Two-state Conformational Changes in Inositol 1,4,5-Trisphosphate Receptor Regulated by Calcium

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Inositol 1,4,5-trisphosphate receptor (IP₃R) is a highly controlled calcium (Ca²⁺) channel gated by inositol 1,4,5-trisphosphate (IP₃). Multiple regulators modulate IP₃-triggered pore opening by binding to discrete allosteric sites within IP₃R. Accordingly we have postulated that these regulators structurally control ligand gating behavior; however, no structural evidence has been available. Here we show that Ca²⁺, the most pivotal regulator, induced marked structural changes in the tetrameric IP₃R purified from mouse cerebella. Electron microscopy of the IP₃R particles revealed two distinct structures with 4-fold symmetry: a windmill structure and a square structure. Ca²⁺ reversibly promoted a transition from the square to the windmill with relocations of four peripheral IP₃-binding domains, assigned by binding to heparin-gold. Ca²⁺-dependent susceptibilities to limited digestion strongly support the notion that these alterations exist. Thus, Ca²⁺ appeared to regulate IP₃ gating activity through the rearrangement of functional domains.

Inositol 1,4,5-trisphosphate receptor (IP₃R) is a tetrameric ion channel that release Ca²⁺ from intracellular stores in response to the binding of 1,4,5-trisphosphate (IP₃), a second messenger generated by various extracellular stimuli, neurotransmitters, neuromodulators, hormones, and lights (1, 2). The IP₃R is widely distributed in living systems and plays pivotal roles in fundamental processes including fertilization, cellular proliferation and differentiation, cellular signaling, and vesicle secretion (2). Molecular cloning studies have revealed that there are three isoforms of IP₃R and that alternative splicing results in several variants of the IP₃R (2). These divergent primary structures of the IP₃R and their differential distributions have been assumed to award the functional diversity of IP₃R by nature.

The most characterized type 1 IP₃R (IP₃R1), a predominant type in rodent cerebellar endoplasmic reticulum (ER) and spine apparatus, plays an integral role in Ca²⁺ signaling (3–5) and neural plasticity (6, 7). The protomer of IP₃R1, a 2749-amino acid polypeptide (M, 313,000), contains the IP₃-binding core (residues 226–578), membrane-spanning domains (residues 2276–2589), and widespread allosteric sites for intracellular effector molecules (Ca²⁺, calmodulin, and ATP) and for phosphorylation by protein kinases (cAMP-dependent protein kinase, protein kinase C, cGMP-dependent protein kinase, Ca²⁺/calmodulin-dependent protein kinase II, and tyrosine kinase) (2). These cumulative allosteric regulations imply a structural paradigm for global conformational changes within the higher ordered structure of IP₃R1.

Because Ca²⁺ rigorously determines the channel activity of IP₃R and Ca²⁺-dependent behavior of IP₃R is considered to be crucial for spatiotemporal organizations of Ca²⁺ signaling (1, 4), the most important regulator for IP₃R is Ca²⁺/per se. Previous functional analysis indicates that a low Ca²⁺ level acts as an essential coagonist for IP₃-gated Ca²⁺ release and a high Ca²⁺ level inversely acts as a feedback repressor (4, 8, 9) via Ca²⁺/calmodulin in part (10). Thereby we assumed that Ca²⁺ could induce alterations in conformational states of IP₃R1 underlying such dynamic regulations. An investigation of this hypothesis requires information about structural rearrangements that has heretofore been unclear because of the structural polymorphism within IP₃R particles, which is partially due to their fragile architectures, presented by previous electron microscopic studies (11–14). To address this issue, we improved rapid purification of the IP₃R1 channel so that we could use electron microscopic study to visualize the domain arrangement and to investigate its structural change by Ca²⁺.

**EXPERIMENTAL PROCEDURES**

Purification of IP₃R—Immunoadsorption purification of IP₃R was performed as described previously (15) with the following modifications. Microsomal membrane (3 mg/ml), prepared from mouse cerebella, was solubilized in 50 mM Tris buffer (pH 7.5) containing 1% (w/v) CHAPS, 150 mM KCl, 2 mM diithiothreitol, 200 mM phenylmethylsulfonyl fluoride, 10 mM pepstatin A, 10 mM leupeptin, 10 µM E-64, and 0.2 mM CaCl₂ or 1 mM EDTA. The solubilized IP₃R was mixed with pepAb-immobilized beads, incubated at 4 °C, washed, and eluted with 20 mM pep 6. The [H]IP₃-binding assay was performed as described previously (11).

Electron Microscopy—The purified IP₃R (0.5 µl) was injected into 9.5 µl of 50 mM Tris buffer (pH 7.5) containing 1 mM CaCl₂, 1 mM EDTA, or 1 mM EGTA and incubated for 30 min on ice. For heparin-gold labeling, purified IP₃R was mixed with a 1–10-fold molar excess of heparin-gold (Sigma) and incubated for 10 min on ice. An aliquot of the mixture (4 µl) was applied onto carbon-coated copper grids. Excess solution was removed by filter paper, and the IP₃R particles were stained with 2 µl of 1% (w/v) uranyl acetate solution. Dried grids were examined on a JEM 1200 EX transmission electron microscope (JEOL).
operated at 80-kV acceleration voltage. Micrographs were taken at magnifications ranging from ×25,000 to ×50,000.

Partial Proteolysis—Partial proteolysis experiments were carried out in the solution containing IP$_3$R1 and lysyl endopeptidase (Lys-C). Reaction mixtures were incubated for 30 min at 37 °C. The proteolysis was stopped by heat treatments at 55 °C for 15 min in the sample buffer including SDS. Proteolytic fragments were analyzed by discontinuous PAGE (5 or 10% gel) and immunoblotting using monoclonal antibodies 18A10 and 4C11 (11, 15).

RESULTS AND DISCUSSION

We purified IP$_3$R1 to apparent homogeneity as judged by gel electrophoresis (Fig. 1A) from mouse cerebella, which was functionally active as estimated by the specific activity of maximum binding to IP$_3$ (3 nmol/mg of protein). The purified IP$_3$R1 was negatively stained and imaged in a transmission electron microscope. Electron microscopy explicitly showed two distinct structures in negatively stained samples. One was a windmill-like structure (Fig. 1B), and another was a square-shaped structure (Fig. 1C). The windmill structure contained four segregated radial wings and a central core. Each wing structure appeared to be composed of two domains: a globular domain, which often exhibited a central spot that was densely stained, and a constricted segment forming a bridge between the globular domain and the central core domain. The dimension of the windmill structure was 31 ± 2 nm ($n$ = 76) from the tip of one wing to the tip of the opposite wing. The globular domain in the wing was 8.1 ± 0.8 nm ($n$ = 30) in diameter, and the central core was 9.8 ± 0.6 nm ($n$ = 15) in diameter. The shape and dimensions of the windmill structure are consistent with those previously reported for IP$_3$R isolated from smooth muscle (12).

The dimensions of the square structure were 19 ± 1 nm ($n$ = 54) on a side and 24 ± 1 nm ($n$ = 54) on a diagonal line (Fig. 1C), similar to that of small dense projections in the smooth ER of rat Purkinje cells (14). Comparison with the dimensions of ryanodine receptor, another intracellular Ca$^{2+}$ channel (16), provides support that the projected size of the square structure in this study is reasonable because of the ratio in the molecular mass of the protomer. We also found that the IP$_3$R1 particles appeared as other forms, suggesting their variances of orientation or intrinsic flexibilities.

Our microscopic data revealed two distinct states of the IP$_3$R1 molecule, leading us to the hypothesis that the conformation of IP$_3$R1 alters. We tried to capture a Ca$^{2+}$-dependent transition between the dual structures by imaging IP$_3$R1 particles injected into 1 mM CaCl$_2$, 1 mM EDTA, or 1 mM EGTA. Significantly the windmill structures were abundantly observed in the presence of Ca$^{2+}$ (Fig. 2A). In contrast, the relative abundance of windmill structures appeared to decrease in specimens prepared in solution containing 1 mM EDTA (Fig. 2B). For statistical evaluation, we counted the windmill particles with more than three identifiable wings and the square forms with a homologous dimension, which had no wing, on electron micrographs. Quantitative analysis clearly indicates a significant difference in the ratio of the two structures in a Ca$^{2+}$-dependent manner (Fig. 2C). Readdition of CaCl$_2$ into the IP$_3$R1 purified with EDTA restored the windmill configuration, and the number of windmill structures showed a marked reduction upon readdition of EDTA into the IP$_3$R1 purified in the presence of Ca$^{2+}$, indicating that the structural rearrangements are reversible (Fig. 2C). IP$_3$ did not induce significant changes in each state at this resolution, thus the binding of IP$_3$ may cause a fine structural change to open the channel.

Our findings provide the first evidence of structural alterations in IP$_3$R1 molecules. The structural alteration could account for the polymorphism in IP$_3$R particles presented by previous electron microscopic studies (11–14). The unique architectures and conspicuous conformational changes within the IP$_3$R1 particle differ remarkably from those in the ryanodine receptor particle (17, 18). These differences might result from intrinsic properties of the gating machinery of IP$_3$R1.

To correlate the structural changes in IP$_3$R1 observed by electron microscopy with changes in solution, we monitored its sensitivity to limited protease digestion. The patterns of degradative intermediates were clearly Ca$^{2+}$-dependent (Fig. 3). In particular, a 38-kDa fragment detected with 4C11 within the IP$_3$-binding domain was markedly generated by cleavage in CaCl$_2$ solution; however, a 48-kDa intermediate and a 38–60-kDa ladder of bands detected with 4C11 were dominantly observed in EDTA solution (Fig. 3B). Furthermore a C-terminal 130-kDa fragment detected with 18A10 was abundantly detected by cleavage of purified IP$_3$R1 in CaCl$_2$ solution compared with 85- and 120-kDa fragments (Fig. 3B). These results suggest structural changes in purified IP$_3$R1 rather than a simple acceleration or regulation of proteolysis by Ca$^{2+}$. The degradation by contaminant protease, such as Ca$^{2+}$-activated cathepsins, was insignificant because of negligible production of digested proteins without Lys-C (Fig. 3). In addition, we also investigated the dynamic property of IP$_3$R1 in crude microsomal membrane. The 38- and 30-kDa fragments detected with 4C11 were evenly precipitated in both CaCl$_2$ and EDTA solutions; however, these fragments were more releasable to supernatant fractions in the presence of CaCl$_2$ than in EDTA (Fig. 3C). We also confirmed the reproducibility of these proteolysis experi-
ments by using 0.5 mM EGTA and 0.2 mM CaCl_2 solution. This heightened ability to release may be related to the structural changes within IP_3.R1 embedded in the microsomal membrane. Taken together, these biochemical data strongly support the presence of structural alterations in IP_3.R1.

To determine how functional domains are arranged, we used colloidal gold conjugated with heparin, which is a competitive inhibitor of IP_3 binding (19) and specifically binds to the N-terminal IP_3-binding region (20). Heparin-gold particles bound not only to the windmill structure but also to the square form (Fig. 4, A and B). In the windmill structure, the gold particles bound to the globular domain of the radial wings (Fig. 4A). In the square form, the gold particles attached at the sites close to the corners (Fig. 4B). We assigned these heparin-binding domains to the N-terminal IP_3-binding domain. Our results provide the first evidence of domain arrangement in quaternary configurations of the IP_3.R1 particle. In both states, the distribution of peripheral IP_3-binding domains occurred away from the center, a plausible Ca^{2+} gateway, by over 5 nm, suggesting that a long range allosteric transmission took place underlying the IP_3-gated Ca^{2+} release.

The comparison between dual structures and mapping of heparin-binding sites indicates that the structural transition from square to windmill is caused by the relocation of functional domains. Therefore, we propose a "flapping model" for the large scale rearrangements in IP_3.R1 (Fig. 4C). The windmill structure may be a consequence of the IP_3-binding domain splitting from the channel domain. The dynamic flapping may be mediated by the bridge domain, which may act as a hinge structure. Additionally, the digested IP_3.R retains the assembly of domains under Ca^{2+}-free conditions (21, 22); thus interdomain coupling may also stabilize the more compact square structure. The Ca^{2+}-dependent cleavage sites and releasable regions presented here are candidates for the apparent hinge and interface structures linking between functional domains.

Our findings show that the functional regulator altered the relative locations of IP_3-binding and channel domains even if there is no IP_3, further emphasizing that the domain arrangement is crucial for the transmission of IP_3 binding cues to Ca^{2+} pores. Two discrete configurations of IP_3.R1 imply dual relay modes controlling the allosteric transmission. Which of two structures is more active? As it appears to have an advantage for direct transferring of conformational changes by IP_3 binding toward the central channel, we cannot exclude the possibility that the compact square form is an active state. Based on single channel analysis, however, high Ca^{2+} only acts as an activator toward purified IP_3.R1 (10). Hence it is favorable that the windmill structure is more active. In this case, it is conceivable that the bridge domain presents an effective relay of ligand binding signals. This notion fascinates us as a new type of mechanical control on ligand gating behavior. The three-dimensional structure at much higher resolution will answer our central question on the precise pathway of allosteric transmission from the IP_3-binding core to the Ca^{2+} gateway underlying IP_3-gated channel opening.

Ca^{2+} dependence of IP_3.R is known to interplay with the allosteric regulation by ATP (23) and the cooperative gating by IP_3 (24). Therefore the Ca^{2+}-dependent global conformational changes may concern primary states for other allosteric ligands. Since IP_3.R is known to interact with phosphatidylinositol 4,5-bisphosphate incorporated in the plasma membrane (25), with the transient receptor potential protein, which is a calcium channel assumed to be involved in capacitative Ca^{2+} entry (26), and with the Homer protein linking with metabotropic glutamate receptor involved in neural plasticity (27), it is
interesting to study how the dramatic conformational change of IP₃R1 alters the association with these signaling molecules. Our novel model for structural rearrangements and the methodology presented here should be useful for understanding of the further biological significance of structural plasticity within IP₃R1 in neural systems.

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