

Calmodulin inhibits inositol 1,4,5-trisphosphate-induced calcium release through the purified and reconstituted inositol 1,4,5-trisphosphate receptor type 1

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Abstract Our previous studies have demonstrated that calmodulin binds to IP₃R type 1 (IP₃R1) in a Ca²⁺ dependent manner, which suggests that calmodulin regulates the IP₃R1 channel. In the present study, we investigated real-time kinetics of interactions between calmodulin and IP₃R1 as well as effects of calmodulin on IP₃-induced Ca²⁺ release by purified and reconstituted IP₃R1. Kinetic analysis revealed that calmodulin binds to IP₃R1 in a Ca²⁺ dependent manner and that both association and dissociation phase consist of two components with time constants of $k_a = 4.46 \times 10^2$ and $> 10^4$ M⁻¹ s⁻¹, $k_d = 1.44 \times 10^{-2}$ and 1.17×10^{-1} s⁻¹. The apparent dissociation constant was calculated to be 27.3 μM. The IP₃-induced Ca²⁺ release through the purified and reconstituted IP₃R1 was inhibited by Ca²⁺/calmodulin, in a dose dependent manner. We interpret our findings to mean that calmodulin binds to IP₃R1 in a Ca²⁺ dependent manner to exert inhibitory effect on IP₃R channel activity. This event may be one of the mechanisms governing the negative feedback regulation of IP₃-induced Ca²⁺ release by Ca²⁺.

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Key words: Calmodulin; Inositol 1,4,5-trisphosphate; Inositol 1,4,5-trisphosphate receptor; Surface plasmon resonance

1. Introduction

Inositol 1,4,5-trisphosphate (IP₃) receptor (IP₃R), an IP₃-induced Ca²⁺ releasing channel located on intracellular Ca²⁺ stores, plays a crucial role in a variety of cell functions, including fertilization, cell proliferation, metabolism, secretion, contraction of smooth muscle and neural signals [1,2]. IP₃-induced Ca²⁺ release (IICR) is regulated by cytosolic free Ca²⁺, in a biphasic manner [3,4]. Open probability of IP₃R induced by IP₃ showed a bell-shaped curve, depending on cytosolic Ca²⁺ concentrations, determined using the cerebellar

microsomal fraction incorporated into lipid bilayer. Increasing cytosolic Ca²⁺ concentration up to 300 nM increased the open probability of IP₃R, whereas higher Ca²⁺ concentration led to inhibition [3]. In addition, rapid increases in Ca²⁺ concentration induced by flash photolysis of the caged Ca²⁺ during IICR immediately altered the rate of IICR, in either a positive or negative manner. This positive or negative feedback regulation depended on increases in cytosolic Ca²⁺. Although the bell-shaped Ca²⁺ dependence of IP₃-induced Ca²⁺ release is considered to be fundamental to generate a complex pattern of spatio-temporal Ca²⁺ increase in cells, such as Ca²⁺ wave and Ca²⁺ oscillation, the mechanism of the bell-shaped dependence of IICR has remained unknown. The inhibitory effects of cytosolic Ca²⁺, however, were absent, when IP₃R was purified and reconstituted [5,6]. In addition, inhibitory effects of cytosolic Ca²⁺ on IICR depend on the isoform of IP₃R, in which high concentrations of Ca²⁺ inhibited IP₃R type 1 (IP₃R1) and IP₃R type 2 (IP₃R2), but not IP₃R type 3 (IP₃R3) [7]. These observations suggest that such effects by Ca²⁺ on IICR may mediate effect of some Ca²⁺ sensing proteins.

Our previous studies demonstrated that IP₃R1 binds calmodulin in a Ca²⁺ dependent manner suggesting that calmodulin regulates the function of IP₃R1 [8,9]. The calmodulin binding domain identified in IP₃R1 is conserved in IP₃R type 2 (IP₃R2) but not in IP₃R3 [9]. To test the thesis that calmodulin may regulate IP₃R1 channel function, we investigated kinetics of interaction between IP₃R1 and calmodulin, using Biomolecular Interaction Analysis system, BIAcore 2000 TM (BIAcore AB). This system is a biosensor which makes use of the phenomenon of the surface plasmon resonance and enables us a real-time analysis of specific protein-protein interaction. Effects of calmodulin on the purified and reconstituted IP₃R1 channel activity were also investigated.

2. Materials and methods

2.1. Materials

The following reagents were purchased. IP₃ from Dojindo Laboratories (Kumamoto, Japan), Chelex-100 from Bio-Rad Laboratories (Hercules, CA, USA) and ⁴⁵Ca²⁺ from NEN (Boston, MA, USA). All the other reagents were of analytical grade or the highest grade available.

2.2. Purification of IP₃R type 1 (IP₃R1) and calmodulin

IP₃R1 was purified type specifically from mouse cerebellar microsomal fractions, using an immunoaffinity column conjugated with a polyclonal antibody against IP₃R1 as described [10]. Calmodulin was

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Abbreviations: IP₃, D-myo-inositol 1,4,5-trisphosphate; IP₃R, IP₃ receptor; IP₃R1, IP₃R type 1; IP₃R2, IP₃R type 2; IP₃R3, IP₃R type 3; HEPES, N-(2-hydroxyethyl) piperazine-N'-2-ethanesulfonic acid; EGTA, ethylene glycol-bis(β-amino-ethyl ether) N,N,N',N'-tetra acetic acid; IICR, IP₃-induced Ca²⁺ release; NHS, N-hydroxysuccinimide; EDC, N-ethyl-N'-(3-diethylaminopropyl)-carbodiimide

purified from bovine brain by TCA precipitation and column chromatography as described previously [11].

2.3. Immobilization of the purified IP₃R1 on a sensor chip

The purified IP₃R1 was covalently immobilized on the surface of a sensor chip through its polysaccharide chain in order to maintain the same orientation against the sensor chip, according to the manufacturer's protocol (BIAcore AB) (Fig. 1). Briefly, the purified IP₃R1 was oxidized with 1 mM sodium metaperiodate in 100 mM sodium acetate buffer pH 5.5 for 20 min on ice to introduce aldehyde groups into the polysaccharide chain. The reaction mixture was passed over the desalting column, NAP5 (Amersham Pharmacia Biotech), to remove unreacted reagents. The NHS/EDC-activated carboxymethylated dextran sensor chip, CM5 (BIAcore AB), was reacted with 5 mM hydrazine hydroxide to introduce hydrazide on the sensor chip, and the residual unreacted esters were inactivated with 1 M ethanol amine hydrochloride. The activated sensor chip was then covalently conjugated with the oxidized IP₃R1, by aldehyde coupling. Following the conjugation, the resulting hydrazone bond was reduced with 100 mM of sodium cyanoborohydride in 10 mM sodium acetate buffer pH 4.5 to form a stable hydrazide bond. All solutions used in these procedures contained 0.5% Triton X-100.

2.4. Kinetic analysis of interaction between IP₃R1 and calmodulin

The interaction between IP₃R1 and calmodulin was investigated using BIAcore 2000 (BIAcore AB) in running buffer (100 mM KCl, 1 mM 2-mercaptoethanol and 20 mM HEPES/KOH 7.4) supplemented with 2 mM CaCl₂ or 2 mM EGTA at 25°C. For the association phase, various concentrations of calmodulin were injected over the surface of the IP₃R1 sensor chip with a flow rate of 30 µl/min for 60 s. Dissociation of the calmodulin from the immobilized IP₃R1 was then observed. The amount of binding protein is represented as an arbitrary unit (resonance unit, RU). There is a linear relationship between the mass of protein bound and the observed RU (1000 RU = 1 ng/mm²).

2.5. Reconstitution of purified IP₃R1 and IP₃-induced ⁴⁵Ca²⁺ influx measurements

The purified IP₃R1 was reconstituted into liposomes as described [10]. IP₃-induced ⁴⁵Ca²⁺ influx into the purified and reconstituted IP₃R1/liposome was measured as described [10] in the presence of 200 µM free Ca²⁺ and 10 µM ⁴⁵Ca²⁺ together with various concentrations of calmodulin. The IP₃R1/liposome was incubated with 1 µM of IP₃ for 1 min at room temperature in order to induce Ca²⁺ influx into the liposome. After removal of extravesicular Ca²⁺ and ⁴⁵Ca²⁺ by Chelex-100, ⁴⁵Ca²⁺ trapped inside the liposome was measured using a liquid scintillation counter.

3. Results and discussion

IP₃-induced Ca²⁺ release (IICR) is regulated by cytosolic free Ca²⁺ in a biphasic manner [3,4], a process considered to

be a fundamental mechanism for generation of a complex spatio-temporal Ca²⁺ increase in cells, such as Ca²⁺ wave and Ca²⁺ oscillation. The inhibitory effects of cytosolic Ca²⁺ were absent or faint when IP₃R1 was purified and reconstituted [5,6], which suggests the possibility that such effects may mediate some Ca²⁺ sensing proteins. Recently, it was reported that the bell-shaped dependence of Ca²⁺ of IICR depended on the isoform of IP₃R, in which inhibitory effects of high concentration of Ca²⁺ were observed in both IP₃R1 and IP₃R2 but not in IP₃R3 [7]. Calmodulin is a Ca²⁺ binding/sensing protein which exerts cellular function in response to Ca²⁺ increase by binding to target proteins. Earlier studies demonstrated that IP₃R1 binds calmodulin in a Ca²⁺ dependent manner [8,9], which suggests that calmodulin regulates the function of IP₃R1. To examine the possibility that calmodulin regulates IP₃R1 channel function, we investigated kinetics of the interaction between IP₃R1 and calmodulin and effects of calmodulin on the purified IP₃R1 channel activity.

To study kinetics of the interaction, we used Biomolecular Interaction Analysis system, BIAcore 2000 TM (BIAcore AB), which allows one to analyze a real-time kinetics of specific protein-protein interactions. We analyzed kinetics of the interaction between IP₃R1 and calmodulin by injecting various concentrations of calmodulin in the presence or absence of Ca²⁺ on the IP₃R1 immobilized sensor chip.

Fig. 1 shows the scheme of preparation of the orientation specific immobilization of IP₃R1 on sensor chip. As the IP₃R1 has *N*-glycosylation sites within the luminal portion (residues 2475 and 2503 of mouse IP₃R1) [12], immobilization of IP₃R1 through its sugar chain could maintain IP₃R1 in the same orientation against the sensor chip. The IP₃R1 sensor chip prepared using this procedure was stable for repetitive use and responses were reproducible.

To examine effects of the concentration of IP₃R1 immobilized on binding responses, we prepared three flow cells with different amounts of IP₃R1. Flow cells (Fc) 1, 2, and 3 were conjugated with different amounts of IP₃R1 by changing reaction time during aldehyde coupling, and Fc4 served as a blank (without IP₃R1). For the association phase, calmodulin was injected into the Fc1–Fc4 for 60 s with a flow rate of 30 µl/min. The dissociation of calmodulin from the immobilized IP₃R1 was then observed. Fig. 2A shows typical sensorgrams of the interaction between the immobilized IP₃R1 and 10 µM

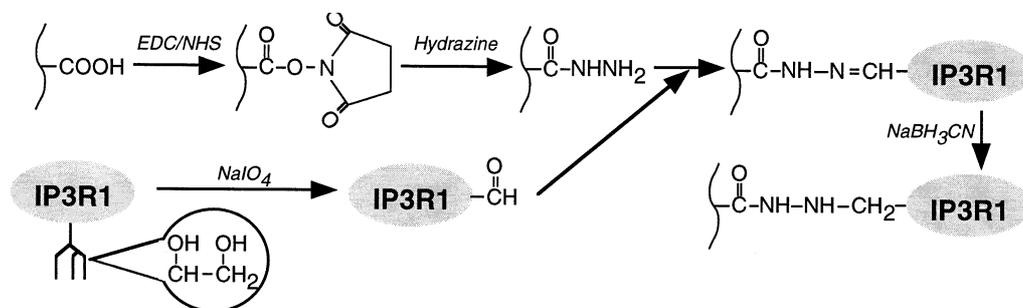


Fig. 1. Schematic representation of the orientation specific immobilization of IP₃R1. Schematic representation of the reaction for the immobilization of IP₃R1 through the polysaccharide chain. Carboxymethylated dextran sensor chip, CM5 (BIAcore AB) was activated by a series of the reactions with EDC/NHS and hydrazine to introduce hydrazide on the sensor chip. The purified IP₃R1 was oxidized with metaperiodate to introduce aldehyde groups to the polysaccharide chain located on the luminal portion of IP₃R1. IP₃R1 was then immobilized on the sensor chip by aldehyde coupling, which followed reduction of hydrazone bond by cyanoborohydride. This procedure allows one to immobilize IP₃R1 in the same orientation.

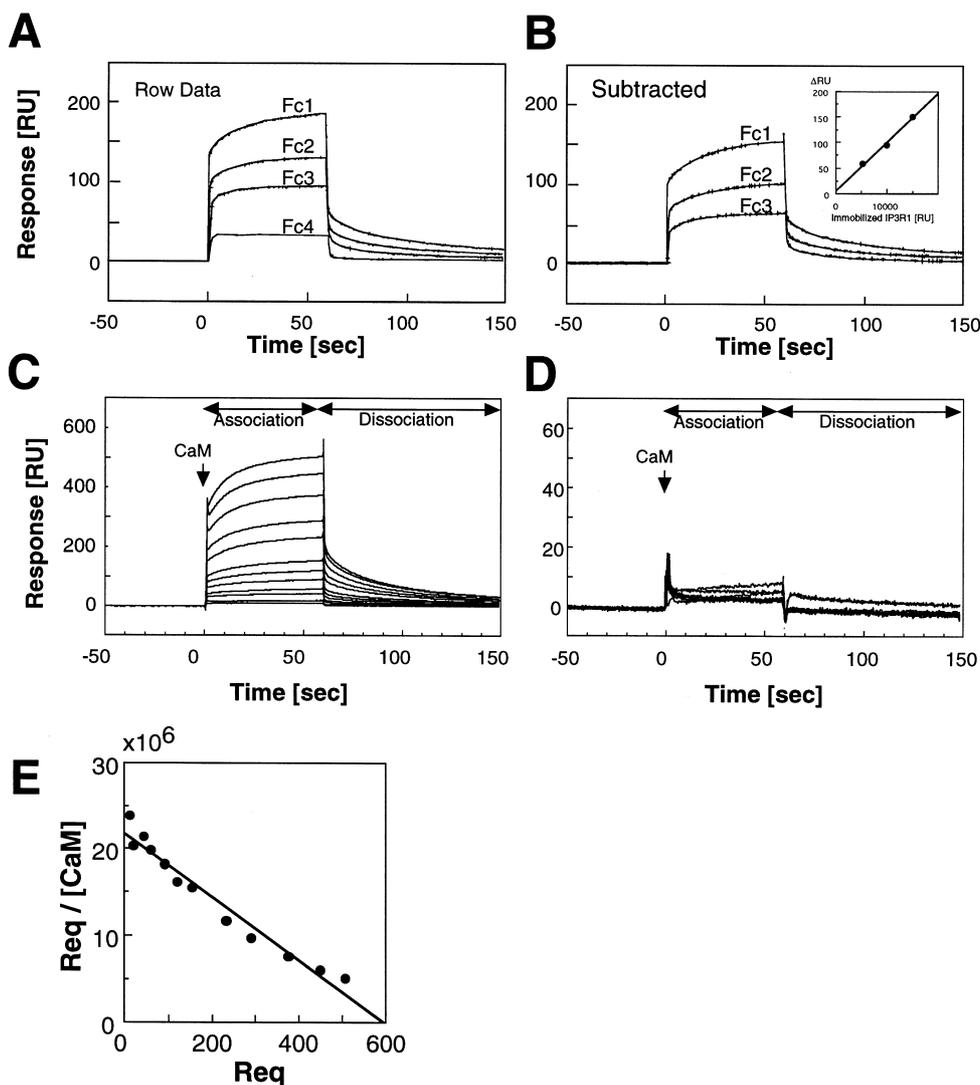


Fig. 2. Sensorgram for calmodulin binding to immobilized IP₃R1. A: Typical sensorgram for calmodulin binding. Calmodulin (10 μM) was injected over three flow cells (Fc1, 2 and 3) immobilized with different concentrations of IP₃R1 and one flow cell (Fc4) without IP₃R1 as blank in the presence of 2 mM Ca²⁺. B: Net sensorgrams were obtained by subtracting the blank (Fc4) from each profile (Fc1, 2 and 3). Inset: The ΔRU values of the end of injections were plotted against the concentration of the immobilized IP₃R1 to examine the crowded effect of immobilized IP₃R1 on the sensor chip. C: Net sensorgram for different concentrations of calmodulin (from bottom to top; 0.5, 1.0, 2.0, 3.0, 5.9, 7.5, 10, 20, 30, 50, 75, 100 μM of calmodulin injected) in the presence of Ca²⁺. D: Net sensorgram for different concentrations of calmodulin (from bottom to top; 0.5, 1.0, 2.0, 3.0, 5.9, 7.5, 10, 20, 30, 50, 75, 100 μM of calmodulin injected) in the absence of Ca²⁺. E: Schatchard plot. Equilibrium responses (Req) of each concentration of calmodulin were obtained by curve fitting of the association phase. Apparent dissociation constant of 27.3 μM for calmodulin IP₃R1 was calculated from the slope of Schatchard plot.

of calmodulin in the presence of Ca²⁺. Net sensorgrams were obtained by subtracting the blank (Fc4) as non-specific binding from each profile (Fc1, 2 and 3) (Fig. 2B). Specific calmodulin binding to the IP₃R1 was observed and the response in resonance unit (RU/arbitrary unit) depended on the amount of IP₃R1 immobilized. The ΔRU values (net response) of the end of injections were plotted against the concentration of the immobilized IP₃R1 (Fig. 2B, inset), in which ΔRU values are proportional to the amounts of IP₃R1 immobilized, confirming there is no crowded effect of immobilized IP₃R1 on the interaction. We then used Fc2 for further kinetic analysis.

Various concentrations of calmodulin (from 0.5 to 100 μM) were injected over the IP₃R1 immobilized flow cell in the presence or absence of Ca²⁺ (Fig. 2C and D). In the presence

of Ca²⁺, calmodulin binds to IP₃R1, in a dose dependent manner (Fig. 2C), whereas, in the absence of Ca²⁺, there was no specific binding of calmodulin to IP₃R1 (Fig. 2D). These results indicate that calmodulin binds to IP₃R1, in a Ca²⁺ dependent manner, thus confirming our previous observation. On the contrary to our observation, Ca²⁺ independent calmodulin binding to IP₃R1 and Ca²⁺ independent inhibition of IP₃ by calmodulin were reported [13,14]. Patel et al. reported that calmodulin binds to partially purified cerebellar IP₃R, in both Ca²⁺ dependent and independent manner [13]. They measured IP₃R/calmodulin binding kinetics by using a scintillation proximity assay system under the condition of a low ionic strength (20 mM PIPES pH 7.2, 1 mM EGTA, 5 mM K₂HPO₄, 0.1% Surfact-Amps X-100, 1% BSA and 0–

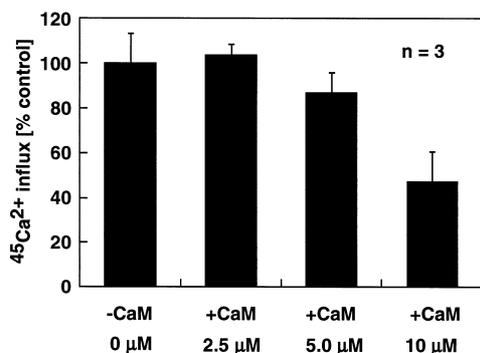


Fig. 3. Calmodulin inhibits IP₃-induced Ca²⁺ release, in a dose dependent manner. Effects of Ca²⁺/calmodulin on IP₃-induced Ca²⁺ release were investigated using purified and reconstituted IP₃R1. IP₃-induced Ca²⁺ influx into the liposome was measured in the presence of various concentrations of calmodulin. The IP₃R1 reconstituted liposome was incubated with 1 μM of IP₃ for 1 min to induce Ca²⁺ influx into liposome. ⁴⁵Ca²⁺ trapped in the liposome was measured after removal of extravesicular Ca²⁺ by Chelex-100.

1 mM CaCl₂). Simpa et al. reported that calmodulin inhibited IP₃ binding to a recombinant ligand binding domain of IP₃R1 (N-terminal 581 amino acids) in the absence of Ca²⁺ [14]. They suggested that the N-terminal IP₃ binding region of IP₃R1 might contain a Ca²⁺ independent calmodulin binding site. However, this effect was completely abolished by changing pH from 7.0 to 7.8. The reason for the discrepancy on the Ca²⁺ dependency of calmodulin binding to the IP₃R1 may be due to a different buffer composition or system used.

Kinetic analysis of the binding curves revealed that both the association and dissociation phases consist of two components with fast and slow time constants. From slopes of dRU/dt versus RU plot of each profile, apparent time constant for slow association phase, $k_{a(slow)}$, was calculated to be $4.46 \times 10^2 \text{ M}^{-1} \text{ s}^{-1}$. Time constant for fast phase, $k_{a(fast)}$, was not determined, because it is too rapid to measure ($> 10^4 \text{ M}^{-1} \text{ s}^{-1}$). In case of the dissociation phase, the sensorgram of each dissociation phase is well fitted by a biexponential curve. Time constants were calculated to be $k_{d(slow)} = 1.44 \times 10^{-2}$ and $k_{d(fast)} = 1.17 \times 10^{-1} \text{ s}^{-1}$ as mean of values at different concentrations of calmodulin. As the precise time constant for fast association phase was not determined, we calculated the apparent dissociation constant for Ca²⁺/calmodulin-IP₃R1 to be 27.3 μM, based on equilibrium responses (Fig. 2E). This value is lower than the previously determined value of 700 nM for the calmodulin binding peptide of IP₃R1 and calmodulin. The reason for this difference may be due to different materials used, i.e. purified and immobilized IP₃R1 was used in this study. The low affinity binding of calmodulin to IP₃R1 but with fast association/dissociation kinetics enables one to speculate that feedback regulation of IP₃R1 by Ca²⁺ could be mediated by Ca²⁺/calmodulin.

To investigate effects of Ca²⁺/calmodulin on IP₃R1 channel activity, IP₃-induced ⁴⁵Ca²⁺ influx was measured in the presence of various concentrations of calmodulin (Fig. 3). In the absence of calmodulin, the IP₃-induced ⁴⁵Ca²⁺ influx by the purified and reconstituted IP₃R1 was observed at high concentration of free Ca²⁺ (210 μM). In the presence of calmodulin, however, this IP₃-induced ⁴⁵Ca²⁺ influx was apparently

inhibited in a calmodulin dose dependent manner, thereby strongly indicating that one of the pathways of Ca²⁺ feedback inhibitory regulation of IP₃R1 could be mediated by Ca²⁺ calmodulin. Miyakawa et al. reported that both IP₃R1 and IP₃R2 mediated Ca²⁺ release exhibited a clear biphasic Ca²⁺ dependence, whereas IP₃R3 showed a flatter Ca²⁺ dependence [7]. If inhibition of IICR by Ca²⁺/calmodulin is a mechanism of negative feedback regulation, this isoform specific inhibitory effect of high Ca²⁺ concentration on IICR might be explained by Ca²⁺/calmodulin binding property to IP₃R. Our previous studies demonstrated that the calmodulin binding domain identified in IP₃R1 was conserved in IP₃R type 2 (IP₃R2) but not in IP₃R3 and that Ca²⁺/calmodulin binds to both IP₃R1 and IP₃R2 but not to IP₃R3 [9,15]. Therefore, we propose that inhibitory effects of high Ca²⁺ concentrations on IICR are mediated by Ca²⁺/calmodulin in an isoform of IP₃R specific manner.

Recently, Missiaen et al. reported that the bell-shaped Ca²⁺ dependence of the IICR is modulated by Ca²⁺/calmodulin [16]. They demonstrated that IICR in permeabilized A7r5 cells is inhibited by calmodulin at cytosolic free Ca²⁺ concentrations of 0.3 μM or higher. At a cytosolic free Ca²⁺ concentration of below 0.3 μM, this inhibitory effect was not observed, indicating that inhibitory effects mediated by calmodulin on IICR are Ca²⁺ dependent. In addition, we observed that the inhibitory effect of high concentration of Ca²⁺ on the open probability of cerebellar IP₃R1 is abolished by addition of calmodulin antagonist, W-7, indicating that inhibitory phase could be mediated by Ca²⁺/calmodulin [17]. Our study using purified and reconstituted IP₃R1 strongly supports these observations of inhibitory effect of Ca²⁺/calmodulin on IICR, and provides new evidence that Ca²⁺/calmodulin directly regulates the IP₃R1 channel activity with rapid binding kinetics. Therefore, inhibitory effects of Ca²⁺/calmodulin could be one of the fundamental mechanisms of immediate negative feedback regulation of IICR by Ca²⁺ which could generate a complex spatio-temporal pattern of Ca²⁺ increase such as Ca²⁺ wave and Ca²⁺ oscillation.

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