Molecular Determinants of Ion Permeation and Selectivity in Inositol 1,4,5-Trisphosphate Receptor Ca\(^{2+}\) Channels*

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We tested the hypothesis that key residues in a putative intraluminal loop contribute to determination of ion permeation through the intracellular Ca\(^{2+}\) release channel (inositol 1,4,5-trisphosphate receptors (IP\(_3\)Rs)) that is gated by the second messenger inositol 1,4,5-trisphosphate (IP\(_3\)). To accomplish this, we mutated residues within the putative pore forming region of the channel and analyzed the functional properties of mutant channels using a 45Ca\(^{2+}\) flux assay and single channel electrophysiological analyses. Two IP\(_3\)R mutations, V2548I and D2550E, retained the ability to release 45Ca\(^{2+}\) in response to IP\(_3\). When analyzed at the single channel level; both recombiant channels had IP\(_3\)-dependent open probabilities similar to those observed in wild-type channels. The mutation V2548I resulted in channels that exhibited a larger K\(_o\) conductance (489 ± 13 pS) for V2548I versus 364 ± 5 pS for wild-type), but retained a Ca\(^{2+}\) selectivity similar to wild-type channels (P\(_{Ca^{2+}}$/P\(_{K^+}\) ≈ 4:1). Conversely, D2550E channels were nonselective for Ca\(^{2+}\) over K\(^+\) (P\(_{Ca^{2+}}$/P\(_{K^+}\) ≈ 0.6:1), while the K\(^+\) conductance was effectively unchanged (391 ± 4 pS). These results suggest that amino acid residues Val\(^{2548}\) and Asp\(^{2550}\) contribute to the ion conduction pathway. We propose that the pore of IP\(_3\)R channels has two distinct sites that control monovalent cation permeation (Val\(^{2548}\)) and Ca\(^{2+}\) selectivity (Asp\(^{2550}\)).

In response to a wide variety of external stimuli, the second messenger inositol 1,4,5-trisphosphate (IP\(_3\)) is generated from the phospholipase C-mediated hydrolysis of phosphatidylinositol bisphosphate (1). IP\(_3\) then diffuses through the cytosol and binds to the IP\(_3\) receptor (IP\(_3\)R), an intracellular ion channel that mediates the release of Ca\(^{2+}\) from the endoplasmic reticulum. IP\(_3\)-mediated increases in cytoplasmic [Ca\(^{2+}\)]modulate a diverse array of cellular processes (2).

The IP\(_3\)R channel is composed of four ~300-kilodaton subunits that together form a single ion conduction pore (3). The C terminus of each subunit is believed to contain six transmembrane helices and is separated from the N-terminal IP\(_3\) binding domain by a large intervening cytoplasmic region (4). The basic six transmembrane domain topology of IP\(_3\)Rs is shared with other cation channels, including the superfamily of voltage-gated ion channels, cyclic nucleotide-gated (CNG) ion channels, vanilloid receptors, and other members of the trp family of ion channels. By analogy, it has been suggested that putative transmembrane helices 5 and 6 and the intervening intraluminal loop constitute the ion permeation pathway of IP\(_3\)Rs (5, 6), a proposal that has been experimentally verified by deletion mutant analysis (7). The intraluminal loop of both IP\(_3\)Rs and ryanodine receptors (RyRs) contain a stretch of amino acids, GVGD and GIGD, respectively, that may be homologous to the selectivity filter of K\(^+\) channels. However, there is no direct evidence that residues within this region contribute to the ion conduction pathway of IP\(_3\)Rs. The present study is the first to identify key residues that regulate ion permeation and selectivity in IP\(_3\)R channels. We mutated three amino acids in this region and examined the functional consequences using a 45Ca\(^{2+}\) flux assay and single channel electrophysiological analyses, carried out by patch clamping nuclei isolated from transfected COS cells. Two mutants (V2548I and D2550E) of the rat type 1 IP\(_3\)R retained the ability to release 45Ca\(^{2+}\) in response to IP\(_3\). When analyzed at the single-channel level, mutant V2548I channels had a larger K\(^+\) conductance compared with wild-type channels with no alteration in the selectivity for divalent cations. However, substitution of Asp\(^{2550}\) with Glu resulted in channels that were nonselective for divalent cations without affecting K\(^+\) conductance. We propose that the molecular mechanism for divalent cation selectivity in IP\(_3\)Rs involves a ring of four aspartic acid residues at position 2550 that form a Ca\(^{2+}\) binding site within the ion conduction pathway, with ion conductance determined by valine at position 2548. Divalent cation interaction with two sites may enable multi-ion occupancy and high ion throughput in the IP\(_3\)R.

**EXPERIMENTAL PROCEDURES**

**Expression Constructs—**The neuronal rat type 1 SI (−), SIH(−), SIH(+) splice variant in pCMV was the kind gift of Dr. Thomas Südhof (University of Texas Southwestern Medical Center). This cDNA was cloned into pCDNA3.1 as described elsewhere (9). Mutations of Asp\(^{2550}\) (rat) to Glu, Asn, and Ala have been described previously (9). All other mutations were carried out using the QuickChange site-directed mutagenesis kit as per manufacturer’s instructions (Stratagene, La Jolla, CA). Sequences of the mutagenic primers used for the polymerase chain reactions are available upon request.

**Cell Culture and Transfection—**COS-7 cells were maintained and transfected as described previously (9).

**45Ca\(^{2+}\) Flux Measurements—**45Ca\(^{2+}\) flux assays were performed ex vivo in 1,2-bis(O-aminophenoyl)ethane-N,N,N’,N”-tetraacetic acid; CNG, cyclic nucleotide-gated; S, siemens.
Molecular Determinants of Ca"2+ Selectivity in IP"3R Channels

Actively as described previously (9, 10). Briefly, micromolar vesicles were prepared from COS-7 cells transiently transfected with either wild-type or mutated IP"3R constructs in conjunction with SERCA-2b (human). The vesicles were then assayed for 44Ca"2+ uptake in the presence of potassium oxalate and MgATP. The inclusion of IP"3 in the assay buffer caused a reduction in the initial rate of SERCA-dependent uptake, giving an indirect measure of IP"3R activity. This assay has been shown to measure 44Ca"2+ flux only through recombinant IP"3Rs (9, 10). The initial rate of uptake in the presence of IP"3 is presented as a percentage of the rate measured without IP"3.

Preparation of Cellular Homogenates for Patch Clamp Recording—Preparations of cellular homogenates for patch clamp recording of nuclei are described in detail elsewhere. Briefly, cells were gently homogenized in a motor-driven glass Teflon homogenizer in a buffer containing 0.25 M sucrose, 0.15 M KCl, 3 mM MgATP, 0.5 mM NaATP, pH 7.5. Cell integrity was monitored by trypan blue exclusion, and homogenization was allowed to proceed until ∼30% of cells were lysed. Cell lysates were stored in the same buffer on ice and used on the same day in patch clamping experiments.

Patch Clamping COS-7 Cell Nuclei—Approximately 10 μl of cellular homogenate was added to a dish containing 1 ml of bath solution (see “Patch Clamp Solutions”) and transferred to the stage of a microscope for patch clamping. Isolated nuclei, visually free of extraneous cellular debris, were localized by trypan blue staining and patch-clamped at room temperature. Single-channel currents were amplified using an Axopatch-1D amplifier (Axon Instruments, Foster City, CA) with anti-aliasing filtering at 1 kHz and transferred to a Power Macintosh 8100 via an ITC-16 interface (Instrutech Corp., Port Washington, NY). Data were digitized at 5 kHz and written directly to hard disc by Pulse + PulseFit software (HEKA Elektronik, Lambrecht/Pfalz, Germany).

Single-channel recordings were analyzed using TAC 3.03 (Bruxton, Seattle, WA) and plotted using Igor Pro 3 (WaveMetrics, Lake Oswego, OR) and SigmaPlot (SPSS Science, Chicago, IL). Permeability ratios were calculated using the experimentally determined reversal potentials as described elsewhere (11).

Patch Clamp Solutions—Bath solution contained 140 mM KCl, 10 mM HEPES, 500 μM BA TPα, 0.001% trypan blue, and 250 mM Ca"2+ adjusted to pH 7.1 with KOH. An additional 5 mM K"+ ions was contributed to the solution by the adjustment of the pH. Pipette solutions contained 140 mM KCl, 10 mM HEPES, 100 μM BA TPα, 0.5 mM NaATP, 10 μM IP"3, and 1.0 μM Ca"2+ adjusted to pH 7.1 with KOH. Free calcium concentrations in all buffers were determined using a Ca"2+-selective mini-electrode as described previously (12). For determinations of ionic selectivity, high Ca"2+ bath buffer contained 50 mM CaCl2, 30 mM KCl, 10 mM HEPES adjusted to pH 7.1 with KOH. Low K"+ pipette solutions contained 14 mM KCl, 10 mM HEPES, 100 μM BA TPα, 0.5 mM NaATP, 10 μM IP"3, and 1.0 μM Ca"2+ adjusted to pH 7.1 with KOH. All selectivity determinations were corrected for the liquid junction potential as described previously (13).

RESULTS AND DISCUSSION

The predicted transmembrane topology of the IP"3R and the putative location of the three residues, Val"2554, Asp"2559, and Asp"2569, examined in the present study are diagrammed in Fig. 1A. All three amino acids are within a predicted intraluminal loop between transmembrane helices 5 and 6, a region that contains the pore domain in other cation channels. Within this region is a sequence GYG D, which is highly reminiscent of the GYG D signature sequence of the selectivity filter of K"+ channels. The Tyr residue in K"+ channels plays a critical role in defining the size of the pore and thereby providing K"+ selectivity (14). In contrast, the selectivity filters of Ca"2+ -selective ion channels lack the corresponding acidic residues (15). The similar permeation properties of IP"3R, CNG, and voltage-gated Ca"2+ channels suggest that the IP"3R channel pore likely contains a high affinity binding site for Ca"2+ ions. In CNG- and voltage-gated Ca"2+ channels, this site is conferred by an acidic residue equivalent in sequence to Asp"2569 in the IP"3R channel (Fig. 1A). Because the IP"3R permeation properties are reminiscent of those of other Ca"2+ channels, but the putative pore sequence is more closely related to K"+ channels, we examined the effects of mutations of Val"2554 and Asp"2559 on the permeation properties of the IP"3R channel. Based on the roles of these residues in K"+ and Ca"2+ channels, respectively, we hypothesized that Val"2554 might influence conduction by its participation in the molecular interactions that control the size of the pore, whereas Asp"2559 may determine divalent cation selectivity. In addition, we also mutated Asp"2569, because mutation of the homologous residue in the RyR influenced its permeation properties (16).

Val"2554 was mutated to Ile or Tyr, the amino acids present at the analogous position in ryanodine receptors and K"+ channels, respectively (Fig. 1A). Asp"2559 and Asp"2569 were mutated to Glu, Asn, or Ala. All the mutant IP"3R channels were expressed at levels comparable with the wild-type channel when transiently transfected into COS-7 cells (Fig. 1B). Mutant channels were first screened for Ca"2+ release activity by measurements of IP"3-dependent 44Ca"2+ flux from microsomes prepared from COS cells co-transfected with both SERCA2b and IP"3R cDNAs (9). Mutating Val"2554 to Ile did not alter channel function in this assay, whereas no Ca"2+ release activity was observed with substitution by Tyr (Fig. 1C). As shown previously, Glu (but not Asn or Ala) substituted for Asp"2559 in the Ca"2+-release assay (9). Asp"2569, predicted to be the final residue before the beginning of the putative transmembrane helix 6 (Fig. 1A), was intolerant to substitutions (Fig. 1C). Mutation of the analogous position (D4917A) in the RyR type 1 channel also abolished Ca"2+ permeability (16).

Patch clamp electrophysiology of transfected COS cell nuclei was performed to examine the effects of the mutations on ion permeation. Our protocol enables recording of recombinant IP"3R channels specifically without contributions from endogenous IP"3R channels. K"+ was used as the permeant ion to minimize Ca"2+-dependent inactivation and maximize single channel conductance (17). Using these recording conditions, IP"3-activated channels were only detected in nuclear membrane patches from cells expressing wild-type, V2548I, or D2550E channels (data not shown). Open probabilities determined at 1 μM free Ca"2+ were similar for both mutant channels and wild-type channels (wild-type, 0.59 ± 0.07; V2548I, 0.65 ± 0.10; D2550E, 0.68 ± 0.13).

From analyses of current-voltage relationships determined in symmetrical 140 mM KCl, the slope conductance of V2548I channels was 489 ± 13 pS, which was significantly larger than that of wild-type channels (364 ± 5 pS). In contrast, the ion selectivity of the V2548I channel was unchanged compared with that of the wild-type channel. The monovalent cation:anion selectivity, determined from reversal potentials measured in the presence of a 10-fold KCl gradient (14 mM KCl pipette, 140 mM KCl bath) (11), was identical for V2548I and wild-type channels (Erev = +44.9 ± 0.5 mV; P"K/"Cl = 22:1; Fig. 1C). To determine the Ca"2+ selectivity, channels were first detected in symmetrical 140 mM KCl, and then the bath was replaced with a high Ca"2+ buffer (50 mM CaCl2, 30 mM KCl). Under these conditions, V2548I channels had a Erev of +19.3 ± 1.8 mV, corresponding to P"K/"Cl = 4:1 (Fig. 3D), not significantly different from that observed for wild-type IP"3R channels (data not shown)². Thus, these results demonstrate that mutation of Val"2554 to Ile increased channel conductance but was without effect on the ion selectivity properties of the pore.

The conductance of D2550E channels was similar to that of wild-type channels (391 ± 4 pS versus 364 ± 5 pS, respectively; Fig. 2B). The mutation was also without effect on the cation:anion selectivity (P"K/"Cl = 22:1; Fig. 1C). In contrast, the mutant channel had a significantly altered cation selectivity, 

Erev for the D2550E channel in the presence of a Ca"2+ gradient
FIG. 1. Mutations in the IP₃R C terminus affect ⁴⁵Ca²⁺ flux. A, each monomer of an IP₃R channel contains six transmembrane helices, with the pore of the channel believed to be formed by contributions from residues located between transmembrane helices 5 and 6 (7, 9). A hydrophobic segment immediately preceding the selectivity filter may be a pore helix, by analogy with the KcsA K⁺ channel (14). Residues Val²⁵⁴⁸, Asp²⁵⁵⁰, and Asp²⁵⁵⁰ were subjected to site-directed mutagenesis and are indicated in bold on the schematic and by arrows and residue number on the expanded sequence. The N terminus, which comprises the majority of the IP₃R protein, has been omitted from the schematic. The sequence alignment of the pore region of the type I inositol trisphosphate receptor (IP₃R-1), rabbit skeletal muscle ryanodine receptor (RyR-1), Shaker potassium channel, vanilloid receptor (VR1), bovine rod cyclic nucleotide-gated channel (bROD), repeat IV of the T-type Ca²⁺ channel (T-type IV), and repeat IV of the L-type Ca²⁺ channel (L-type IV) is shown. Darker shading indicates identity between all seven ion channels, and the gray shaded residues indicate homology. The putative selectivity filter of all channel families is indicated by a solid line. GenBank accession numbers (from top to bottom) are J05510, X15750, XM_008512, M17211, X16804, NM_018896, and L04569. B, COS cells were transfected with wild-type (WT) and mutant IP₃R constructs, and 20 μg of total cellular lysate was loaded onto a 5% SDS-polyacrylamide gel electrophoresis gel. 20 μg of cerebellum microsomes were measured in the presence of 1.0 m⁴⁵CaCl₂. Uptake rates were plotted as a function of control rate of uptake in the absence of IP₃. Therefore, Ca²⁺ release through the recombinant IP₃R channel would be indicated by a reduction in the initial rate of uptake when compared with control. Absence of response to IP₃ is indicated by a solid line. The ⁴⁵Ca²⁺ release activities of mutations D2550E, D2550N, and D2550A have been reported previously (9). *, significantly different from control (p < 0.001).

The present study has identified two residues (Val²⁵⁴⁸ and Asp²⁵⁵⁰) that, when mutated, affect the conductance or divalent cation selectivity of the IP₃R channel. Thus these two residues are likely to be part of the ion conduction pathway of IP₃Rs. This result, taken together with analyses of IP₃R topology (19, 20) and the sequence homology with the selectivity filter of K⁺ channels, confirms previous predictions that the pore region of the IP₃R is constructed in a manner analogous to that of several classes of voltage- and ligand-gated cation channels with the pore located between the last two transmembrane segments (5, 20). A similar conclusion was reached for the RyR channel (21).

In CNG- and voltage-gated Ca²⁺ channels, a conserved glutamate in the pore region is critical for Ca²⁺ selectivity (22, 23). Cysteine accessibility and pH sensitivity studies of these channels have suggested that the side chains of four of these residues (contributed by each repeat or monomer) likely project into the pore to coordinate the permeant Ca²⁺ ion and regulate divalent cation permeation (24, 25). The location of Asp²⁵⁵⁰ in the analogous position in the IP₃R channel and the effects of its mutation on divalent cation selectivity indicate that this residue functions similarly in the IP₃R channel. The fact that mutation of this residue to Glu with preservation of its charge affected the Ca²⁺ selectivity suggests that steric considerations as well as electrostatic forces contribute to the positioning of the ring of carboxylate groups in the pore. Functional non-equivalence of Glu and Asp residues at analogous positions in voltage-gated Ca²⁺ channels and CNG channels has also been noted previously (23, 26). One of the findings of the present study is that although the D2550E mutant exhibited diminished divalent cation selectivity, permeation of monovalent cations was unaffected, suggesting that a high affinity interaction at position 2550 is not rate-limiting for monovalent ion permeation. Similarly, monovalent ion permeability is not impaired when all four glutamates within the EEEE locus are mutated to Ala or Gln in voltage-gated Ca²⁺ channels (27). This is also the observed result when the analogous Asp is mutated to Asn in vanilloid receptors (28).

The conserved acidic residue that is critical for divalent cation selectivity and permeation in Ca²⁺-selective channels is adjacent to the GXG signature sequence in other cation channels (Fig. 1A). The crystal structure of the Streptomyces lividans K⁺ channel indicates that this acidic residue is exposed at the outer mouth of the pore (14). A distinction between the IP₃R channel on one hand, and CNG channels and voltage-gated Ca²⁺ channels on the other, is that the latter have deletions of two amino acids (Fig. 1A) between the initial glycine and the acidic residue of the signature sequence, whereas the IP₃R channel contains them. The crystal structure of the K⁺ channel has shown that the rigid arrangement of backbone carbonyl oxygen atoms of the selectivity filter within the GYG sequence is responsible for the precise coordination of K⁺ ion in the pore (14). In this structure the side chain of the central tyrosine residue is directed away from the pore and makes specific interactions with other parts of the protein that contribute to holding the pore at a fixed size (14). Mutation of the GYG sequence in IP₃R to the GYG sequence of K⁺ channels resulted in an absence of detectable IP₃R channel activity. On the other hand, mutation to GIG increased the K⁺ conductance. It is interesting to note that the single channel conductance of RyR, which contains the GIG sequence, is greater than that of IP₃R (16, 18) and that the reverse mutation of Ile in the RyR to...
Val, as in IP$_3$R, confers a lower conductance on RyR channels (16). Thus, this residue likely plays a role in IP$_3$R channels in contributing to the mechanisms that determine the size of the pore, which in turn contribute to the magnitude of alkali metal cation conductance.

We propose a model in which the pore of IP$_3$R channels has two distinct sites that control K$^+$ ion permeation (Val$_{2548}$) and divalent cation selectivity (Asp$_{2550}$). The interaction of multi-ion occupancy of the pore, which could promote high cation conductance.

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**REFERENCES**

1. Berridge, M. J., and Irvine, R. F. (1989) *Nature* **341**, 197–205
2. Clapham, D. E. (1995) *Cell* **80**, 259–268
3. Patel, S., Joseph, S. K., and Thomas, A. P. (1999) *Cell Calcium* **35**, 247–264
4. Joseph, S. K., Boehning, D. B., and Lin, C. (1999) in *Methods in Calcium Signaling Research* (Putney, J. W., ed) CRC Press, Boca Raton, FL
5. Mignery, G., and Sudhof, T. C. (1993) *Methods Neurosci.* **18**, 247–265
6. Michikawa, T., Hamanaka, H., Otsu, H., Yamamoto, A., Miyawaki, A., Furutchi, T., Tashiro, Y., and Mikoshiba, K. (1994) *J. Biol. Chem.* **269**, 9184–9199
7. Ramos-Franco, J., Galvan, D., Mignery, G., and Fill, M. (1999) *J. Gen. Physiol.* **114**, 243–250
8. Joseph, S., and Samanta, S. (1993) *J. Biol. Chem.* **268**, 6477–6486
9. Boehning, D., and Joseph, S. K. (2000) *J. Biol. Chem.* **275**, 21492–21499
10. Boehning, D., and Joseph, S. K. (2000) *EMBO J.* **19**, 5450–5459
11. Hille, B. (1992) * Ionic Channels of Excitable Membranes*, Sinauer Associates, Sunderland, MA
12. Sigel, E., and Affolter, H. (1987) *Methods Enzymol.* **141**, 25–36
13. Neher, E. (1995) in *Single Channel Recording* (Sakmann, B., and Neher, E. eds) pp. 147–153, Plenum Press, New York
14. Doyle, D. A., Cabral, J. M., Pfuetzner, R. A., Kuo, A., Galbis, J. M., Cohen, S. L., Chait, B. T., and MacKinnon, R. (1998) *Science* **280**, 69–77
15. Mak, D. O., and Foskett, J. K. (1998) *Ann. J. Physiol.* **275**, C179–C188
16. Gao, L., Balshaw, D., Xu, L., Tripathy, A., Xin, C., and Meissner, G. (2000) *Biophys. J.* **79**, 828–840
17. Mak, D. O., and Foskett, J. K. (1994) *J. Biol. Chem.* **269**, 28375–28378
18. Tinker, A., and Williams, A. J. (1992) *J. Gen. Physiol.* **100**, 479–493
19. Joseph, S. K., Boehning, D., Piersson, S., and Nichitica, C. V. (1997) *J. Biol. Chem.* **272**, 1579–1588
20. Galvan, D. L., Borrego-Diaz, E., Perez, P., and Mignery, G. A. (1999) *J. Biol. Chem.* **274**, 28483–28492
21. Zhao, M. C., Li, P., Li, X. L., Zhang, L., Winkfein, R. J., Chen, and SRW. (1999) *J. Biol. Chem.* **274**, 25971–25974
22. Park, C. S., and MacKinnon, R. (1995) *Science* **341**, 13328–13333
23. Ellinor, P. T., Yang, J., Sather, W. A., Zhang, J., and Tsien, R. W. (1995) *Neuron* **15**, 1121–1132
24. Wu, X., Edwards, H. D., and Sather, W. A. (2000) *J. Biol. Chem.* **275**, 31778–31785
25. Root, M. J., and MacKinnon, R. (1994) *Science* **265**, 1852–1856
26. Seifert, R., Eismann, E., Ludwig, J., Baumann, A., and Kaupp, U. B. (1999) *EMBO J.* **18**, 119–130
27. Cibulsky, S. M., and Sather, W. A. (2000) *J. Gen. Physiol.* **116**, 349–362
28. Garcia-Martinez, C., Morellina-Palao, C., Planells-Cases, R., Merino, J. M., and Ferrier-Montel, A. (2000) *J. Biol. Chem.* **275**, 32552–32558