Temperature-dependence and conformational basis of inositol 1,4,5-trisphosphate receptor regulated by Ca^{2+}

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Abstract The inositol 1,4,5-trisphosphate ($InsP_3$) receptor was purified from bovine cerebellum and reconstituted in liposomes composed of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) (1:1) successfully. No effect of Ca^{2+} concentration on [^{3}H]-$InsP_3$ binding to unreconstituted $InsP_3$ receptor could be observed either at 4℃ or at 25℃, whereas the effect of [Ca^{2+}] on reconstituted $InsP_3$ receptor depended on the temperature. The Ca^{2+} concentration outside the proteolipsome ($[Ca^{2+}]_o$) had no detectable effect on $InsP_3$ binding to $InsP_3$ receptor at 4℃. In contrast, with increase of $[Ca^{2+}]_o$ from 0 to 100 nmol/L at 25℃, the $InsP_3$ binding activity increased gradually. Then the $InsP_3$ binding activity was decreased drastically at higher $[Ca^{2+}]_o$ and inhibited entirely at 50 μmol/L $[Ca^{2+}]_o$. Conformational studies on intrinsic fluorescence of the reconstituted $InsP_3$ receptor and its quenching by KI and HB indicated that the global conformation of reconstituted $InsP_3$ receptor could not be affected by $[Ca^{2+}]_o$ at 4℃. While at 25℃, the effects of 10 μmol/L $[Ca^{2+}]_o$ on global, membrane and cytoplasmic conformation of the reconstituted $InsP_3$ receptor were different significantly from that of 100 nmol/L $[Ca^{2+}]_o$.

Keywords: Ca^{2+}, inositol 1, 4, 5-trisphosphate receptor, reconstitution, temperature, conformation.

Inositol 1,4,5-trisphosphate receptor, as an indispensable member in the inositol phosphates signal transduction across membrane, played an important role in Ca^{2+} release from endoplasmic reticulum (ER) and subsequently complicated cellular Ca^{2+} signal such as Ca^{2+} spike and Ca^{2+} oscillation[1]. The regulation of inositol 1, 4, 5-trisphosphate ($InsP_3$) receptor by Ca^{2+} was very complex according to current studies. Most results obtained in cells and microsomes showed that the $InsP_3$ receptor was activated in the lower concentration range (200—500 nmol/L) of cytoplasmic Ca^{2+} and was strongly inhibited when the Ca^{2+} concentration exceeded 1 μmol/L[2,3]. Fewer studies on regulation of purified $InsP_3$ receptor by Ca^{2+} could be found in literatures and the results were controversial[4,5]. The effects of Ca^{2+} on the $InsP_3$ binding were examined carefully after purification and reconstitution of $InsP_3$ receptor, and the intrinsic fluorescence was further measured to study the underlying conformational basis.

1 Materials and methods

1.1 Materials

The bovine cerebella were purchased from Dachang County, Hebei Province. After being

slaughtered, the cerebella were removed out and put in ice immediately.

Tris was purchased from Bohringer. β-mercaptoethanol was from Serva. ^{3}H-InsP_3 was from Dupont NEN. Triton X-100 (TX-100) was from FARCO. Con A Sepharose and Heparin Sepharose were products of Pharmacia. EGTA and α-methylmannoside were from Fluka. SM2 Bio-Beads was from Bio-Rad. Human γ-globulin and InsP_3 were from Sigma. PEG 6000 was imported from Japan and subfractioned. PC and PE were from Avanti Polar-Lipids. Hypocrellin B (HB) was kindly provided by Prof. Yue Jiachang. All the other reagents were of analytic grade or above.

1.2 Purification of InsP_3 receptor

The purification of InsP_3 receptor was similar to the method of Supattapone[4] with some modifications.

1.3 Reconstitution of InsP_3 receptor

The method of Lévy[6] was used to prepare proteoliposomes containing InsP_3 receptors and PC/PE (1:1), in which TX-100 was removed by SM2 Bio-Beads. The ratio of lipid to protein was 10∶1. The obtained proteoliposomes were resuspended in buffer A (50 mmol/L Tris, 50 mmol/L NaCl, 50 mmol/L KCl, 1 mmol/L EGTA, 1 mmol/L β-mercaptoethanol, pH 8.0). The maximal InsP_3 binding of proteoliposomes was not changed after being treated with 0.2% TX-100, which indicated that all the InsP_3 binding sites located at the cytoplasmic domain of InsP_3 receptor were oriented outside of the proteoliposomes.

1.4 Control of free Ca^{2+} concentration

The free Ca^{2+} concentrations in all buffers were controlled by adding adequate $CaCl_2$ and EGTA. The amount of $CaCl_2$ and EGTA was calculated with WinMAXC[7].

1.5 InsP_3 binding assay

The InsP_3 binding was determined by PEG precipitation method[8]. Briefly, 10 nmol/L [^{3}H]-InsP_3 was added to each 50 μL sample (the nonspecific binding was determined in the presence of 1 μmol/L unlabeled InsP_3). After being incubated for 10 min at 4℃ (or 2 min at 25℃), 2 μL γ-globulin (50 mg/mL) and 50 μL 30% PEG 6000 in buffer B (50 mmol/L Tris, 1 mmol/L mercaptoethanol, pH 8.0) were added. The mixture was then centrifuged at 5 000 r/min for 5 min. The pellet was solubilized in 50 μL 10% SDS and counted in 5 mL TX-100/Xylene scintillation cocktail.

1.6 Intrinsic fluorescence of InsP_3 receptor

All intrinsic fluorescence of InsP_3 receptor was measured with an F-4010 spectrofluorometer (Hitachi). Proteoliposomes containing 25 μg InsP_3 receptor were suspended in 0.8 mL buffer A with indicated free [Ca^{2+}] and under constant stir. Temperature was controlled by circulated water bath. Excitation wavelength was 295 nm. The range of emission wavelength was from 300 to 400 nm. The band-pass of excitation and emission was set to 5 nm. The photomultiplier worked in

high-energy mode. The ratio of signal to noise was increased by CAT eight times.

1.7 The quenching of intrinsic fluorescence by KI

The proteoliposomes were suspended in buffer C (50 mmol/L Tris, 250 mmol/L KCl+KI, 5 mmol/L $Na_2S_2O_3$, 1 mmol/L EGTA, 1 mmol/L β-mercaptoethanol, pH 8.0) containing indicated concentration of KI and free Ca^{2+}. The fluorescence emission was measured under conditions described in sec. 1.6. All quenching data were fitted to modified Stern-Volmer equation by Levenberg-Marquardt method to obtain Ksv and fa. The equation is showed below:

$$\frac{\Delta F}{F_0} = \frac{\mathrm{faKsv}[Q]}{1 + \mathrm{Ksv}[Q]},$$

where F_0 is the relative fluorescence intensity (RFI) without quencher; ΔF, the difference between RFI with and without quencher (F_0-F_i); [Q], concentration of quencher.

1.8 The quenching of intrinsic fluorescence by HB

The HB concentrations in samples were controlled by adding 5 mmol/L HB to DMSO. The conditions for sample measuring and data process are the same as decribed in sec. 1.7.

2 Results and discussion

2.1 Purification of $InsP_3$ receptor

The purified $InsP_3$ receptor was a single band with apparent molecular weight of 240 ku in 7.5% SDS-PAGE after silver staining (fig. 1b), which was consistent with other reports[4].

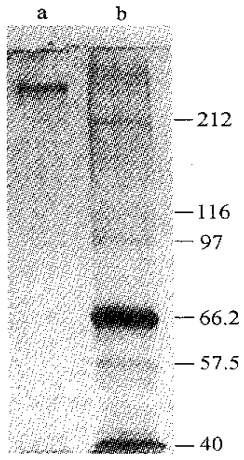

Fig. 1. 7.5% SDS-PAGE of purified $InsP_3$ receptor (Ag stained). a, purified $InsP_3$ receptor; b, Promega high MW marker (ku).

2.2 Effect of [Ca^{2+}] on purified $InsP_3$ receptor binding to [^{3}H]-$InsP_3$

Some properties of $InsP_3$ receptor could only be observed at mild temperature and disappeared when the temperature was lower, such as quantal Ca^{2+} release and $InsP_3$-induced inactivation[9—11]. So it implied that the status of $InsP_3$ receptor was also varied at different temperatures. Much different from the physiological condition, the $InsP_3$ binding was assayed at 0—4℃ in all published works up to now, which may be unfavorable to observing the effects of some factors on $InsP_3$ receptor. Therefore, the activity of purified $InsP_3$ receptor binding to ^{3}H-$InsP_3$ was studied at both 4 and 25℃. The results in table 1 showed that no changes could be detected in the [Ca^{2+}] range from 50 nmol/L to 50 μmol/L we observed. This agreed well with the results of Supattapone et al., who found that the [Ca^{2+}] sensitivity of $InsP_3$ binding to $InsP_3$ receptor from mouse cerebellum disappeared after purification[4].

Table 1 Effect of [Ca^{2+}] on [^{3}H]-$InsP_3$ binding to purified $InsP_3$ receptor at 4℃ and 25℃

Temp.	IP_3 binding/fmol				
	0 nmol/L	50 nmol/L	100 nmol/L	1 μmol/L	50 μmol/L
4℃	95.2±13	105.7±5.7	109.3±2.9	103.8±8.1	102.6±21
25℃	105.9±7.5	114.9±4.9	109.6±3.8	116.7±5.5	117.1±5.0

All data are represented as mean±SD of four experiments. No significant difference between the data groups can be observed after analysis by single factor Anova analysis. $P >> 0.05$.

2.3 Regulation of reconstituted $InsP_3$ receptor by $[Ca^{2+}]_o$ at 25℃

The effect of $[Ca^{2+}]$ outside the proteoliposome ($[Ca^{2+}]_o$) on [^{3}H]-$InsP_3$ binding was studied after the purified $InsP_3$ receptor was reconstituted into liposomes composed of PC:PE (1:1). The results in table 2 showed that the $[Ca^{2+}]_o$ had no effect on reconstituted $InsP_3$ receptor binding to $InsP_3$ at 4℃, which was consistent with the incapability of Ca^{2+} in regulation of $InsP_3$ binding to mouse cerebellar $InsP_3$ receptor reconstituted by CHAPS dialysis method as observed by Ferris[12]. In contrast, at 25℃, $InsP_3$ binding to reconstituted $InsP_3$ receptor increased significantly when the $[Ca^{2+}]_o$ rose from 0 to 100 nmol/L, and was inhibited greatly and disappeared eventually when the $[Ca^{2+}]_o$ was further increased above 1 μmol/L (table 2). Thrower reported that the open probability of Ca^{2+} channel of reconstituted $InsP_3$ receptor was reduced at high $[Ca^{2+}]_o$, which would suggest that the Ca^{2+} channel of reconstituted $InsP_3$ receptor was closed at high $[Ca^{2+}]_o$. It was also notable that the $InsP_3$ binding to reconstituted $InsP_3$ receptor at 25℃ was only about 1/5 of that at 4℃, which implied that the conformations of $InsP_3$ receptor under these two conditions were different. We also observed that $InsP_3$ binding to reconstituted $InsP_3$ receptor decreased with $[Ca^{2+}]$ inside the proteoliposome falling (results not shown). All these results indicated that increase of $[Ca^{2+}]_o$ can regulate the $InsP_3$ binding of reconstituted $InsP_3$ receptor, but the effect could be observed only at relatively high temperature, and only in the case of reconstitution.

Table 2 The effect of $[Ca^{2+}]_o$ on ^{3}H-IP_3 binding of the reconstituted IP_3 receptor at 4 and 5℃

Temp.	IP_3 binding/fmol					
	0 nmol/L	50 nmol/L	100 nmol/L	1 μmol/L	50 μmol/L	500 μmol/L
4℃	111.8±3.9	104.4±7.4	99.8±10.9	110.1±4.7	108.5±6.5	110.0±7.9
25℃	18.0±2.4	33.1±5.9	58.0±3.9	12.0±2.9	0.3±1.1	1.1±2.6

All data are represented as mean±SD of four experiments. Significant difference between the data groups can be observed after analysis by single factor Anova analysis. $P<<0.01$.

Twenty one tryptophane residues can be found in the mouse cerebellar $InsP_3$ receptor, sixteen of which were located in cytosolic domain and five in transmembrane domain[13]. Because of the efficiency and convenience of measuring the tryptophane fluorescence and its quenching, it was used to study the conformational change underlying the regulation of $InsP_3$ receptor by $[Ca^{2+}]_o$.

2.4 Effect of $[Ca^{2+}]_o$ on intrinsic fluorescence of the reconstituted $InsP_3$ receptor

No substantial changes of intrinsic fluorescence of the reconstituted $InsP_3$ receptor could be detected at 4℃ after addition of 100 nmol/L or 10 μmol/L $[Ca^{2+}]_o$ (fig. 2(a)), in consistence with the effect of $[Ca^{2+}]_o$ on the $InsP_3$ binding to $InsP_3$ receptor. At 25℃, however, addition of 100

nmol/L $[Ca^{2+}]_o$ decreased the fluorescence intensity slightly (fig. 2(b)), while 10 μmol/L $[Ca^{2+}]_o$ increased the fluorescence intensity considerably. Such a conformational difference between low $[Ca^{2+}]_o$ and high $[Ca^{2+}]_o$ may reflect the transition of $InsP_3$ receptor from high $InsP_3$ binding state to low one, which may provide conformation basis for regulation of $InsP_3$ receptor by the low $[Ca^{2+}]_o$ and high $[Ca^{2+}]_o$ at 25℃.

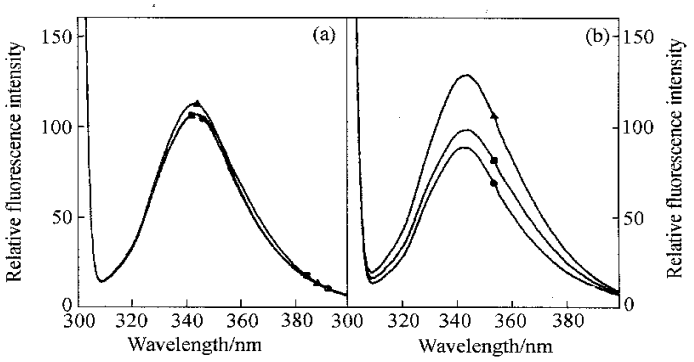

Fig. 2. The effect of $[Ca^{2+}]_o$ on intrinsic fluorescence of reconstituted IP_3 receptor at 4℃ (a) and 25℃ (b) in the presence of 1 μmol/L IP_3. ■, Control; ●, 100 nmol/L Ca^{2+}; ▲, 10 μmol/L Ca^{2+}.

The above results showed that high $[Ca^{2+}]_o$ and low $[Ca^{2+}]_o$ had different effects on both the activity and global conformation of the reconstituted $InsP_3$ receptor. Then the quenching of intrinsic fluorescence of the $InsP_3$ receptor by KI and HB was further compared to investigate the conformational changes in the regulation of reconstituted $InsP_3$ receptor by high and low $[Ca^{2+}]_o$.

2.5 Effect of $[Ca^{2+}]_o$ on quenching of intrinsic fluorescence of reconstituted $InsP_3$ receptor by KI and HB

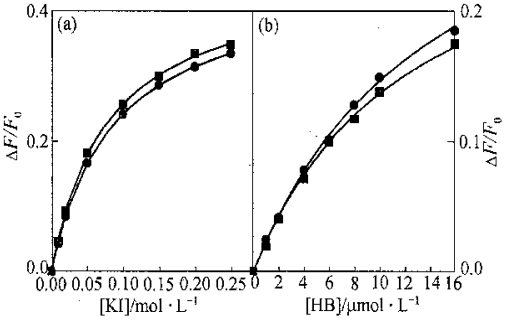

Fig. 3. The effect of high and low $[Ca^{2+}]_o$ on intrinsic fluorescence of reconstituted IP_3 receptor quenched by KI (a) and HB (b) in the presence of 1 μmol/L IP_3 at 25℃. The Ksv of KI and HB were expressed in L/mol and 10^4 L/mol, respectively. (a) ■, 100 nmol/L Ca^{2+}, Ksv: 12.5, fa: 46.2%; ●, 10 μmol/L Ca^{2+}, Ksv: 11.6, fa: 45.1%; (b) ■, 100 nmol/L Ca^{2+}, Ksv: 8.1, fa: 30.5%; ●, 10 μmol/L Ca^{2+}, Ksv: 6.9, fa: 35.8%.

KI, as a collisional quencher, extinguishes the fluorescence emission of tryptophane primarily in hydrophilic environment. Therefore, the conformation of cytoplasmic domain of the $InsP_3$ receptor can be studied separately by this method. The results (fig. 3(a)) did not show much difference in quenching of intrinsic fluorescence of reconstituted $InsP_3$ receptor by KI between high $[Ca^{2+}]_o$ and low $[Ca^{2+}]_o$. The fa decreased only slightly from 46.2% to 45.1%. Accordingly, the conformation of the cytoplasmic domain of $InsP_3$ receptor at low $[Ca^{2+}]_o$ was similar to that at high $[Ca^{2+}]_o$, keeping up with that neither high $[Ca^{2+}]_o$ nor low $[Ca^{2+}]_o$ can affect the $InsP_3$ binding to $InsP_3$ receptor at 4℃.

Hypocrellin B (HB), as a peryloquinine derivative, was used as hydrophobic fluorescence

quencher to study the conformational changes of membrane proteins[14]. Slight difference was found between effects of low $[Ca^{2+}]_o$ and high $[Ca^{2+}]_o$ on quenching of intrinsic fluorescence of $InsP_3$ receptor by HB (fig.3(b)). fa increased from 30.5% to 35.8% and Ksv decreased from 8.1×10^4 L/mol to 6.9×10^4 L/mol. Thus the conformation of membrane domain changed slightly although high $[Ca^{2+}]_o$ has little effect on the global or cytoplasmic conformation of the reconstituted $InsP_3$ receptor at 4℃.

2.6 Comparison between the effects of high and low $[Ca^{2+}]_o$ on conformations of membrane and cytoplasmic domains at 25℃

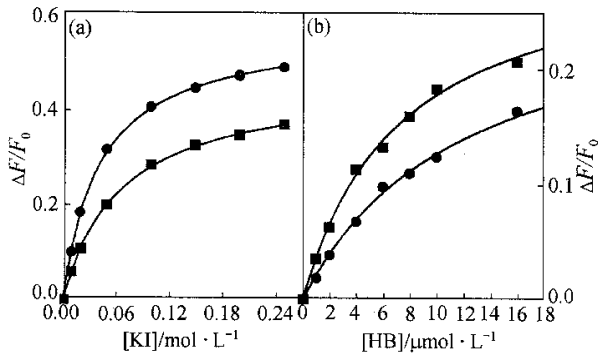

Fig. 4. The effect of high and low $[Ca^{2+}]_o$ on intrinsic fluorescence of reconstituted IP_3 receptor quenched by KI (a) and HB (b) in the presence of 1 μmol/L IP_3 at 25℃. The Ksv of KI and HB were expressed in L/mol and 10^4 L/mol, respectively. (a) ■, 100 nmol/L Ca^{2+}, Ksv: 15.3, fa: 46.3%; ●, 10 μmol/L Ca^{2+}, Ksv: 23.8, fa: 57.1%; (b) ■, 100 nmol/L Ca^{2+}, Ksv: 13.7, fa: 30.9%; ●, 10 μmol/L Ca^{2+}, Ksv: 8.4, fa: 27.9%.

When conformation of the reconstituted $InsP_3$ receptor was examined at 25℃ in the presence of high $[Ca^{2+}]_o$, quenching by either KI or HB differed remarkably from that at 4℃ (fig. 4). KI quenching in the presence of high $[Ca^{2+}]_o$ changed greatly compared with low $[Ca^{2+}]_o$, the fa increased from 46.3% to 57.1%, and Ksv increased from 15.3 to 23.8 (fig. 4(a)), which indicated that tryptophane residues in cytoplasmic domain of $InsP_3$ receptor were exposed to a greater extent and the quenching efficiency was also improved. It indicated that only at 25℃ can high $[Ca^{2+}]_o$ modulate the conformation of cytoplasmic domain of the reconstituted $InsP_3$ receptor. In view of the localization of $InsP_3$ binding site in cytoplasmic domain, the results provided the conformation basis for inhibition of $InsP_3$ binding to $InsP_3$ receptor by high $[Ca^{2+}]_o$.

At 25℃, the efficiency for HB quenching at high $[Ca^{2+}]_o$ decreased obviously and the extent of change was greater than that at low $[Ca^{2+}]_o$ or 4℃ (fig. 4(b)). The fa and Ksv were 27.9% and 8.4×10^4 L/mol, respectively. These results indicated that the tryptophane residues in membrane domain of the reconstituted $InsP_3$ receptor were exposed to a less extent at high $[Ca^{2+}]_o$. Taken together, high $[Ca^{2+}]_o$ could not affect the global and cytoplasmic conformations of reconstituted $InsP_3$ receptor at 4℃, therefore was incapable of regulating the $InsP_3$ binding, whereas at 25℃, the conformations of global, cytoplasmic or the membrane domain were all changed significantly, thereby the function of $InsP_3$ receptor was inhibited remarkably.

In many tissues, the response of $InsP_3$ receptor to $InsP_3$ was biphasically modulated by physiological changes in cytosolic Ca^{2+}[1—3]. The inhibitory effect of Ca^{2+} on $InsP_3$ binding lost after receptor purification[4]. Calmedin, as a possible candidate responsible for the modulation of

$InsP_3$ receptor by Ca^{2+}, has not been purified up to now, properties of which were also uncharacterized[4,15,16]. Our results pointed out that Ca^{2+} had no effect on unreconstituted or reconstituted $InsP_3$ receptor at 4℃, whereas at 25℃, low $[Ca^{2+}]_o$ activated the binding of $InsP_3$ to the reconstituted $InsP_3$ receptor while higher $[Ca^{2+}]_o$ inhibited it (table 2). Furthermore, higher $[Ca^{2+}]_o$ can also induce obvious conformational changes of the reconstituted $InsP_3$ receptor. It may suggest that only at a mild temperature and after the reconstitution can the $InsP_3$ receptor display a suitable conformation, which is essential for the regulation of the reconstituted $InsP_3$ receptor by Ca^{2+}. Importance of those factors in regulation of purified $InsP_3$ receptor by Ca^{2+} has been paid little attention to in many observations in literature, so the effect of Ca^{2+} and temperature on regulation of purified $InsP_3$ receptor could not be observed. Our results also indicated that the regulation by Ca^{2+} was an inherent property of $InsP_3$ receptor and was the basis for regulation of $InsP_3$ receptor by Ca^{2+} *in vivo*, while other related proteins may act as fine-tuners.

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References

1. Dawson, A. P., Calcium signalling: How do IP_3 receptors work? Curr. Biol., 1997, 7: R544.
2. Bezprozvanny, I., Watras, J., Ehrlich, B. E., Bell-shaped calcium-response curves of Ins(1,4,5)P_3- and calcium-gated channels from endoplasmic reticulum of cerebellum, Nature, 1991, 351: 751.
3. Finch, E. A., Turner, T. J., Goldin, S. M., Calcium as a coagonist of inositol 1,4,5-trisphosphate- induced calcium release, Science, 1991, 252: 443.
4. Supattapone, S., Worley, P. F., Baraban, J. M. et al., Solubilization, purification and characterization of an inositol trisphosphate receptor, J. Biol. Chem., 1988, 263: 1530.
5. Thrower, E. C., Lea, E. J. A., Dawson, A. P., The effects of free $[Ca^{2+}]$ on the cytosolic face of the inositol (1,4,5)-trisphosphate receptor at the single channel level, Biochem. J., 1998, 330: 559.
6. Lévy, D., Bluzat, A., Seigneuret, M. et al., A systematic study of liposome and proteoliposome reconstitution involving Bio-Bead-mediated Triton X-100 removal, Biochim. Biophys. Acta, 1990, 1025: 179.
7. Bers, D. M., Patton, C. W., Nuccitelli, R., A practical guide to the preparation of Ca^{2+} buffers, Methods Cell Biol., 1994, 40: 3.
8. Maeda, N., Niinobe, M., Mikoshiba, K., A cerebellar Purkinje cell marker P400 protein is an inositol 1,4,5-trisphosphate ($InsP_3$) receptor protein, Purification and characterization of $InsP_3$ receptor complex, EMBO J., 1990, 9: 61.
9. Meyer, T., Stryer, L., Transient calcium release induced by successive increments of inositol 1,4,5-trisphosphate, Proc. Natl. Acad. Sci. USA, 1990, 87: 3841.
10. Kindman, L. A., Meyer, T., Use of intracellular Ca^{2+} stores from rat basophilic leukemia cells to study the molecular mechanism leading to quantal Ca^{2+} release by inositol 1,4,5-trisphosphate, Biochemistry, 1993, 32: 1270.
11. Hajnóczky, G., Thomas, A. P., The inositol trisphosphate calcium channel is inactivated by inositol trisphosphate, Nature, 1994, 370: 474.
12. Ferris, C. D., Huganir, R. L., Supattapone, S. et al., Purified inositol 1,4,5-trisphosphate receptor mediates calcium flux in reconstituted lipid vesicles, Nature, 1989, 342: 87.
13. Furuichi, T., Yoshikawa, S., Miyawaki, A. et al., Primary structure and functional expression of the inositol 1, 4, 5-trisphosphate-binding protein P400, Nature, 1989, 342: 32.
14. Yue, J. C., Pang, S. Z., Quenching of fluorescence in membrane protein by hypocrellin B, Science in China, Ser. C, 1997, 40(2): 194.
15. Danoff, S. K., Supattapone, S., Snyder, S. H., Characterization of a membrane protein from brain mediating the inhibition of inositol 1,4,5-trisphosphate receptor binding by calcium, Biochem. J., 1988, 254: 701.
16. Mignery, G. A., Johnston, P. A., Südhof, T. C., Mechanism of Ca^{2+} inhibition of inositol 1,4,5-trisphosphate ($InsP_3$) binding to the cerebellar $InsP_3$ receptor, J. Biol. Chem., 1992, 267: 7450.