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Regulation of AP-2-Synaptotagmin Interaction by Inositol High Polyphosphates

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The inositol high-polyphosphate series (IHPS) inhibits its neurotransmission through binding to the second C2 domain of synaptotagmins I and II(Syt), synaptic vesicle membrane proteins. We have revealed that several proteins, including α adaptins which are specific subunits of clathrin assembly protein, AP2, were eluted from mouse brain by affinity elution chromatography from the C2 domains of Syt II-immobilized Sepharose using 50 μM of $InsP_6$. The interaction between Syt II and AP2 was more markedly inhibited by IHPS than by the same concentration of $InsP_3$. Limited digestion of mouse crude synaptosomal fractions with trypsin revealed different cleavage patterns in the presence and absence of 50 μM $InsP_6$. These results suggest that IHPS-binding to the C2B domain of synaptotagmin alters the state of protein–protein interaction including the synaptotagmin-AP2 interaction, possibly resulting in the inhibition of events involved in the synaptic vesicle trafficking.

Synaptotagmins (Syts) are a family of intrinsic membrane proteins and share the common structural character of a single trasnsmembrane region with a short intravesicular amino terminus and tandem cytoplasmic repeats homologous to the C2 regulatory region of protein kinase C(1). Syt I, the best characterized isoform, is considered to be a Ca^{2+} sensor which functions in Ca^{2+} regulated synaptic vesicle exocytosis(2).

Recently, we found that Syt I and II are inositol high-phosphate series (Inositol-1,3,4,5-tetrakisphosphate-($InsP_4$), Inositol-1,3,4,5,6-pentakisphosphate($InsP_5$), and inositol hexakisphosphate($InsP_6$); IHPS) binding proteins(3) and that the central region of the second C2(C2B) domain of Syt II is essential for IHPS binding(4). A study using squid giant synapses revealed that microinjection of the members of IHPS into the presynaptic terminal blocks synaptic transmission(5) and that this blockade is released by co-injection of the specific IgG which recognizes the Syt II C2B domain and inhibits IHPS binding to Syt(6). These evidences strongly suggest that IHPS blocks synaptic transmission through binding to the C2B domain of synaptotagmin and that IHPS acts as a modulator of neurotransmitter release(reviewed in ref.7).

In this study, to clarify the molecular mechanism of synaptic transmission inhibition induced by IHPS, we tried to isolate Syt-binding proteins which binding is regulated by IHPS. Herein, we carried out affinity elution chromatography from the C2 domain of Syt II-immobilized Sepharose using InsP6 as the eluent and examined the effects of IHPS against the binding between synaptotagmin II and AP2 which was a component of the eluate. The IHPS-effect on the patterns of partial synaptotagmin digestion in crude synaptosomes was also examined.

MATERIALS AND METHODS

Materials. Inositol-1,4,5-trisphosphate ($InsP_3$) and $InsP_4$ were obtained from Dojindo Laboratories, Japan. $InsP_5$ and $InsP_6$ were purchased from Calbiochem. Monoclonal antibodies that recognize α-adaptin were generously provided by Dr. Margaret S. Robinson (the University of Cambridge, UK). TPCK-treated trypsin was obtained from Sigma. All other reagents used were of analytical grade or the highest grade available.

Construction, expression and purification of fusion proteins. The construction of a glutathion S-transferase (GST) fusion protein containing the mouse Syt III C2AB domain (GST-STIIIC2AB), mouse Syt II C2AB domain (GST-STIIC2AB), Syt II C2A domain (GST-STIIC2A), Syt II C2B domain (GST-STIIC2B) or its mutant (GST-STII C2BΔ325-341) which abolishes the ability to bind to IHPS(8) was described previously(4,8). GST fusion proteins were expressed and purified essentially as described(4,8).

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Abbreviations: Syt, synaptotagmin; $InsP_4$, inositol-1,3,4,5-tetrakisphosphate; $InsP_5$, inositol-1,3,4,5,6-pentakisphosphate; $InsP_6$, inositol hexakisphosphate; IHPS, inositol high polyphosphate series; $InsP_3$, inositol-1,4,5-trisphosphate; GST, glutathion S-transferase.

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Isolation of Syt II C2AB binding proteins. Mouse whole brain(5g) was homogenized in a 5×vol. of buffer A (20 mM Tris-HCl pH 8.0, 2 mM EDTA, 0.5 M NaCl, 0.5% Triton X-100, 1 mM DTT, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml Pepstatin A) and centrifuged at 100,000 ×g for 1h at 4°C. The supernatant was diluted with a 4× vol. of buffer A minus NaCl Triton X-100. Forty ml of the diluted solution was incubated with 1ml (50% slurry) of the glutathione Sepharose (Pharmacia) beads retaining GST-STIIC2AB (1mg protein/ml beads) at 4°C, for 4h. After the beads had been packed into mini-column and thoroughly washed with buffer B (20 mM Tris-HCl pH 8.0, 2 mM EDTA, 0.1 M NaCl, 0.1% Triton X-100, 1 mM DTT, 1 mM PMSF), proteins were sequentially eluted with 10 ml each of buffer B containing first 50 μM InsP3, second 50 μM InsP6, and finally 10 mM glutathione. Eluted proteins were detected using a Bio-Rad protein assay kit.

Protein binding assay. One hundred microliters of GST-STII-C2AB fusion proteins immobilized onto glutathione Sepharose(50% slurry, 1mg protein/ml beads)were incubated with 1 ml of crude mouse brain extract(750 μg/ml proteins) at 4°C for 4h. After removing the unbound fraction by centrifugation (5,000 rpm ×1min. at 4°C), the resins were washed with buffer B. Proteins bound to the resins were eluted by adding 2×SDS-PAGE sample buffer and then examined by 12.5% SDS-PAGE and western blotting.

The effects of IHPS on the binding of AP2 to synaptotagmin were studied as follows. The washed resins containing GST fusion proteins preincubated with crude mouse brain extract were prepared as above and were suspended in 100 μl of buffer B (50% slurry). After the suspensions had been incubated with various inositol phosphates at the indicated concentrations for 1h at 4°C, the supernatants containing the proteins released from the resins were collected by centrifugation. Equal aliquots of the supernatants were analyzed on 10% SDS-PAGE, followed by western blotting using anti α-adaptin specific antibodies. Quantitative analysis was carried out using ^{125}I Protein A(DuPont) as the secondary antibody and a Fujix BAS 2000 system.

Limited digestion of synaptotagmin II. Murine cerebellar crude synaptosomes were prepared as described by Huttner et al.(9). The synaptosomal suspension (1.3 mg protein/ml, 10 mM Hepes, pH 7.4, 150 mM NaCl, 2mM EGTA) was incubated for 25 min at 25°C with TPCK-treated trypsin at the indicated concentrations and with 50 μM of $InsP_6$ or H_2O alone. Reactions were halted by adding of SDS-PAGE sample buffer and analyzed by SDS-PAGE and western blotting using anti synaptotagmin II C2B polyclonal antibodies(6).

RESULTS AND DISCUSSION

To better understand the molecular mechanism of IHPS-induced synaptic transmission blockade, we attempted to isolate the Syt-binding proteins of which the binding is regulated by IHPS. GST-STIIC2AB Sepharose beads were incubated with mouse whole brain extract, the binding proteins were sequentially eluted with 50 μM $InsP_3$, 50 μM $InsP_6$, and 10 mM glutathione (Fig. 1, closed circle). No significant proteins were eluted by 50μM $InsP_3$ (Fig.1, Fractions 1-8). On the other hand, the proteins peak appeared by elution with 50 μM $InsP_6$ (Fig.1, Fractions 9-15). The peak was significantly higher than that composed of proteins eluted with 50 μM $InsP_6$ from GST-STIIC2AB Sepharose which was not incubated with the brain extract (Fig.1, open circle). No proteins were eluted from GST Sepharose, as a control, by either 50 μM $InsP_3$ or 50 μM $InsP_6$ (Fig.1, open triangle). These lines of

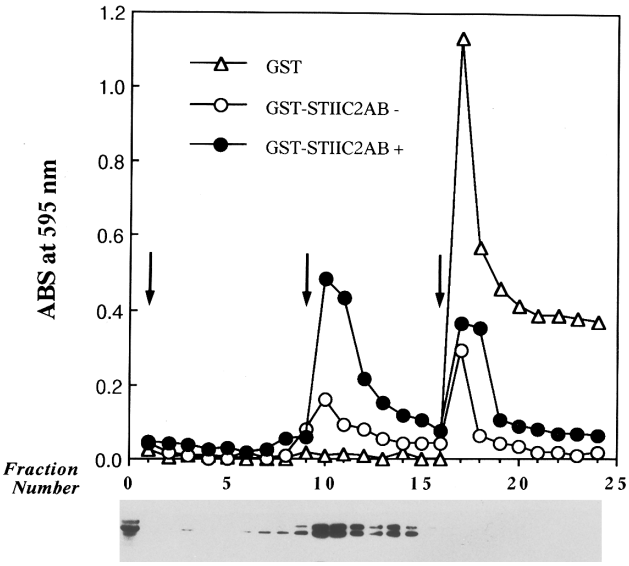

FIG. 1. Elution profile from GST-STIIC2AB-conjugated Sepharose. Whole brain extracts were incubated with glutathione Sepharose preadsorbed with GST alone(open triangles) or GST-STIIC2AB fusion protein(solid circles). Open circles indicate the elution profile from GST-STIIC2AB-conjugated Sepharose without incubation with the extract. Arrows indicate the start of each eluent, from the left, 50 μM $InsP_3$, 50 μM $InsP_6$, 10 mM glutathione. Each fraction was analyzed by western blotting for α-adaptin(bottom panel).

evidence indicate the existence of proteins which associate with the Syt II C2AB domain and which are released from it by $InsP_6$, but not by $InsP_3$. SDS-PAGE of the fractions eluted wiht 50 μM $InsP_6$ from GST-STIIC2AB Sepharose revealed that these fractions contained at least ten different polypeptides, i.e. 120 kDa, 100kDa, 80 kDa, 66 kDa, 50 kDa, 43 kDa, 40 kDa 33 and 32 kDa, 29 kD, and 25 kDa(Fig.2A). It is reported that clathrin assembly protein AP2 complex bound to C2B domain of Syt I, III-VIII(10,11,12, reviewed in ref. 17). Next, we examined which fractions contained AP2 by western blotting using monoclonal antibodies against the α subunit of AP2(Fig.1 and Fig.2B). AP2, indeed, bound GST-STIIC2AB Sepharose and was eluted mainly with 50 μM $InsP_6$ as a doublet band of 100 and 110 kDa polypeptides corresponding to αC and αA subunit, respectively in western blotting. β-SNAP was also reported to bind the C2B domain of synaptotagmin and the binding was inhibited by IHPS(13). However, in this experiment, we could not detect β-SNAP binding to GST-STIIC2AB even in immunoblotting (data not shown), possibly due to our using different Syt isoform or different GST fusion construct from that of Schivao, G. et al.(13). Previously, we demonstrated that $InsP_4$, $InsP_5$, and $InsP_6$, blocked synaptic transmission while $InsP_3$ did not. To test the possibility that the AP2-Syt interaction is related to this phenomenon, we examined the effects of various concentrations of IHPS on the interaction. In general, as the number

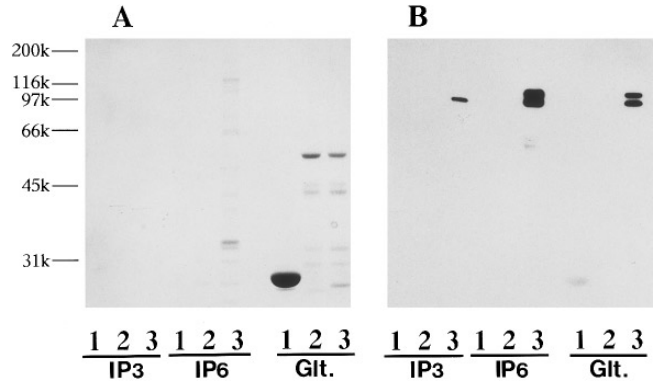

FIG. 2. Syt C2AB domain-binding proteins. The fractions of each eluate, 50 μM $InsP_3$(IP3), 50 μM $InsP_6$(IP6), and 10 mM glutathione(Glt.), were collected and concentrated, and were processed for SDS-PAGE(A) and western blotting for α-adaptin(B). Lanes 1, 2, and 3 indicate the fractions from the glutathione beads preadsorbed with GST alone, GST-STIIC2AB without incubation with the brain extract, and GST-STIIC2AB incubated with the extract, respectively.

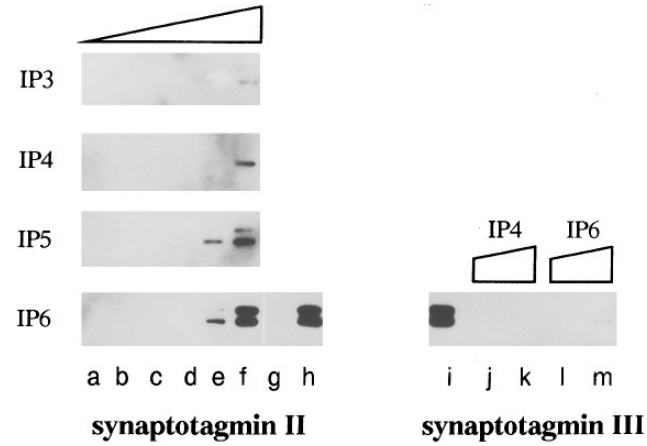

FIG. 3. Effect of IHPS on the interaction between Syt and AP2. The effects of various concentrations of IHPS(1 nM(a), 10 nM(b), 100 nM(c), 1μM(d), 10 μM(e, j, l), 100 μM(f, k, m)) on the interaction between Syt II and AP2(left panel) or Syt III and AP2(right panel) were examined. AP2 released from GST-STIIC2AB or GST-STIII-C2AB Sepharose by IHPS was detected by western blotting for α-adaptin. AP2 interacted equally with GST-STIIC2AB(h) and GST-STIIIC2AB(i). 100 μM inositolhexakissulfate did not inhibit the interaction between Syt II and AP2(g).

of phosphoryl groups increases, the ability dissociating the Syt-AP2 interaction also increase(Fig.3; left panel). For example, 100 μM of $InsP_3$, $InsP_4$, $InsP_5$, or $InsP_6$ dissociates 5.2%, 7.7%, 83.4%, or 94.1% of AP2 from Syt, respectively. Since 100 μM inositol hexakissulfate did not affect the binding(Fig.3; left panel, h), the effects of these compounds are not due to these highly negative charge, indicating inositol polyphosphates specific events. This order was the same as that of the affinity of the recombinant GST-STIIC2B fusion protein for IHPS examined previously(4). However, the apparent I.C. 50 value of $InsP_4$ against the binding between GST-STIIC2AB and AP2(I.C.50 $\gg$ 100 μM) was apparently much higher than the Kd value of GST-STIIC2B for $InsP_4$(Kd=117 nM,(4)). Furthermore, the AP2 specific subunits, αA and αC adaptin subunits were themselves reported to be $InsP_6$-binding proteins(14,15,16) and IHPS inhibited the clathrin assembly properties(14,16). These evidences suggested the possibility that th effects of IHPS on the interaction between GST-STIIC2AB and AP2 was not through IHPS-binding to the C2B domain of Syt II. To rule out this possibility, the same experiments was carried out using GST-STIIIC2AB which still has the capacity to bind to AP2(10) but does not significantly bind to $InsP_4$(8). AP2 bound to GST-STIIIC2AB to the same extent as to GST-STIIC2AB(Fig.3; right panel, lane i) but 100 μM $InsP_4$ or $InsP_6$ showed no or only 5% inhibition of Syt-AP2 binding, respectively(Fig.3; lanes j,k, and l,m). These findings showed that IHPS-binding to the Syt C2B domain inhibited the Syt-AP2 interaction.

Our previous studies(8) showed that the IHPS binding site of Syt was mapped on the central region of the C2B domain, which contains a cluster of positively charged amino acids (8 Lys and 1 Arg). To compare the binding regions of AP2 and IHPS on the Syt C2B domain, we examined both the AP2-binding to the mutant fusion protein (mut 13) lacking the central region of the C2B domain(Δ325-341) which completely abolished the ability to bind IHPS(8) and the effect of IHPS on the binding. The identical amounts of each fusion protein, wild and mut 13, were prepared(Fig.4 A), and the AP2 binding to each fusion protein was detected by western blotting. The amount of AP2-binding to mut 13 was still 40.3%

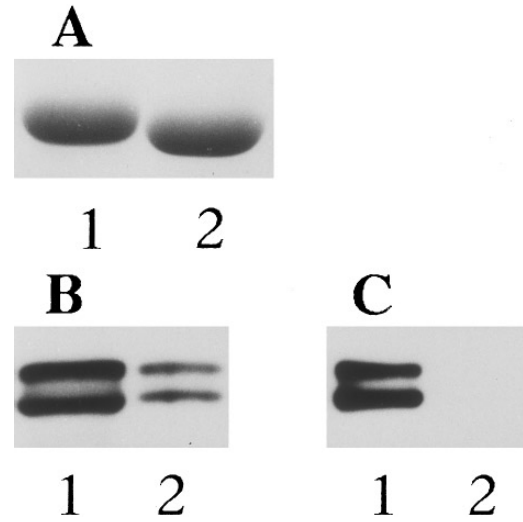

FIG. 4. Binding of AP2 to a mutant Syt. (A)Wild(lane,1) and mutant(lane,2, mut13(7)) GST-STIIC2B fusion proteins were expressed. (B)AP2 binding to each fusion protein was examined by western blotting for α-adaptin. lane,1; wild, lane,2; mut13. (C)AP2 released from wild(lane1) or mut13(lane2) Sepharose by 100 μM $InsP_6$ was analyzed by western blotting.

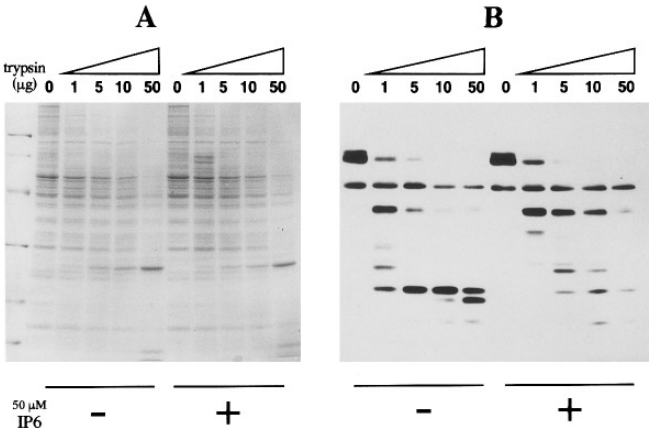

FIG. 5. Effect of 50 μM InsP6 on the tryptic digestion of Syt in cerebellar synaptosomes. Crude murine cerebellar synaptosomes suspensions were treated with the indicated amounts of TPCK-trypsin with or without 50 μM $InsP_6$. Each sample was analyzed by protein staining(A) and western blotting for synaptotagmin(B). The left lane of panel A represents the molecular standard; 97.4kDa, 66kDa, 45kDa, 31kDa, 21.5kDa, and 14.5kDa.

of that to the wild type protein(Fig.4 B), and the binding was specific because no AP2 binding was detected for the control, GST alone(data not shown). One hundred μM of $InsP_6$ eluted AP2 from the wild type protein(Fig.4 C, lane1) with nearly 100% recovery. On the other hand, no AP2 was eluted from mut 13(Fig.4C, lane2) with the same concentration of $InsP_6$. These results suggested that the AP2-binding region on the Syt II C2B domain overlapped the IHPS-binding domain, though this overlap was incomplete. It is likely that the AP2 molecule is in contact with the C2B domain, which has a broader surface or multiple contact points than IHPS. This, as well as the high affinity of AP2 for synaptotagmin($EC50 \approx 10^{-10}$,(10,11)), may partially explain that the much higher concentration of IHPS compared to their affinity for synaptotagmin is necessary for inhibiting the interaction between AP2 and synaptotagmin.

Finally, to test whether it occur on the native synaptotagmin that IHPS-dependent changes of conformation or protein-protein interaction state, the crude synaptosomal fractions were prepared from mouse brain and limited tryptic digestion was carried out in both the presence and the absence of 50 μM $InsP_6$. Fifty μM $InsP_6$ has almost no effect on trypsin activity since there was no significant difference in the overall protein digestion pattern determined by protein staining(Fig.5, A). In the presence of 50 μM $InsP_6$, two obvious changes in the proteolytic pattern of synaptotagmin were observed; 1)a tryptic fragment of approximately 43 kDa was stabilized, 2)an approximately 25 kD fragment which was relatively resistant to trypsin in the absence of 50 μM $InsP_6$ was destabilized(Fig.5, B). These findings suggested that the conformation or protein-protein interaction state of synaptotagmin *in situ* was altered by IHPS.

In this study, several proteins which bind the C2AB domain of synaptotagmin, and are then released from synaptotagmin by IHPS were detected. Since AP2 was one of these proteins, the inhibitory effect of IHPS in neurotransmitter release might be partially due to the inhibition of the interaction between synaptotagmin and AP2 resulting in blocking endocytosis. However, as the apparent I.C.50 values are so high, some other proteins or factors are presumably involved in the mechanism of the IHPS-induced neurotransmission blockade. The proteins isolated herein are candidates and the work of identifying these proteins constitutes a key approach to elucidating the mechanism of IHPS-induced neurotransmission inhibition.

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