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

Junji Hirota[a,b,*], Takayuki Michikawa[b], Tohru Natsume[a], Teiichi Furuichi[b], Katsuhiko Mikoshiba[a,b]

[a] *Mikoshiba Calciosignal Net Project, Exploratory Research for Advanced Technology (ERATO), Japan Science and Technology Corporation (JST), 2-28-8 Honkomagome, Bunkyo-ku, Tokyo 113-0021, Japan*

[b] *Department of Molecular Neurobiology, Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108, Japan*

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Abstract Our previous studies have demonstrated that calmodulin binds to IP_3R type 1 (IP_3R1) in a Ca^{2+} dependent manner, which suggests that calmodulin regulates the IP_3R1 channel. In the present study, we investigated real-time kinetics of interactions between calmodulin and IP_3R1 as well as effects of calmodulin on IP_3-induced Ca^{2+} release by purified and reconstituted IP_3R1. Kinetic analysis revealed that calmodulin binds to IP_3R1 in a Ca^{2+} 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 IP_3-induced Ca^{2+} release through the purified and reconstituted IP_3R1 was inhibited by Ca^{2+}/calmodulin, in a dose dependent manner. We interpret our findings to mean that calmodulin binds to IP_3R1 in a Ca^{2+} dependent manner to exert inhibitory effect on IP_3R channel activity. This event may be one of the mechanisms governing the negative feedback regulation of IP_3-induced Ca^{2+} release by Ca^{2+}.

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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 (IP_3) receptor (IP_3R), an IP_3-induced Ca^{2+} releasing channel located on intracellular Ca^{2+} 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]. IP_3-induced Ca^{2+} release (IICR) is regulated by cytosolic free Ca^{2+}, in a biphasic manner [3,4]. Open probability of IP_3R induced by IP_3 showed a bell-shaped curve, depending on cytosolic Ca^{2+} concentrations, determined using the cerebellar microsomal fraction incorporated into lipid bilayer. Increasing cytosolic Ca^{2+} concentration up to 300 nM increased the open probability of IP_3R, whereas higher Ca^{2+} concentration led to inhibition [3]. In addition, rapid increases in Ca^{2+} concentration induced by flash photolysis of the caged Ca^{2+} 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 Ca^{2+}. Although the bell-shaped Ca^{2+} dependence of IP_3-induced Ca^{2+} release is considered to be fundamental to generate a complex pattern of spatio-temporal Ca^{2+} increase in cells, such as Ca^{2+} wave and Ca^{2+} oscillation, the mechanism of the bell-shaped dependence of IICR has remained unknown. The inhibitory effects of cytosolic Ca^{2+}, however, were absent, when IP_3R was purified and reconstituted [5,6]. In addition, inhibitory effects of cytosolic Ca^{2+} on IICR depend on the isoform of IP_3R, in which high concentrations of Ca^{2+} inhibited IP_3R type 1 (IP_3R1) and IP_3R type 2 (IP_3R2), but not IP_3R type 3 (IP_3R3) [7]. These observations suggest that such effects by Ca^{2+} on IICR may mediate effect of some Ca^{2+} sensing proteins.

Our previous studies demonstrated that IP_3R1 binds calmodulin in a Ca^{2+} dependent manner suggesting that calmodulin regulates the function of IP_3R1 [8,9]. The calmodulin binding domain identified in IP_3R1 is conserved in IP_3R type 2 (IP_3R2) but not in IP_3R3 [9]. To test the thesis that calmodulin may regulate IP_3R1 channel function, we investigated kinetics of interaction between IP_3R1 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 IP_3R1 channel activity were also investigated.

2. Materials and methods

2.1. Materials

The following reagents were purchased. IP_3 from Dojindo Laboratories (Kumamoto, Japan), Chelex-100 from Bio-Rad Laboratories (Hercules, CA, USA) and $^{45}Ca^{2+}$ from NEN (Boston, MA, USA). All the other reagents were of analytical grade or the highest grade available.

2.2. Purification of IP_3R type 1 (IP_3R1) and calmodulin

IP_3R1 was purified type specifically from mouse cerebellar microsomal fractions, using an immunoaffinity column conjugated with a polyclonal antibody against IP_3R1 as described [10]. Calmodulin was

*Corresponding author. Dept. of Molecular Neurobiology, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108, Japan. Fax: +81 (3) 5449-5420.
E-mail: jhirota@ims.u-tokyo.ac.jp

Abbreviations: IP_3, D-*myo*-inositol 1,4,5-trisphosphate; IP_3R, IP_3 receptor; IP_3R1, IP_3R type 1; IP_3R2, IP_3R type 2; IP_3R3, IP_3R type 3; HEPES, *N*-(2-hydroxyethyl) piperizine-*N*′-2-ethanesulfonic acid; EGTA, ethylene glycol-bis(β-amino-ethyl ether) *N*,*N*,*N*′,*N*′-tetra acetic acid; IICR, IP_3-induced Ca^{2+} release; NHS, *N*-hydroxysuccinimide; EDC, *N*-ethyl-*N*′-(3-diethylaminopropyl)-carbodiimide

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

2.3. Immobilization of the purified IP_3R1 on a sensor chip

The purified IP_3R1 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 IP_3R1 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 IP_3R1, 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 IP_3R1 and calmodulin

The interaction between IP_3R1 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 $CaCl_2$ or 2 mM EGTA at 25°C. For the association phase, various concentrations of calmodulin were injected over the surface of the IP_3R1 sensor chip with a flow rate of 30 μl/min for 60 s. Dissociation of the calmodulin from the immobilized IP_3R1 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/mm^2).

2.5. Reconstitution of purified IP_3R1 and IP_3-induced $^{45}Ca^{2+}$ influx measurements

The purified IP_3R1 was reconstituted into liposomes as described [10]. IP_3-induced $^{45}Ca^{2+}$ influx into the purified and reconstituted IP_3R/liposome was measured as described [10] in the presence of 200 μM free Ca^{2+} and 10 μM $^{45}Ca^{2+}$ together with various concentrations of calmodulin. The IP_3R1/liposome was incubated with 1 μM of IP_3 for 1 min at room temperature in order to induce Ca^{2+} influx into the liposome. After removal of extravesicular Ca^{2+} and $^{45}Ca^{2+}$ by Chelex-100, $^{45}Ca^{2+}$ trapped inside the liposome was measured using a liquid scintillation counter.

3. Results and discussion

IP_3-induced Ca^{2+} release (IICR) is regulated by cytosolic free Ca^{2+} in a biphasic manner [3,4], a process considered to be a fundamental mechanism for generation of a complex spatio-temporal Ca^{2+} increase in cells, such as Ca^{2+} wave and Ca^{2+} oscillation. The inhibitory effects of cytosolic Ca^{2+} were absent or faint when IP_3R1 was purified and reconstituted [5,6], which suggests the possibility that such effects may mediate some Ca^{2+} sensing proteins. Recently, it was reported that the bell-shaped dependence of Ca^{2+} of IICR depended on the isoform of IP_3R, in which inhibitory effects of high concentration of Ca^{2+} were observed in both IP_3R1 and IP_3R2 but not in IP_3R3 [7]. Calmodulin is a Ca^{2+} binding/sensing protein which exerts cellular function in response to Ca^{2+} increase by binding to target proteins. Earlier studies demonstrated that IP_3R1 binds calmodulin in a Ca^{2+} dependent manner [8,9], which suggests that calmodulin regulates the function of IP_3R1. To examine the possibility that calmodulin regulates IP_3R1 channel function, we investigated kinetics of the interaction between IP_3R1 and calmodulin and effects of calmodulin on the purified IP_3R1 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 IP_3R1 and calmodulin by injecting various concentrations of calmodulin in the presence or absence of Ca^{2+} on the IP_3R1 immobilized sensor chip.

Fig. 1 shows the scheme of preparation of the orientation specific immobilization of IP_3R1 on sensor chip. As the IP_3R1 has *N*-glycosylation sites within the luminal portion (residues 2475 and 2503 of mouse IP_3R1) [12], immobilization of IP_3R1 through its sugar chain could maintain IP_3R1 in the same orientation against the sensor chip. The IP_3R1 sensor chip prepared using this procedure was stable for repetitive use and responses were reproducible.

To examine effects of the concentration of IP_3R1 immobilized on binding responses, we prepared three flow cells with different amounts of IP_3R1. Flow cells (Fc) 1, 2, and 3 were conjugated with different amounts of IP_3R1 by changing reaction time during aldehyde coupling, and Fc4 served as a blank (without IP_3R1). 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 IP_3R1 was then observed. Fig. 2A shows typical sensorgrams of the interaction between the immobilized IP_3R1 and 10 μM

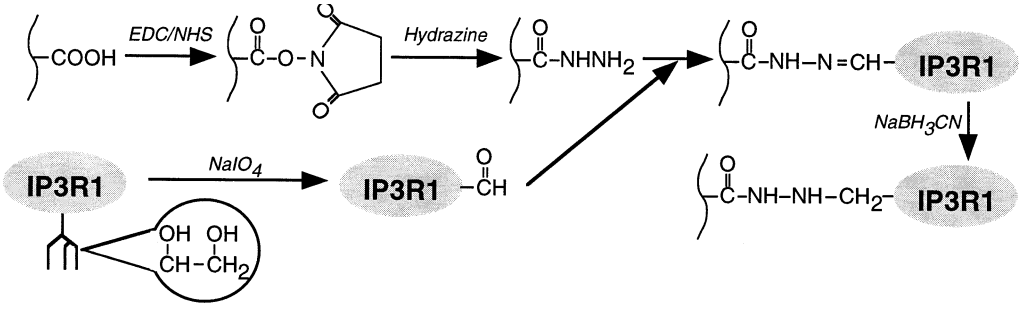


Fig. 1. Schematic representation of the orientation specific immobilization of IP_3R1. Schematic representation of the reaction for the immobilization of IP_3R1 through the polysaccharide chain. Carboxymethylated dextran sensor chip, CM5 (BIAcore AB) was activated by a series of the reactions with EDC/NHS and hydrazine to introduce hydrazide on the sensor chip. The purified IP_3R1 was oxidized with metaperiodate to introduce aldehyde groups to the polysaccharide chain located on the luminal portion of IP_3R1. IP_3R1 was then immobilized on the sensor chip by aldehyde coupling, which followed reduction of hydrazone bond by cyanoborohydride. This procedure allows one to immobilize IP_3R1 in the same orientation.

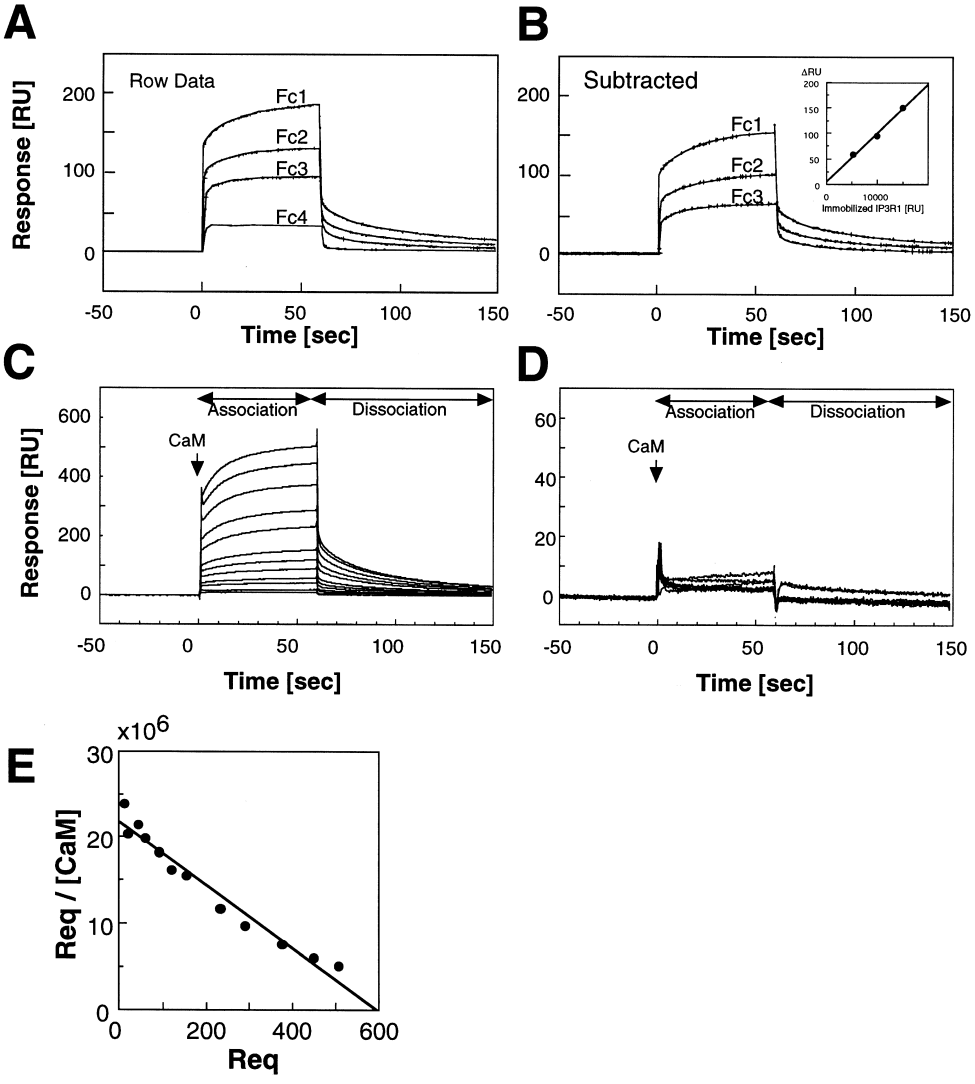


Fig. 2. Sensorgram for calmodulin binding to immobilized IP_3R1. A: Typical sensorgram for calmodulin binding. Calmodulin (10 μM) was injected over three flow cells (Fc1, 2 and 3) immobilized with different concentrations of IP_3R1 and one flow cell (Fc4) without IP_3R1 as blank in the presence of 2 mM Ca^{2+}. 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 IP_3R1 to examine the crowded effect of immobilized IP_3R1 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 Ca^{2+}. 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 Ca^{2+}. 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 IP_3R1 was calculated from the slope of Scatchard plot.

of calmodulin in the presence of Ca^{2+}. 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 IP_3R1 was observed and the response in resonance unit (RU/arbitrary unit) depended on the amount of IP_3R1 immobilized. The ΔRU values (net response) of the end of injections were plotted against the concentration of the immobilized IP_3R1 (Fig. 2B, inset), in which ΔRU values are proportional to the amounts of IP_3R1 immobilized, confirming there is no crowded effect of immobilized IP_3R1 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 IP_3R1 immobilized flow cell in the presence or absence of Ca^{2+} (Fig. 2C and D). In the presence of Ca^{2+}, calmodulin binds to IP_3R1, in a dose dependent manner (Fig. 2C), whereas, in the absence of Ca^{2+}, there was no specific binding of calmodulin to IP_3R1 (Fig. 2D). These results indicate that calmodulin binds to IP_3R1, in a Ca^{2+} dependent manner, thus confirming our previous observation. On the contrary to our observation, Ca^{2+} independent calmodulin binding to IP_3R1 and Ca^{2+} independent inhibition of IP_3 by calmodulin were reported [13,14]. Patel et al. reported that calmodulin binds to partially purified cerebellar IP_3R, in both Ca^{2+} dependent and independent manner [13]. They measured IP_3R/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 K_2HPO_4, 0.1% Surfact-Amps X-100, 1% BSA and 0–

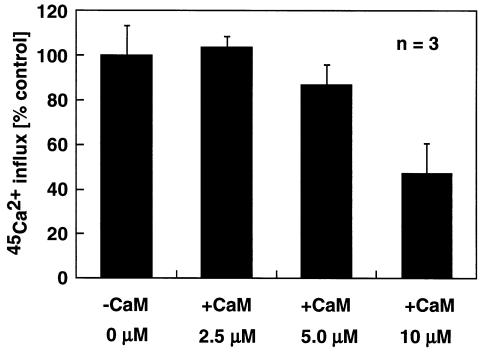


Fig. 3. Calmodulin inhibits IP_3-induced Ca^{2+} release, in a dose dependent manner. Effects of Ca^{2+}/calmodulin on IP_3-induced Ca^{2+} release were investigated using purified and reconstituted IP_3R1. IP_3-induced Ca^{2+} influx into the liposome was measured in the presence of various concentrations of calmodulin. The IP_3R1 reconstituted liposome was incubated with 1 μM of IP_3 for 1 min to induce Ca^{2+} influx into liposome. $^{45}Ca^{2+}$ trapped in the liposome was measured after removal of extravesicular Ca^{2+} by Chelex-100.

1 mM $CaCl_2$). Simpa et al. reported that calmodulin inhibited IP_3 binding to a recombinant ligand binding domain of IP_3R1 (N-terminal 581 amino acids) in the absence of Ca^{2+} [14]. They suggested that the N-terminal IP_3 binding region of IP_3R1 might contain a Ca^{2+} 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 Ca^{2+} dependency of calmodulin binding to the IP_3R1 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 4.46×10^2 M^{-1} s^{-1}. Time constant for fast phase, $k_{a(fast)}$, was not determined, because it is too rapid to measure ($>10^4$ M^{-1} 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 $k_{d(slow)} = 1.44\times10^{-2}$ and $k_{d(fast)} = 1.17\times10^{-1}$ 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 Ca^{2+}/calmodulin-IP_3R1 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 IP_3R1 and calmodulin. The reason for this difference may be due to different materials used, i.e. purified and immobilized IP_3R1 was used in this study. The low affinity binding of calmodulin to IP_3R1 but with fast association/dissociation kinetics enables one to speculate that feedback regulation of IP_3R1 by Ca^{2+} could be mediated by Ca^{2+}/calmodulin.

To investigate effects of Ca^{2+}/calmodulin on IP_3R1 channel activity, IP_3-induced $^{45}Ca^{2+}$ influx was measured in the presence of various concentrations of calmodulin (Fig. 3). In the absence of calmodulin, the IP_3-induced $^{45}Ca^{2+}$ influx by the purified and reconstituted IP_3R1 was observed at high concentration of free Ca^{2+} (210 μM). In the presence of calmodulin, however, this IP_3-induced $^{45}Ca^{2+}$ influx was apparently inhibited in a calmodulin dose dependent manner, thereby strongly indicating that one of the pathways of Ca^{2+} feedback inhibitory regulation of IP_3R1 could be mediated by Ca^{2+} calmodulin. Miyakawa et al. reported that both IP_3R1 and IP_3R2 mediated Ca^{2+} release exhibited a clear biphasic Ca^{2+} dependence, whereas IP_3R3 showed a flatter Ca^{2+} dependence [7]. If inhibition of IICR by Ca^{2+}/calmodulin is a mechanism of negative feedback regulation, this isoform specific inhibitory effect of high Ca^{2+} concentration on IICR might be explained by Ca^{2+}/calmodulin binding property to IP_3R. Our previous studies demonstrated that the calmodulin binding domain identified in IP_3R1 was conserved in IP_3R type 2 (IP_3R2) but not in IP_3R3 and that Ca^{2+}/calmodulin binds to both IP_3R1 and IP_3R2 but not to IP_3R3 [9,15]. Therefore, we propose that inhibitory effects of high Ca^{2+} concentrations on IICR are mediated by Ca^{2+}/calmodulin in an isoform of IP_3R specific manner.

Recently, Missiaen et al. reported that the bell-shaped Ca^{2+} dependence of the IICR is modulated by Ca^{2+}/calmodulin [16]. They demonstrated that IICR in permeabilized A7r5 cells is inhibited by calmodulin at cytosolic free Ca^{2+} concentrations of 0.3 μM or higher. At a cytosolic free Ca^{2+} concentration of below 0.3 μM, this inhibitory effect was not observed, indicating that inhibitory effects mediated by calmodulin on IICR are Ca^{2+} dependent. In addition, we observed that the inhibitory effect of high concentration of Ca^{2+} on the open probability of cerebellar IP_3R1 is abolished by addition of calmodulin antagonist, W-7, indicating that inhibitory phase could be mediated by Ca^{2+}/calmodulin [17]. Our study using purified and reconstituted IP_3R1 strongly supports these observations of inhibitory effect of Ca^{2+}/calmodulin on IICR, and provides new evidence that Ca^{2+}/calmodulin directly regulates the IP_3R1 channel activity with rapid binding kinetics. Therefore, inhibitory effects of Ca^{2+}/calmodulin could be one of the fundamental mechanisms of immediate negative feedback regulation of IICR by Ca^{2+} which could generate a complex spatio-temporal pattern of Ca^{2+} increase such as Ca^{2+} wave and Ca^{2+} oscillation.

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References

[1] Berridge, M.J. (1993) Nature 361, 315–325.
[2] Mikoshiba, K. (1997) Curr. Opin. Neurobiol. 7, 339–345.
[3] Bezprozvanny, I., Watras, J. and Ehrlich, B.E. (1991) Nature 351, 751–754.
[4] Iino, M. and Endo, M. (1992) Nature 360, 76–78.
[5] Hirota, J., Michikawa, T., Miyawaki, A., Furuichi, T., Okura, I. and Mikoshiba, K. (1995) J. Biol. Chem. 270, 19046–19051.
[6] Thrower, E.C., Lea, E.J. and Dawson, A.P. (1998) Biochem. J. 330, 559–564.
[7] Miyakawa, T., Maeda, A., Yamazawa, T., Hirose, K., Kurosaki, T. and Iino, M. (1999) EMBO J. 18, 1303–1308.
[8] Maeda, N., Kawasaki, T., Nakade, S., Yokota, N., Taguchi, T., Kasai, M. and Mikoshiba, K. (1991) J. Biol. Chem. 266, 1109–1116.
[9] Yamada, M., Miyawaki, A., Saito, K., Nakajima, T., Yamamoto-Hino, M., Ryo, Y., Furuichi, T. and Mikoshiba, K. (1995) Biochem. J. 308, 83–88.
[10] Nakade, S., Rhee, S.K., Hamanaka, H. and Mikoshiba, K. (1994) J. Biol. Chem. 269, 6735–6742.
[11] Yazawa, M., Sakuma, M. and Yagi, K. (1980) J. Biochem. (Tokyo) 87, 1313–1320.

[12] Michikawa, T., Hamanaka, H., Otsu, H., Yamamoto, A., Miyawaki, A., Furuichi, T., Tashiro, Y. and Mikoshiba, K. (1994) J. Biol. Chem. 269, 9184–9189.
[13] Patel, S., Morris, S.A., Adkins, C.E., O'Beirne, G. and Taylor, C.W. (1997) Proc. Natl. Acad. Sci. USA 94, 11627–11632.
[14] Sipma, H., De Smedt, P., Sienaert, I., Vanligen, S., Missiaen, L., Parys, J.B. and De Smedt, H. (1999) J. Biol. Chem. 274, 12157–12162.
[15] Cardy, T.J. and Taylor, C.W. (1998) Biochem. J. 334, 447–455.
[16] Missiaen, L., Parys, J.B., Weidema, A.F., Sipma, H., Vanlingen, S., De Smedt, P., Callewaert, G. and De Smedt, H. (1999) J. Biol. Chem. 274, 13748–13751.
[17] Michikawa, T., Hirota, J., Kawano, S., Hiraoka, M., Yamada, M., Furuichi, T. and Mikoshiba, K. (1999) Neuron, in press.