Mutational Analysis of the Ligand Binding Site of the Inositol 1,4,5-Trisphosphate Receptor*

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To define the structural determinants for inositol 1,4,5-trisphosphate (IP$_3$) binding of the type I inositol 1,4,5-trisphosphate receptor (IP$_3$R$_1$), we developed a means of expressing the N-terminal 734 amino acids of IP$_3$R$_1$ (T734), which contain the IP$_3$ binding region, in Escherichia coli. The T734 protein expressed in E. coli exhibited a similar binding specificity and affinity for IP$_3$ as the native IP$_3$R from mouse cerebellum. Deletion mutagenesis, in which T734 was serially deleted from the N terminus up to residue 215, markedly reduced IP$_3$ binding activity. However, when deleted a little more toward the C terminus (to residues 220, 223, and 225), the binding activity was retrieved. Further N-terminal deletions over the first 228 amino acids completely abolished it. C-terminal deletions up to residue 579 did not affect the binding activity, whereas those up to residue 568 completely abolished it. C-terminal deletions up to residue 579 did not affect the binding activity, whereas those up to residue 568 completely abolished it. C-terminal deletions were performed on 41 basic Arg and Lys residues within the N-terminal 650 amino acids of T734. We showed that single amino acid substitutions for 10 residues, which were widely distributed within the binding core and conserved among all members of the IP$_3$R family, significantly reduced the binding activity. Among them, three (Arg-265, Lys-508, and Arg-511) were critical for the specific binding, and Arg-568 was implicated in the binding specificity for various inositol phosphates. We suggest that some of these 10 residues form a basic pocket that interacts with the negatively charged phosphates of IP$_3$.

Many cellular responses to hormones, neurotransmitters, growth factors, etc. are mediated by the intracellular second messenger inositol 1,4,5-trisphosphate (IP$_3$ or (1,4,5)IP$_3$) ($^1$). IP$_3$ releases Ca$^{2+}$ from intracellular stores by binding to the IP$_3$ receptor (IP$_3$R) (2), which is a tetrameric IP$_3$-gated Ca$^{2+}$ release channel (3–5). There are at least three types of IP$_3$R derived from distinct genes in mammals (6–12). Structural and functional studies on type I IP$_3$R (IP$_3$R$_1$) (2749 amino acids, 313 kDa) have revealed that it is structurally divided into three parts: a large N-terminal cytoplasmic arm (83% of the receptor molecule); a putative six membrane-spanning domains clustered near the C terminus, which are thought to constitute an ion channel by forming a tetramer; and a short C-terminal cytoplasmic tail (13, 14).

The binding of IP$_3$ to this receptor purified from mouse cerebellum is stoichiometric ($K_d$ = ~100 nM, Hill coefficient = ~1.0) (2, 15). To localize the IP$_3$ binding site, deletion mutagenesis studies showed that IP$_3$R$_1$ binds IP$_3$ within the N-terminal 650 amino acids independently of the tetramer formation (16, 17). Newton et al. (18) have reported that the N-terminal 576 amino acids fused to glutathione S-transferase specifically bound IP$_3$ with high affinity, whereas further N- or C-terminal deletions of this region completely abolished the specific binding. Furthermore, Mourey et al. (19) have reported that residues 471-501 in this region were labeled with a photaffinity ligand. These results indicated that the IP$_3$ binding site is localized within the N-terminal 576 amino acids and consists of some distantly separated motifs.

IP$_3$ is characterized by three negatively charged phosphate groups at equatorial positions 1, 4, and 5 of an inositol ring. Ca$^{2+}$ release experiments using various synthetic inositol phosphate analogues showed that the IP$_3$ binding site is markedly stereospecific (20–22). The ability of IP$_3$ to release Ca$^{2+}$ depends critically upon the positional distribution of the phosphate groups around the inositol ring, suggesting that binding sites for these three phosphate groups make major contributions to the recognition and binding of IP$_3$. Thus, it has been assumed that there is a pocket of positive charges that facilitate ionic interactions with the negative charges on these three phosphate groups. This hypothetical model is supported by the following evidence. IP$_3$ binding to the platelet membrane is blocked by the specific Arg-modifying reagent, p-hydroxyphenylglyoxal, suggesting the involvement of Arg in the IP$_3$ binding (23). IP$_3$ binding to the receptor is competitively blocked by heparin (24), and the IP$_3$R protein has been purified by heparin affinity chromatography (2, 15).

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‡ The abbreviations used are: IP$_3$ and (1,4,5)IP$_3$, inositol 1,4,5-trisphosphate; IP$_3$R, inositol 1,4,5-trisphosphate receptor; IP$_3$R$_1$, type 1 inositol 1,4,5-trisphosphate receptor; mAb, monoclonal antibody; (1)P$_3$, inositol 1-phosphate; (1,4)P$_2$, inositol 1,4-bisphosphate; (4,5)P$_2$, inositol 4,5-bisphosphate; (2,4,5)P$_3$, inositol 2,4,5-trisphosphate; (1,3,4,5)P$_4$, inositol 1,3,4,5-tetrakisphosphate; RyR, ryanodine receptor.
The known binding site for heparin in antithrombin III is highly basic because of enriched Arg or Lys residues (25). The IP$_3$ binding to the receptor is augmented with increasing pH over the range 5–9 (24). A study using NMR spectroscopy showed that IP$_3$ dissociates protons from three phosphate groups over this pH range, indicating that the negative charges of IP$_3$ contribute its binding to the receptor (26). Finally, x-ray crystallographic studies of the pleckstrin homology domain of $\beta$-spectrin and phospholipase C-$\beta$ showed that IP$_3$ dissociates protons from three phosphate residues of this domain, respectively, interact with the phosphate groups of IP$_3$ (27, 28).

Despite the above evidence, the detailed molecular structure of the IP$_3$ binding site of the IP$_3$R remains to be studied. In this study, we developed an Escherichia coli expression system for various recombinant IP$_3$R binding sites of mouse IP$_3$R1. The N-terminal 734 amino acids of IP$_3$R1 (T734) expressed in E. coli exhibited similar binding characteristics to those of the native cerebellar IP$_3$R. Eighteen deletion mutants of T734 showed that 353 amino acids (residues 226–578) are directly responsible for the IP$_3$ binding. Furthermore, we performed site-directed mutagenesis on 41 basic amino acid residues within the N-terminal 650 amino acids of T734 and showed that 10 amino acid substitutions markedly reduced the IP$_3$ binding activity. They were scattered within residues 226–578 and conserved among all members of the IP$_3$R family. Of these, three were critical for IP$_3$ binding and one was involved in binding specificity. We discuss the structure of the IP$_3$ binding site of the IP$_3$R.

**EXPERIMENTAL PROCEDURES**

Materials—Recombinant Pfu DNA polymerase, Taq DNA polymerase, restriction and other modification enzymes were obtained from Stratagene, Takara Shuzo (Otsu, Japan), and New England Biolabs. o-myo-inositol1,4,5-triphosphate (Sigma; position 328)–o-myo-inositol1,3,4,5-tetrakisphosphate (Sigma; position 338)–o-myo-inositol1,3,4,5,6,7,8,9-octakisphosphate (Sigma; position 350), and o-myo-inositol2,3,4,5,6,7,8,9-nonakisphosphate (Sigma; position 362) were from Dojindo (Kumamoto, Japan). (1,3,4,5)IP$_4$ were from Calbiochem. To clone the cDNA encoding the N-terminal 734 amino acids of mouse IP$_3$R1 into the pET-3a vector (29) in frame, a site for NdeI (CATATG) was introduced into the pUC119 vector. From the resultant plasmid, the PCR fragment was digested with BglII and SacI (nucleotide positions 1,882 and 2,029–2,009), containing the new SacI site (underlined). The BglII (nucleotide position 1,882)–SacI (nucleotide position 2,068 or 2,036) portion of the PCR product was then subcloned into the BglII (nucleotide position 1,882) and SacI (nucleotide position 2,281) sites of pET-T734.

The plasmid pET–224–579, expressing 356-amino acid polypeptides (nucleotide 224–579) was also produced by PCR using the sense primer 5'-GCCCAATTCAGAGGCGGCAAGCGT-3'–nucleotide position 1,831–1,854 and the antisense primer 5'-GCGGATCTTTAACCAGAATCCGGGTGACGCCGAGAATTCGATTACAAATTGCTTGCGTAC-3'; position 2,064–2,040, plus an additional 45-base pair sequence (underlined) containing a BamHI site, stop codon (double underline), and the epitope sequence for streptavidin (Arg-His-Pro-Gln-Phe-Gly-Gly-C) (30, and EcoRV site). The resultant PCR fragment was digested with BglII and BamHI and then subcloned into the BglII–1,882 and BamHI (in pET vector) sites of the expression plasmid pET-D1–223.

The codons CAG and AAA, GCA and GCG, CGA, and AAG were used for the substitution for Gin, Ala, Arg, and Lys, respectively, on the basis of codon usage and RNA contents in E. coli (31). Site-directed mutagenesis (K1000/K101Q, K235Q, K249Q, K257Q/K258Q/K259Q, R265Q, R269Q, R293Q, R304Q/K306Q, K350Q, R376Q, K412O, K424Q, K427Q, K441Q, K459Q, K470Q/R471Q, K501Q, R504Q/K506Q, K508Q, K508A, K508R, K511Q, K511K, R537Q, R545Q, R554Q, R566Q, R568Q/K569Q, R569Q, K569R, K580Q/K582O, K581O, K583Q, E589Q, R602Q, R603Q, R604Q, R606Q, R623Q, R626Q, R629Q) was performed by means of two-step PCR (32). In R504Q/R506Q, for example, two overlapping fragments (nucleotide positions 1,542–1,854 and 1,831–2,339) were separately amplified with two pairs of oligonucleotides (fragment 1,542–1,854, sense primer S, 5'-AGCTTTCCGTCGGTACGGTGGG-GG-3' (1,854–1,831); fragment 1,831–1,854, mutagenic sense primer SmS, 5'-GCCCAATTCAGAGGCGGCAAGCGT-3' (1,854–1,831), fragment 1,831–2,009, mutagenic sense primer Sm1, 5'-GCCCAATTCAGAGGCGGCAAGCGT-3' (1,831–1,854) and mutagenic antisense primer SmA, 5'-AGCTTTCCGTCGGTACGGTGGG-GG-3' (33, 2,339–2,320)). The two resulting PCR fragments were purified, annealed, and reamplified with the primers Sm1 and AS. The second PCR fragment carrying the mutation was subcloned into the NdeI–SacI (nucleotide positions 1,588 and 1,882, respectively), and then the wild-type counterpart was displaced. All mutagenized fragments were verified by dideoxy chain termination (33).

Expression of Recombinant Ligand Binding Site in E. coli—A single colony of E. coli BL21(DE3) (33) transformed with pET-T734 was selected into 1.5 ml of Luria-Bertani medium containing 100 μg/ml ampicillin and incubated at 27°C for 13–14 h (full growth). One hundred microliters of the culture was inoculated in 10 ml of Luria-Bertani medium containing 100 μg/ml ampicillin and incubated at 21°C for 9–10 h to an A$_{600}$ of ~1.5. After addition of isoproplthio-$\beta$-d-galactoside to a final concentration of 0.5 mM, the culture was incubated at 14°C for another 20 h. Cells were harvested by centrifugation, washed with 1 ml of phosphate-buffered saline, and stored at −80°C. The same procedures were performed on E. coli cells harboring other mutant plasmids or pET-3a.

Preparation of Soluble Fraction of E. coli—Cell pellets were resuspended in 1 ml of binding buffer (50 mM Tris-HCl (pH 8.0 at 4°C), 1 mM 2-mercaptoethanol, 1 mM EDTA) containing protease inhibitors (10 μM pepstatin A, 10 μM leupeptin, and 0.6 mM phenylmethylsulfonyl fluoride), incubated with 0.1 mg/ml of lysozyme at 4°C for 30 min, then subjected to six cycles of freezing in liquid nitrogen followed by thawing in a water bath at 37°C. Chromosomal DNAs were sheared with a sonicator (Astron XL2020) in the presence of 32 μg/ml DNase I for 10 s at 4°C twice. The suspension was centrifuged at 3000 × g for 60 min at 2°C. The supernatant was Western blotted and IP$_3$ binding was assayed after the protein concentration was determined using a kit for bovine serum albumin as the standard.

Polyclonal Rabbit Antiserum Preparation—A polyclonal antiserum was raised in a rabbit to a synthesized peptide corresponding to residues 501–518 of mouse IP$_3$R1, which was coupled via an additional N-terminal cysteine to keyhole limpet hemocyanin using the cross-linking agent 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC). The AUC was assayed after the protein concentration was determined using a kit for bovine serum albumin as the standard.
9.6 nM ([3H]IP₃ (or 0.96 nM for Scatchard analyses of the expressed residues 224–579) in 100 µl of binding buffer for 10 min at 4°C. The mixture was then added to 4 µl of γ-globulin (50 mg/ml) and 100 µl of a solution containing 30% (w/v) polyethylene glycol 6000, 50 mM Tris-HCl (pH 8.0 at 4°C), 1 mM 2-mercaptoethanol, and 1 mM EDTA. After incubation at 4°C for 5 min, the protein-polyethylene glycol complex was collected by centrifugation at 10,000 × g for 5 min. We confirmed by Western blotting that all expressed recombinant proteins were precipitated under these conditions (data not shown). The pellets were dissolved in 180 µl of Solvable (DuPont NEN). After neutralization with 18 µl of acetic acid, the radioactivity was measured in 5 ml of Atolmount (DuPont NEN) with a liquid scintillation counter. The specific binding was defined by subtracting the nonspecific binding in the presence of 2 or 10 µM of cold IP₃ from the total. Specific ([3H]1,4,5)IP₃ binding was also inhibited in the reaction mixture described above, except for the presence of various inositol phosphates.

**RESULTS**

Characterization of T734 Expressed in E. coli—To define the structural determinants of the IP₃ binding site of IP₃R₁, we developed an E. coli expression system for the N-terminal 734 amino acids (named T734) that contain the IP₃ binding region (16–18) and the epitope of anti-IP₃R₁ mAb 4C11 (residues 678–699) (6). The constructs were designed so that expressed recombinant proteins had no foreign sequences, such as fusion proteins, to avoid interference with the IP₃ binding, except for the 5 additional amino acids at the C terminus. The recombinant proteins expressed in E. coli were almost all found in aggregates (inclusion bodies) when cells were grown at 37°C, as judged by Western blotting using mAb4C11. We did not detect any significant IP₃ binding activity in the soluble fraction. On the other hand, when cells were grown at 14–28°C, some fractions of the expressed proteins became soluble. A specific immunoreactive band of about 80 kDa was detected by mAb4C11 in the soluble fraction from T734-expressing cells (Fig. 1A). The soluble fraction from T734 cells grown at the low temperature bound significantly high levels of IP₃ compared with the control cells, which showed no activity (Fig. 1B). Thus, the IP₃ binding properties of the soluble fraction containing T734 were further characterized without purification. The binding specificity of T734 to various inositol phosphates was examined by competition for [3H]1,4,5)IP₃ binding (Fig. 1C). The competitive potency was in the following order: (1,4,5)IP₃ > (2,4,5)IP₃ > (1,3,4,5)IP₄ > (4,5)IP₂ > (1,4)IP₂ > (1)IP₁. This binding specificity was very similar to that of the native IP₃R₁ from mouse cerebellum (data not shown). Scatchard analysis indicated that the dissociation constant (Kᵦ) of the IP₃ binding to T734 was 50 ± 2.4 nM (n = 6) (Fig. 1D), which was also consistent with that of the cerebellar IP₃R (37 nM). When the soluble fraction from T734-expressing cells was applied to a heparin-agarose column, the T734 protein was retained on the column in low salt (0.25 M NaCl) and eluted in high salt (0.5 M NaCl) (data not shown). The IP₃ binding activity of T734 was completely inhibited by heparin, as was cerebellar IP₃R. These results demonstrated that the bacterially expressed T734, even in the crude soluble fraction, had similar IP₃ binding characteristics to those of cerebellar IP₃R.

**Determination of IP₃ Binding Site by Deletion Mutagenesis**—To define the boundary of the IP₃ binding site, we constructed 2 internal deletion mutants and 15 N-terminal deletion mutants based on the T734 construct (Fig. 2A). Deletion mutants were named D(deleted amino acid positions).”

We confirmed the expression of these mutant proteins by Western blotting using mAb4C11 (Fig. 2C). As shown in Fig. 2B, one of the internal deletion mutants, D(579–649) retained IP₃ binding activity, whereas that with a further 11-amino acid deletion, D(568–649), completely lost the activity, indicating that the C-terminal boundary of the binding site was located between amino acid positions 568 and 578. This agrees with the C-terminal border at the 576-amino acid position of rat IP₃R₁, reported by Newton et al. (18). An N-terminal deletion mutant D(1–31) in the region showed that the first 31 amino acids were deleted lost almost all the IP₃ binding activity. Similarly, serial N-terminal deletions up to position 215 markedly reduced the IP₃ binding activity (D(1–62), D(1–146), D(1–183), D(1–199), and D(1–215)). On the contrary, with deleting further toward the C terminus up to amino acid positions 220, 223, and 225 (D(1–220), D(1–223), and D(1–225), respectively), the activity was retrieved. The D(1–223) mutant exhibited binding specificity similar to that of T734 ([1,4,5)IP₃ > (2,4,5)IP₃ > (1,3,4,5)IP₄].) IP₃, data not shown). However, further N-terminal deletions over the first 228 amino acids (D(1–228), D(1–238), D(1–261), D(1–283), and D(1–307)) completely abolished it again, indicating that the N-terminal boundary of the specific IP₃ binding site was located between amino acid positions 226 and 228.

Characterization of the 356-Amino Acid Polypeptide (Residues 224–579) Close to the IP₃ Binding Core—We expressed the 356-amino acid polypeptide (residues 224–579), which appeared to be about the minimum required for specific IP₃ binding. An expressed recombinant protein of approximately 42 kDa was detected using polyclonal rabbit antiserum raised against a synthesized peptide corresponding to amino acids 501–518 of mouse IP₃R₁ (Fig. 3A). The soluble fraction containing this 42-kDa protein exhibited significant IP₃ binding activity (Fig. 3B).
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from their Gln mutation counterparts. Therefore, the mutational effects shown in Fig. 5 are not attributable to the substituted Gln itself.

To further verify these findings, we mutated Lys-508, Arg-511, and Lys-569 to other positively charged residues (Lys→Arg and Arg→Lys) (Table I). The mutants K508R and R511K completely lost the activity. The other mutant, K569R, retrieved a little but retained only −20% activity of T734. As a result, even these functionally conservative mutations markedly reduced the IP3 binding of R568Q.

Binding Characteristics of R568Q—To evaluate the mutational effect on the IP3 binding properties of R568Q, which retained partial binding activity (Fig. 5), we performed a Scatchard analysis and inhibition studies (Fig. 6). The Kd value of R568Q (200 nM) (Fig. 6A) for (1,4,5)IP3 increased 4 times over that of wild-type T734 (50 nM) (Fig. 1D). R568Q exhibited different binding specificity ((1,4,5)IP3 = (2,4,5)IP3 > (1,3,4,5)IP4) (Fig. 6B) from that of the wild type ((1,4,5)IP3 > (2,4,5)IP3 > (1,3,4,5)IP4) (Fig. 1C). This difference in R568Q could be explained by the decrease in affinity for (1,4,5)IP3 and (1,3,4,5)IP4 and the increase in that for (4,5)IP2 and (2,4,5)IP3. On the other hand, other two mutants with partial binding activity, R504Q and R506Q, exhibited similar binding specificity to that of T734 ((1,4,5)IP3 > (2,4,5)IP3 > (1,3,4,5)IP4; data not shown).

DISCUSSION

It has been assumed that IP3R has a pocket with a highly restricted structure that specifically recognizes the IP3 molecule (20). What is the minimum number of amino acids required to assure the binding conformation, and which residues are present on the surface of the pocket? To address these questions by molecular biological means, we developed an E. coli expression system in which the N-terminal 734-amino acid residues of mouse IP3R1 (T734) are expressed as soluble protein. The binding affinity for (1,4,5)IP3 and specificity for various inositol phosphates of the expressed T734 proteins were similar to those of cerebellar IP3R variants, indicating that the binding sites expressed in this system form a functional conformation resembling the native binding site.

Structure of the IP3 Binding Site (Residues 226–578) —We found that, at most, 353 amino acid residues (residues 226–578) are present on the surface of the pocket. To address these questions by molecular biological means, we developed an E. coli expression system in which the N-terminal 734-amino acid residues of mouse IP3R1 (T734) are expressed as soluble protein. The binding affinity for (1,4,5)IP3 and specificity for various inositol phosphates of the expressed T734 proteins were similar to those of cerebellar IP3R variants, indicating that the binding sites expressed in this system form a functional conformation resembling the native binding site.

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substitutions of Gln for Arg-241, Lys-249, Arg-265, Arg-269, Arg-504, Arg-506, Lys-508, Arg-511, Arg-568, and Lys-569 markedly reduced the IP$_3$ binding activity. All 10 residues were identical among all IP$_3$Rs, suggesting that they are functionally important. Three of them (Arg-265, Lys-508, and Arg-511) were critical, since the IP$_3$ binding was completely abolished, even by substitution with a single amino acid. The reduction in IP$_3$ binding activity caused by these site-directed substitutions is possibly due to loss of the original side chain, which directly interacts with IP$_3$, or to disruption of a local or global conformation for the binding. These 10 residues are scattered within the binding core, which is consistent with the results from the deletion studies, and can be classified into four segments (the first containing Arg-241 and Lys-249; the second, Arg-265 and Arg-269; the third, Arg-504, Arg-506, Lys-508, and Arg-511; and the fourth, Arg-568 and Lys-569). We suggest that on the tertiary structure of IP$_3$R, these separated segments are positioned close to each other and form a positively charged pocket for binding to the negatively charged phosphate groups of IP$_3$.

Arg-269; the third, Arg-504, Arg-506, Lys-508, and Arg-511; and the fourth, Arg-568 and Lys-569. We suggest that on the tertiary structure of IP$_3$R, these separated segments are positioned close to each other and form a positively charged pocket for binding to the negatively charged phosphate groups of IP$_3$. The third segment is close to the residues 476–501, which were labeled by photoaffinity ligand, suggesting that it is in the proximity of the ligand binding site (19). Even functionally conservative mutations between Arg and Lys (K508R, R511K, and K569R) markedly reduced the IP$_3$ binding activity like Gln or Ala substitutions, suggesting that the restricted basic amino acid residues in the higher order structure are requisite for the binding.

![Fig. 5](image-url) Specific IP$_3$ binding activity of site-directed mutants carrying Gln substitutions for Arg and/or Lys residues. The specific IP$_3$ binding (B) of site-directed mutants that have Gln substitutions for Arg and/or Lys residues were normalized with that of the wild-type T734, taking into consideration the expression level (E) measured by densitometry (Millipore), as follows. The specific [{$^3$H}]IP$_3$ binding activity of the mutant (%) = (B of the mutant/B of the T734) × 100. Thirty- and three-μg soluble proteins prepared from transformed E. coli were used for the IP$_3$ binding assay and the Western blotting using mAb4C11, respectively. Nonspecific binding was measured in the presence of 2 μM IP$_3$. Values are the means ± S.D. of three separate experiments.

![Fig. 6](image-url) Binding characteristics of R569Q. A, Scatchard analysis of the inhibition of specific [{$^3$H}]IP$_3$ binding by (1,4,5)IP$_3$ to the soluble proteins prepared from transformed E. coli. The result of a typical experiment is shown. The mean K$_d$ from two separate experiments performed in duplicate was 200 nM. B, inhibition of specific [{$^3$H}]IP$_3$ binding to the soluble proteins (30 μg) from transformed E. coli by various inositol phosphates: (1,4,5)IP$_3$ (●), (2,4,5)IP$_3$ (○), (1,3,4,5)IP$_4$ (△), (4,5)IP$_2$ (□). Values are normalized to 100% of the control measured without competitor. Nonspecific binding was measured in the presence of 10 μM IP$_3$. Each point is the mean of two separate experiments performed in duplicate.

### Table I

| Mutant   | [{$^3$H}]IP$_3$ binding activity (pmol/mg) | % of control |
|----------|------------------------------------------|-------------|
| K508Q    | 2.1 ± 1.0                                 | 2.1 ± 1.0   |
| K508A    | 1.8 ± 0.46                                | 1.8 ± 0.46  |
| K508R    | 2.2 ± 0.90                                | 2.2 ± 0.90  |
| R511Q    | 3.1 ± 0.44                                | 3.1 ± 0.44  |
| R511A    | 2.6 ± 0.94                                | 2.6 ± 0.94  |
| R511K    | 1.8 ± 0.58                                | 1.8 ± 0.58  |
| K569Q    | 7.4 ± 3.0                                 | 7.4 ± 3.0   |
| K569A    | 12 ± 0.81                                 | 12 ± 0.81   |
| K569R    | 21 ± 1.6                                  | 21 ± 1.6    |
specific interaction with IP$_3$. In comparison with the wild-type T734, the mutant R568Q exhibited lower affinity for (1,4,5)IP$_3$ and a different binding specificity for various inositol phosphates, suggesting that Arg-568 is involved in not only high affinity binding with (1,4,5)IP$_3$, but also determination of binding specificity. Therefore, Arg-568 may be involved in recognition of the functional group at the equatorial position-1 of the inositol ring, since R568Q recognizes (4,5)IP$_2$ and (2,4,5)IP$_3$ with higher affinity but (1,4,5)IP$_3$ and (1,3,4,5)IP$_4$ with lower affinity than the wild type.

The function of the N terminus (residues 1-225)—This study indicated that the first 225 amino acids are not requisite for the specific IP$_3$ binding, although there is significant sequence homology (64% identity and 76% similarity) in this region of the IP$_R$ family. On the other hand, the deletion of only the first 31 amino acids resulted in a severe reduction in the binding activity. Such contradictory mutational effects were also found in serial N-terminal deletions up to residue 215. However, a deletion of 5 amino acids more toward the C terminus recovered the binding activity (D(1-220)). The binding activity was also recovered in the deletions as far as the residue 225 (D(1-223) and D(1-225)). These can be explained by two models. Serial deletions of the N terminus up to the residue 215 interfere with the higher order structure of the IP$_3$ binding site formed by the residues 226-576, or the residues 216-220 act as a part of the inhibitory determinant when deletions up to residue 215 are performed. Therefore, we tested whether the synthetic peptide (CNTSWKIVLFMK) corresponding to the residues 214-225 inhibits the IP$_3$ binding of the cerebellar IP$_R$, T734, or the mutant D(1-223). However, as far as we tested, this peptide had no significant inhibitory effect (data not shown) although we could not rule out the possibility that it does not inhibit the binding in an intermolecular manner.

The binding affinity for IP$_3$ of the core region (residues 224-579) was more than 10-fold higher than that of T734. This augmented affinity was probably due to deleting the N-terminal 223 amino acids from T734, since the mutant D(1-223) showed similar higher binding affinity (data not shown). Thus, we suggest that the N-terminal 225 amino acids are not directly responsible for, but may modulate, IP$_3$ binding (for example, the binding affinity).

Comparison of the N-terminal Portions between IP$_R$ and the Ryanodine Receptor (RyR)—RyR is another intracellular Ca$^{2+}$ release channel originally identified in the sarcoplasmic reticulum of skeletal muscle or cardiac muscle. IP$_R$ has fragmentary sequence homology with RyR in the N-terminal portion, suggesting involvement of this region in common receptor-channel function(s) (6, 36). This seems to be supported by the fact that a single mutation of Arg-615 of the type 1 RyR (RyR1) to Cys causes porcine malignant hyperthermia (37). The porcine malignant hyperthermia RyR1 channels are hypersensitive to various modulators, suggesting that the region around Arg-615 is a regulatory domain of channel opening, or that it is involved in binding an unknown channel activator. This region of RyR1 is fragmentarily homologous with the corresponding region (residues 621-659) of IP$_R$1 (Fig. 4). Besides this region, there are four homologous fragments within the N-terminal 580 amino acids of IP$_R$1 (residues 115-197, 227-252, 276-319, and 472-516) (6), of which three are within the IP$_3$ binding region defined in this study. Among 10 important basic residues for the IP$_3$ binding, three (Arg-241, Arg-504, and Arg-506) are conserved, but three critical residues (Arg-265, Lys-508, and Arg-511) are diverse in the RyR family (Fig. 4). Therefore, these homologous regions may be required for receptor-channel functions common in the intracellular Ca$^{2+}$ release channel superfamily, such as sensing activation signal(s), modulation, or the transduction of activating signal(s) to channel opening.

We defined the importance of basic amino acids for the binding of IP$_R$1 to IP$_3$, which is similar to that reported for some pleckstrin homology domains that bind IP$_3$ (27, 28). However, the overall constitution of the binding site seems to be different from these, which is reflected in the binding affinity and selectivity. On the other hand, IP$_R$ may recognize the hydroxy groups and the inositol ring of IP$_3$ (20, 38, 39). The amino acids involved in these interactions in the critical region defined here require further analysis.

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Ligand Binding Site of the IP$_3$ Receptor

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