIP₃R, Xenopus IP₃R (D14400); rIP₃R2, rat type 2 IP₃R (X61677); hIP₃R2, human type 2 IP₃R (D26350); rIP₃R3, rat type 3 IP₃R (L06096); hIP₃R3, human type 3 IP₃R (D26351); DIP₃R, Drosophila IP₃R (D90403); CIP₃R, Caenorhabditis elegans IP₃R (AJ243179); rbRyR1, rabbit type 1 RyR (X15750); rbRyR2, rabbit type 2 RyR (U50465); rbRyR3, rabbit type 3 RyR (X68650).

Figure 6. Western blot analysis and IP₃-dependent Ca²⁺-release activity of the cysteine mutants. (A) Western blot analysis of mutants C1976S, C2610S, C2613S, and C2610/2613S with antibody 1ML1. Lane 1: R23-11 (10 µg/ml); Lane 2: high-dose wild-type IP₃R1-expressing cells (KMN 13) (1 µg); Lane 3: low-dose wild-type IP₃R1-expressing cells.
(KMN 107) (10 µg); Lane 4 to 7: cells expressing C1976S, C2610S, C2613S, and C2610/2613S, respectively (10 µg each). Molecular size markers are shown on the left (x 10³). (B) The time courses of the Ca²⁺ release from the microsomal fractions containing cysteine mutants are shown. Ca²⁺ release was monitored in the presence of 2 µM thapsigargin. IP₃ (2.5 µM) was added at the times indicated by the arrowheads. Constant leakage was subtracted from each trace.

Figure 7. Limited trypsin digestion of the cysteine mutants of IP₃R1. Crude microsomal fractions (0.25–5 mg protein/ml) were treated with various concentrations of trypsin. Tryptic fragments I, III, IV, and V were detected by Western blot using antibodies N1, 10A6, anti(1718-31), and 1ML1, respectively. Each major tryptic fragment is indicated by a solid arrow. (A) C1976S, (B) C2610S, (C) C2613S, (D) C2610/2613S. Trypsin concentrations are indicated on each lane (µg/ml). Molecular size markers are shown on the left (x 10⁻³).

Figure 8. A five-domain structure model of the IP₃R1. In this model, the structure of the IP₃R1 is divided into five functional domains, namely, an N-terminal coupling domain (amino acid residues 1-225), ligand-binding domain (226-578), internal coupling domain (579-2275), transmembrane domain (2276-2589), and gatekeeper domain (2590-2749). The signal of IP₃ binding is transferred through both the N-terminal and internal coupling domains to the gatekeeper domain, which triggers conformational change of the activation gate formed within the transmembrane domain.
<table>
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<tr>
<th>Recombinant Proteins</th>
<th>Affinity (Kd [nM]) †</th>
<th>Bmax [pmol/mg protein]) †</th>
<th>Hill Coefficient†</th>
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<td>wild type (KMN13)</td>
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<td>13 ± 9</td>
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<td>D2736-2749</td>
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<td>7.2 ± 0.4</td>
<td>0.95 ± 0.07</td>
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† Values are expressed as means ± S.D. (n = 3).
*, p<0.05, **, p<0.01 by Student’s t test
**Table 2**

**IP₃ Binding Properties of the Cysteine Mutants**

<table>
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<th>Recombinant Proteins</th>
<th>Affinity (Kₐ [nM])</th>
<th>Binding Capacity (Bₘₐₓ [pmol/mg protein])</th>
<th>Hill Coefficient</th>
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<td>C1976S</td>
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<td>C2610S</td>
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<td>C2613S</td>
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<td>C2610, 2613S</td>
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<td>0.99 ± 0.1</td>
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</table>

Values are expressed as mean ± S.D. (n = 3).
Fig. 1
Fig. 2
Fig. 5
Fig. 7
Fig. 8
Critical regions for activation gating of inositol 1,4,5-trisphosphate receptor
Keiko Uchida, Hiroshi Miyauchi, Teiichi Furuichi, Takayuki Michikawa and Katsuhiko Mikoshiba

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