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Inositol hexakisphosphate (InsP6) can weaken the Ca^{2+}-dependent membrane binding of C2AB domain of synaptotagmin I

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Abstract The synaptic vesicle protein synaptotagmin I has been proposed to serve as a Ca^{2+} sensor for rapid exocytosis. In the present work, two fragments of the large cytoplasmic domain of synaptotagmin I, C2A and C2AB, were compared by combining surface plasmon resonance with circular dichroism and fluorescence techniques. C2AB and C2A had almost identical membrane binding constants, indicating that C2A is the predominate domain to bind to negatively charged phospholipids. After reacting with inositol hexakisphosphate (InsP6) a conformational change of C2AB was detected in the presence of liposome. The InsP6 binding notably weakened the Ca^{2+}-dependent C2AB-membrane interaction, which suggests that InsP6 may act as a modulator of neurotransmitter release by altering the state of synaptotagmin–phospholipid interaction. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Synaptotagmin; Inositol hexakisphosphate; Surface plasmon resonance; Lipid-protein interaction

1. Introduction

Synaptotagmin I is a member of the transmembrane proteins which are in synaptic vesicles and large secretory granules of neurons and endocrine cells [1–3]. It spans the vesicle membrane once, has a short carboxyl-terminal intravesicular domain (residues 1–53 in rat) and a large cytoplasmic region (residues 80–421 in rat) that contains two C2 domains homologous to the C2 regulatory region of protein kinase C [4]. Because it is difficult to study membrane protein in solution, glutathione *S*-transferase (GST) fusion proteins containing the first (C2A), the second (C2B) or both C2 domains (C2AB) were the main constituents used to study the physiological role of synaptotagmin [5–8]. The C2A domain has several Ca^{2+} binding sites [9,10]. The equilibrium and kinetic Ca^{2+} binding properties of C2A were consistent with the Ca^{2+} requirement and speed of secretion [7], therefore synaptotagmin has been considered as a Ca^{2+} sensor which functions in Ca^{2+} regulated synaptic vesicle exocytosis [2]. C2A may partially insert into anionic phospholipid membranes in the presence of Ca^{2+} [6–8]. This interaction has been proposed to contribute to the lipid rearrangements that underlie membrane fusion [11].

There are several protein binding sites in the C2B domain, such as clathrin assembly protein-2 (AP-2) [12] and N-type calcium channels [13,14], but the exact function of C2B domain is still not clear. It was reported that the tandem C2 domains cooperate to form complexes with components of the soluble *N*-ethyl maleimide-sensitive factor attachment protein receptor complex [7,15–17]. In addition, synaptotagmin I was found to form both homo- and heterodimers in a Ca^{2+}-dependent manner [11,16]. Thus, synaptotagmin I has been postulated to not only act as a Ca^{2+} sensor but also play an important role in the process leading to exocytosis of synaptic vesicles.

It was reported that C2B domain would bind inositol highphosphate series (IHPS, including inositol-1,3,4,5-tetrakisphosphate (InsP4), inositol-1,3,4,5,6-pentakisphosphate (InsP5) and inositol hexakisphosphate (InsP6)) [18–21]. This binding was not Ca^{2+}-dependent, but a Ca^{2+} concentration-dependent switch of binding affinity was reported using the liposome model system [22]. A study using squid giant synapses revealed that microinjection of the members of IHPS into the presynaptic terminal blocked synaptic transmission and this blockage could be released by co-injection of the specific IgG that recognizes the C2B domain of synaptotagmin [23]. This finding suggests that IHPS may act as a modulator of neurotransmitter release. A further experiment showed that IHPS binding to the C2B domain of synaptotagmin altered the status of protein–protein interaction including the synaptotagmin–AP-2 interaction [24], which gives a possible explanation of how IHPSs perform their function.

C2A and C2B domains are connected with a short tether. Hence the binding of IHPS to the C2B domain may have a definite effect on the function of the C2A domain which is essential for synaptotagmin's physiological role. In the present work, the effects of IHPS on the interaction of C2A or C2AB with phospholipid membrane are studied with both supported lipid layer and liposome systems. These studies provide a possible approach to understanding the complex interactions among synaptotagmin, lipid membrane and the related factors, occurring in the process of exocytosis of synaptic vesicles.

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Abbreviations: DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DMPS, 1,2-dimyristoyl-*sn*-glycero-3-phosphoserine; PS, phosphatidylserine; PC, phosphatidylcholine; IHPS, inositol highphosphate series; InsP6, inositol hexakisphosphate; CD, circular dichroism; SPR, surface plasmon resonance

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2. Materials and methods

2.1. Materials

1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-dimyristoyl-*sn*-glycero-3-phosphoserine (DMPS), egg phosphatidylcholine (PC), phosphatidylserine (PS; from bovine brain) and InsP6 were purchased from Sigma Chemical Co., St. Louis, MO, USA. Glutathione–Sepharose 4B beads and thrombin were purchased from Amersham Pharmacia Biotech, Co., Piscataway, NY, USA. All the other chemicals were of analytical grade and purchased locally.

2.2. Purification of synaptotagmin I

cDNA encoding rat synaptotagmin I was donated by Edwin R. Chapman (University of Wisconsin School of Medicine, USA). GST fusion cytoplasmic domain of synaptotagmin C2AB domain (residues 96–421), which can be considered as the intact cytoplasmic domain [25] and C2A domain (96–265), were transferred into DH5α *Escherichia coli* cells. Proteins were purified by glutathione–Sepharose chromatography with a minor modification to the previous protocol [26]. After the protein had bound to glutathione–Sepharose, 1 ml buffer containing 1μg/ml DNase and RNase was added into the column, then the beads were incubated for 36 h at 4°C to get rid of DNA and RNA from the protein. After a wash with 20 ml PBS buffer, GST fusion proteins were eluted out by PBS buffer containing 5 mM glutathione.

2.3. Surface plasmon resonance (SPR) binding assays

The binding of synaptotagmin C2AB and C2A to lipid monolayer was carried out on a homemade SPR instrument [27–29]. A mixture of 1:1 DMPC/DMPS (dissolved in 3:1 chloroform/methanol, v/v) was spread on a Langmuir–Blodgett trough and compressed to a surface pressure of 44 mN/m, then the monolayer was horizontally transferred to a glass slide covered by a 50 nm thick gold layer. For each experiment, the monolayer surface was first blocked with 200 μg/ml bovine serum albumin, then rinsed with buffer. Series concentrations (40–500 nM) of C2AB or C2A were added into sample chamber to perform the binding process. The dissociation constant of C2AB and C2A were calculated using a double inverse linear rearrangement of adsorption isotherm [29,30]. For the experiments of the InsP6 effects on synaptotagmin binding to lipid, proteins were first incubated with 1.6–160 μM InsP6 for 1 h at 4°C.

2.4. Liposome preparation

Small unilamellar vesicles were prepared according to Ji et al. with minor modification [31]: lipids of 1:1 mixture of PS (bovine brain) and egg PC were dissolved in chloroform/methanol (3:1, v/v) and dried under a stream of nitrogen. Residual solvents were removed under high vacuum for 2 h. The lipid films were then resuspended in PBS and sonicated at 30°C using a probe sonicator to near optical clarity.

2.5. Circular dichroism (CD) spectroscopy

CD measurements were carried out on a Jasco J-715 spectropolarimeter. Samples containing 5 μM C2AB and C2A were scanned at least four times at the rate of 100 nm/min and averaged. The temperature of the sample compartment was maintained at 25 ± 0.2°C. The path length of the quartz cell was 0.1 mm. In the experiments, a blank run of buffer alone was subtracted from the

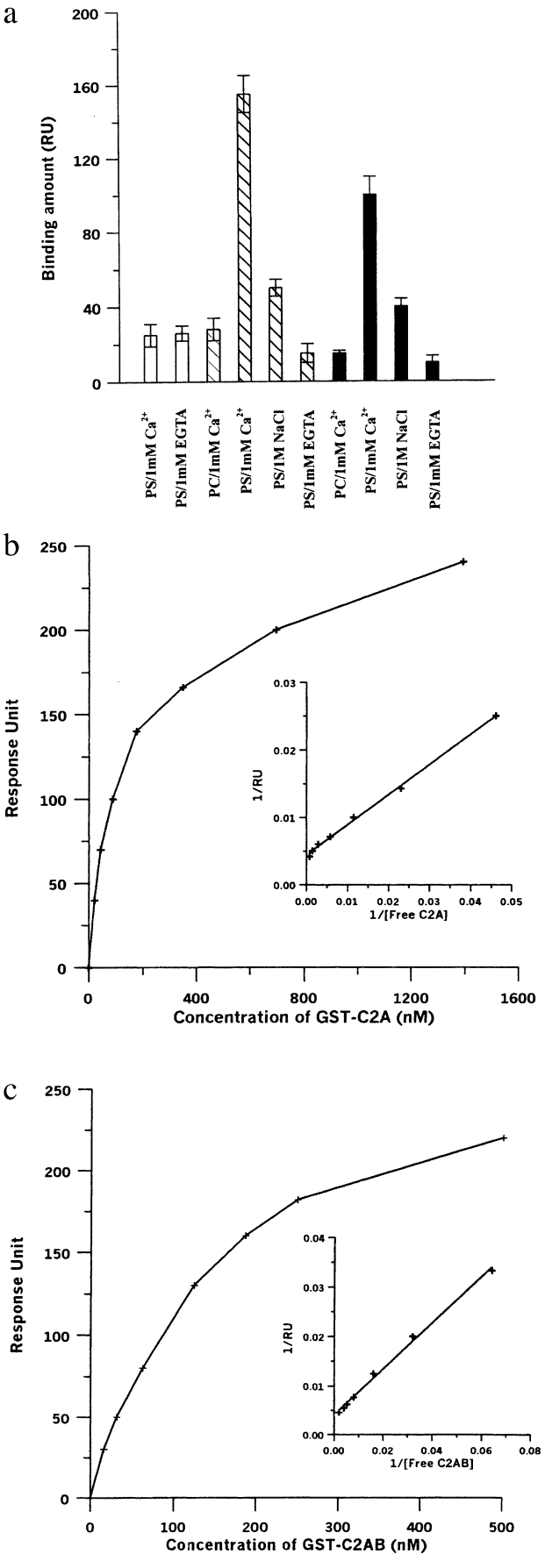

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Fig. 1. PS and Ca^{2+} dependence of GST-C2A and GST-C2AB binding to phospholipid monolayer. a: Binding amount of GST (open bars) on 50% PS/50% PC monolayer, GST-C2A (hatched bars) and GST-C2AB (closed bars) on pure PC and 50% PS/50% PC monolayers. Ions added into buffer (Tris–HCl, pH 7.4, 100 mM NaCl) are indicated at the bottom of the figure. The monolayer surface was first blocked with 200 μg/ml bovine serum albumin, then 0.008 mg/ml GST, GST-C2A or GST-C2AB was added into sample chamber to carry out the binding process. Assays were carried out in triplicate. b,c: Derivation of dissociation constant of GST-C2A (b) and GST-C2AB (c) to negatively charged phospholipids. The running buffer is Tris–HCl, pH 7.4, 100 mM NaCl, 1 mM Ca^{2+}. The two double inverse linear rearrangements of adsorption isotherms both have coefficient of curve fitness of $R^2 = 0.99$.

experimental spectra for correction. The 200–250 nm spectra were used for analysis and calculation. All spectra were converted into mean residue ellipticity, $[\theta]$, in degrees$\times$cm$^2\times$dmol^{-1}, using a mean residue molecular mass of 110 Da.

2.6. Fluorescence measurements

Fluorescence was measured with a HITACHI F-2500 fluorescence spectrophotometer using a 1 cm^2 quartz fluorescence cuvette. The emission and excitation slit widths were set at 5 nm. The excitation wavelength was set at 295 nm and 300–400 nm emission spectrum was scanned. The background spectra of the buffers were subtracted, and the data of the emission peaks and fluorescence intensities were determined from the corrected spectra.

3. Results

3.1. Interactions of C2A and C2AB with supported phospholipid monolayers

SPR is a useful tool in obtaining thermodynamics and kinetics information of biochemical reactions occurring at or near interfaces due to its label-free and real time capability [32]. Both the binding constant and the stoichiometry of the interaction can be obtained by SPR. The Ca^{2+}-dependent binding of GST, GST-fused C2A and C2AB domains of synaptotagmin I to negatively charged PS containing lipid monolayer, was measured by SPR, as shown in Fig. 1a. In the presence of 1 mM Ca^{2+}, the binding amounts of C2A and C2AB to pure DMPC monolayer were 28 ± 6 RU and 15 ± 5 RU, which is much less than that of a mixture of 1:1 DMPS/DMPC monolayer (155 ± 10 RU and 100 ± 9.8 RU). The control experiment showed that GST only had a binding amount of 25 ± 6 RU on the DMPS/DMPC monolayer. This indicates that synaptotagmin prefers to bind to negatively charged monolayer, which is in accord with the results obtained in the liposome model system [11]. Adding 1 M NaCl or 1 mM EGTA into the buffer inhibited the binding of synaptotagmin to the DMPS/DMPC monolayer. The presence of 1 M NaCl did not eliminate all the binding of C2AB and C2A to the mixture, and a considerable amount (about 30%) of C2AB or C2A still remained on the DMPS/DMPC monolayer. However, EGTA can eliminate almost all binding of synaptotagmin to lipids, which may be due to the chelation effect of EGTA on Ca^{2+} from synaptotagmin. Such phenomenon suggests that as well as electrostatic force, the hydrophobic interaction may also be involved in the Ca^{2+}-dependent membrane binding of C2A and C2AB.

Further analysis was carried out to obtain numerical binding constants of the two proteins to the negatively charged lipid mixture. As shown in Fig. 1b,c, the dissociation constants were obtained in terms of the method described in Section 2. The two parameters obtained were 115 nM and

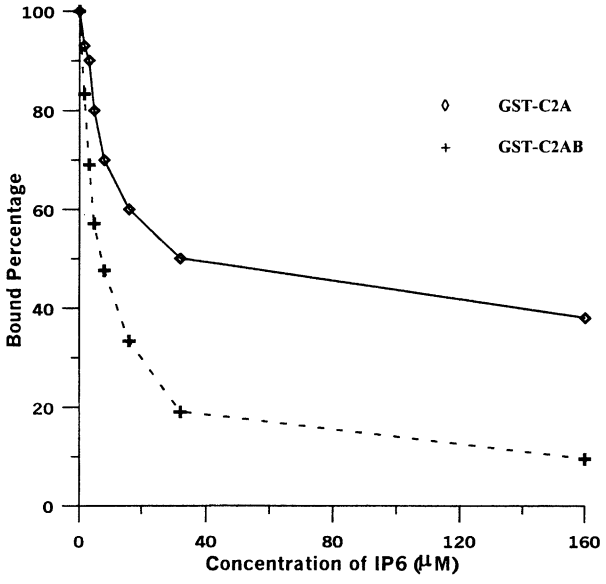

Fig. 2. The inhibitory effect of InsP6 on the phospholipid affinity of GST-C2A (◊) and GST-C2AB (+). Before the binding process is carried out, GST-C2A or GST-C2AB was incubated with different concentration of InsP6 for 1 h at 4°C. All the experiments were carried out at 25 ± 1°C

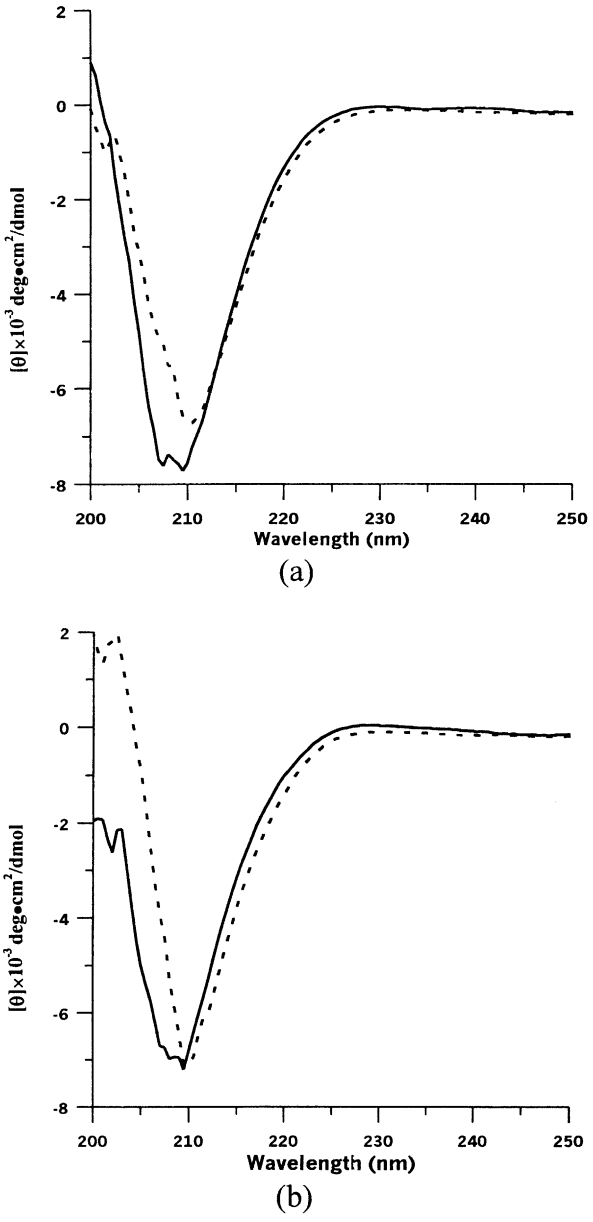

Fig. 3. CD spectroscopy of GST-C2AB with different concentration of InsP6. a: The effect of InsP6 on C2AB without liposome. The solid line is in the absence of InsP6, and the dotted line is with 8 μM InsP6. b: The effect of InsP6 with 200 μM liposome (50% PS/50% PC). The solid line is in the absence of InsP6, and the dotted line is with 8 μM InsP6. The buffer used is PBS with 1 mM Ca^{2+}, pH 7.4. All the experiments were carried out at 25 ± 1°C.

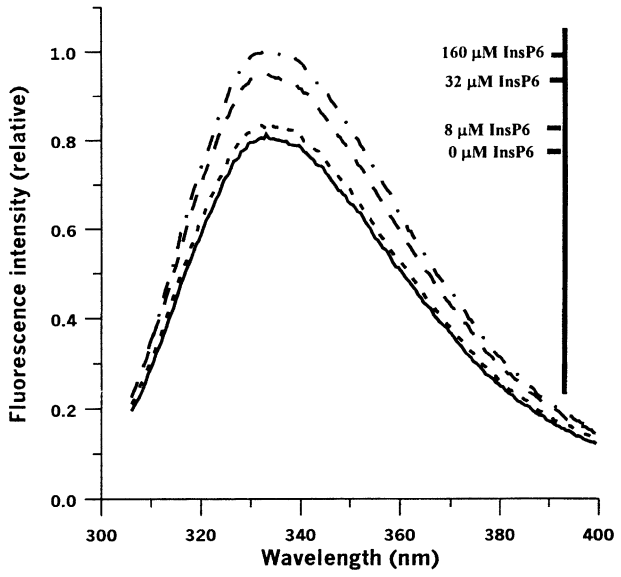

Fig. 4. Effect of InsP6 on Trp fluorescence of GST-C2AB in the presence of 50% PS/50% PC liposome. The InsP6 concentration from the bottom curve to top is 0, 8, 32, 160 μM. The buffer is PBS with 1 mM Ca^{2+}, and the concentration of liposome is 200 μM. All the experiments were carried out at 25 ± 1°C

101 nM for C2AB and C2A, respectively. The C2AB domain had almost the same binding affinity as that of C2A, indicating that the binding site of synaptotagmin to negatively charged lipids is more probably located in the C2A domain.

3.2. *The influence of InsP6 on the synaptotagmin/phospholipid interactions*

The role of InsP6 plays in synaptotagmin/lipids interactions was investigated by analyzing the effect of InsP6 on the binding of C2AB to a lipid mixture. Since it was reported that InsP6 does not bind to the C2A domain [19], C2A was used as a reference protein for comparison. The results in Fig. 2 show that the adsorption amounts of the two proteins remarkably decreased after incubation of InsP6 with the two proteins. The distinct effect of InsP6 on the membrane adsorption between C2A and C2AB is an intriguing phenomenon. The inhibitory effect of InsP6 on C2AB was more significant than that on C2A. At low concentration of InsP6 (1.6 μM), there was little difference in the binding percentage between C2AB and C2A, while increasing InsP6 concentration decreased the binding amount of C2AB more rapidly. After the proteins were incubated with 32 μM InsP6, the binding amount of C2AB decreased to approximately 20% while that of C2A decreased to 50%.

Table 1
The percentages of various secondary structures in GST-C2AB with different concentration of InsP6

	Without InsP6	With 8 μM InsP6
(a) GST-C2AB without liposome		
Helix:	8.9	8.9
Beta:	32.9	34.0
Turn:	30.1	29.3
Random:	28.1	27.8
Total:	100.0	100.0
(b) GST-C2AB with 200 μM 50% PS/50% PC liposome		
Helix:	6.9	12.4
Beta:	31.4	27.2
Turn:	30.6	34.8
Random:	31.1	25.5
Total:	100.0	100.0

The 200–250 nm spectrum was used for fitting. The fitting software used was J-700 for Windows Secondary Structure Estimate, version 1.10.00, provided by the JASCO Corporation, Hachioji City, Tokyo, Japan.

The binding affinity change in C2AB adsorption may be associated with its structural change. CD and fluorescence measurements were performed to verify this idea. If a certain change occurs in the secondary structure of C2AB after InsP6 binding, it should be detected by CD spectroscopy. The CD spectra (200–250 nm) of C2AB reacting with InsP6 in the absence and presence of PS/PC (1:1) liposome are shown in Fig. 3a,b. The correspondent contents of α-helices, β-sheets and random-coils listed in Table 1 were estimated using the method programmed by Yang [33]. Comparing the CD spectrum before and after the addition of InsP6 shows that in the absence of liposome the change in the secondary structure of C2AB was minimal while in the presence of liposome it was significant. After reacting with 8 μM InsP6 the α-helix content increased from 7% to 12% in liposome solution, while in absence of liposome the change in α-helix was nearly zero.

Synaptotagmin has three tryptophan residues, one is in the C2A domain (Trp259), and the other two are in the C2B domain (Trp380 and Trp404). None of these three is near the membrane insertion loop [8,34]. Tryptophan fluorescence was strongly influenced by the environment of the indole side chain. Fig. 4 shows the measurements of the intrinsic Trp fluorescence of C2AB in the presence of PS/PC (1:1) liposome with different concentration of InsP6. The fluorescence spectra had no wavelength shift, but have notable intensity increase after reacting with InsP6.

4. Discussion

4.1. *C2A is the predominant membrane-binding domain*

Genetic studies have provided compelling evidence that synaptotagmin I functions as the major Ca^{2+} sensor in neuronal exocytosis [35–38]. It has been reported that the ability of C2A to bind Ca^{2+}, and thus trigger exocytosis, is strongly potentiated by anionic phospholipids [11]. The C2B domain was found to bind IHPS and some other proteins, although its exact function is still unclear. Thus, to understand the interaction of the large cytoplasmic domain of synaptotagmin with phospholipids is of essential importance to reveal the molecular mechanism involved in the neuronal exocytosis.

In the present study, the interactions of synaptotagmin C2AB and C2A domains with supported monolayer were analyzed. The results show that C2AB and C2A can specifically bind to negatively charged lipid containing monolayers in a calcium-dependent manner. The high concentration of NaCl can significantly inhibit, but not totally eliminate, the Ca^{2+}-dependent protein adsorption, indicating that the electrostatic interaction is the major force, and that the hydrophobic interaction may also be involved. The fact that EGTA can totally eliminate the protein adsorption indicates that calcium may be involved in the hydrophobic interaction.

The obtained dissociation constants of C2AB and C2A to DMPS/DMPC monolayer are almost identical. This suggests that the affinity to lipids of C2A and C2AB is similar. Because C2AB includes C2A and C2B, it leads to a conclusion that the

C2A domain is the predominate fragment to bind to the phospholipid membrane.

4.2. InsP6 binding can weaken the C2AB/membrane interaction

It was reported that InsP6 could block synaptic transmission, therefore InsP6 was assumed to be a potential regulator in neurotransmitter release [23]. It was suggested that InsP6 might regulate the synaptotagmin–AP-2 interaction [24]. One InsP6 molecule carries 12 negative charges in a fully dissociation state, so InsP6 in solution may compete with negatively charged membrane surface for the adsorption of C2A and C2AB. Thus, addition of InsP6 may inhibit the membrane adsorption of the proteins to a certain extent. Such inhibitory effect will work on both C2A and C2AB, as shown in Fig. 2.

Importantly, these results show a different effect of InsP6 on C2A and on C2AB (see Fig. 2) which is obviously different from the effects of Ca^{2+}, NaCl and EGTA on membrane binding. For the factors of Ca^{2+}, NaCl and EGTA, they all have the similar influence on C2A and C2AB (see Fig. 1a). This reflects the fact that the factors of Ca^{2+}, NaCl and EGTA are mainly working on the C2A domain, since C2AB most probably binds to the membrane through its C2A domain. The different effect of InsP6 on the membrane binding of C2A from that of C2AB, thus, reflects that C2B is the major target of InsP6. This result coincides with the previous published data [19].

Structural changes occur during InsP6 binding to C2AB. It was reported that in the presence of 50 μM InsP6, the tryptic digestion pattern of synaptotagmin in cerebellar synaptosomes has notable changes, which suggests the conformation of synaptotagmin in situ is changed by the addition of InsP6 [24]. Here, a considerable structural change has been detected by both CD and fluorescence measurements in the presence of liposome, but the mechanism by which InsP6 affects the membrane binding of C2AB is still unknown. It was reported that synaptotagmin C2AB had a certain intramolecular association between the two C2 domains in the trigger of Ca^{2+} [39]. Therefore, InsP6 binding to C2B domain may change the association of the two domains. Such change may not be in its secondary structure (in the absence of liposome, as shown in Fig. 3a), but can weaken the C2AB/membrane interaction (as shown in Fig. 2). Therefore, the IHPS-induced inhibitory effect on neurotransmitter release might be partially caused by the inhibitory role of InsP6 in synaptotagmin–lipid interaction.

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