Evidence for synaptotagmin as an inhibitory clamp on synaptic vesicle release in Aplysia neurons

KELSEY C. MARTIN*, YINGHE HU**, BETH A. ARMITAGE*, STEVEN A. SIEGELBAUM, ERIC R. KANDEL, AND BONG-KIUN KAANG‡

Howard Hughes Medical Institute and Center for Neurobiology and Behavior, College of Physicians and Surgeons of Columbia University, 722 West 166th Street, New York, NY 10032

Contributed by Eric R. Kandel, August 22, 1995

ABSTRACT While previous studies have demonstrated that synaptotagmin plays an essential role in evoked neurotransmitter release, it has been difficult to determine whether it acts to facilitate or inhibit release. To address this question, we used acute genetic manipulations to alter the expression of synaptotagmin in Aplysia neurons. Transient overexpression of synaptotagmin in acutely dissected cholinergic neurons and in cultured glutamatergic neurons decreased the amplitude of the excitatory postsynaptic potential (EPSP) by 32% and 26%, respectively. In contrast, treatment of cultured presynaptic neurons with synaptotagmin antisense oligonucleotides increased the amplitude of the EPSP by 50–75%. These results are consistent with a role of synaptotagmin as an inhibitor of release.

Neurotransmitter release is distinguished from other forms of exocytosis by its tight spatial and temporal regulation. Since calcium influx is the signal for release, there must be one or more calcium-sensing proteins present at the active zone, acting either to facilitate fusion in the presence of calcium or to inhibit fusion in its absence. Synaptotagmin has many of the characteristics expected of such a calcium sensor. It contains two sequences homologous to the C2 domain of protein kinase C and has been found to bind calcium in a phospholipid-dependent manner in vitro (1–3). Synaptotagmin coprecipitates with calcium channels (4) and with several plasma membrane proteins, including syntaxin (5) and the neurexins (6). Synaptotagmin is also a receptor for clathrin AP-2, suggesting that it may also be involved in the endocytosis of synaptic vesicles (3, 7).

Several previous studies have addressed the function of synaptotagmin in neurotransmitter release. Synthetic C2 domain peptides and antibodies against synaptotagmin inhibit release when microinjected into individual cells (8, 9). Caenorhabditis elegans, Drosophila, and mice bearing mutations in synaptotagmin all had impaired synaptic function (10–13). Biochemical studies of the interaction of synaptotagmin with other synaptic membrane proteins suggest that it functions prior to the final fusion step (14, 15). The results of these studies have led to three alternative views of the function of synaptotagmin: it docks vesicles at the plasma membrane, it promotes vesicle fusion, or it inhibits vesicle fusion.

We have designed experiments using acute genetic manipulations to increase or decrease synaptotagmin expression in Aplysia Californica neurons. First, we cloned the Aplysia homolog of synaptotagmin and then overexpressed it in cholinergic neurons of acutely dissected Aplysia ganglia and in glutamatergic sensory neurons cocultured with a single motor neuron. Second, we used antisense oligonucleotides to reduce the expression of endogenous synaptotagmin in Aplysia sensory-motor neuron cocultures. In both cases, we measured the effect of our manipulations on synaptic strength. Our results are consistent with synaptotagmin acting as an inhibitor of neurotransmitter release.§

MATERIALS AND METHODS

Cloning. The coding region of synaptotagmin A from the marine ray Discopyge omnata was amplified by PCR (Perkin–Elmer/Cetus) and used to screen an Aplysia nervous system cDNA library cloned in the λ-ZAP vector (Stratagene) as described (16).

Antibodies. Antibodies were generated by Berkeley Antibody (Richmond, CA) against a maltose binding protein–synaptotagmin fusion protein (New England Biolabs) containing residues 1–67 fused to residues 93–426 of synaptotagmin. The antiserum was purified using a fusion protein affinity column (Pierce).

Overexpression. The entire coding region of Aplysia synaptotagmin was amplified from the cDNA by PCR (Perkin–Elmer/Cetus) and ligated into the pNEX8 expression vector (17) to create pNEX8-pδ65. Two complementary synthetic oligonucleotides encoding the influenza hemagglutinin (HA) epitope YPYDVPDYA were ligated into Sac I-digested pNEX-8-pδ65 to insert the tag between Arg-411 and Pro-412 of Aplysia synaptotagmin. Generation of pNEX8-lacZ has been described (18).

DNA was prepared for microinjection as described (17) and injected by brief pressure pulses applied to microelectrodes leveled to a resistance of 20 MΩ. In cultured Aplysia neurons, overexpression of synaptotagmin was verified by indirect immunofluorescence using affinity-purified anti-synaptotagmin antibodies and expression of lacZ was verified by enzymatic staining for β-galactosidase as described (17). Immunofluorescence was visualized on a Bio-Rad MRC1000 confocal microscope mounted on a Zeiss Axiosvert and images were taken using fixed settings. In the buccal ganglia, pNEXδ-lacZ was coexpressed with pNEXδ-pδ65 and enzymatic staining of β-galactosidase was used as an indication of synaptotagmin overexpression.

Antisense Oligonucleotides. Oligonucleotides were synthesized by Oligos Etc. (Wilsonville, OR). The following sequences were used: antisense, 5′-GAG AAG CGG TCA AAG TC-3′; control (reverse order), 5′-CTG AAA CTG GCG AAG AG-3′ (base pairs 718–734 after the initiating ATG). Oligos containing three thiol groups at the 3′ and 5′ ends

Abbreviations: EPSP, excitatory postsynaptic potential; HA, hemagglutinin.

*K.C.M., Y.H., and B.A.A. made equal contributions to this work.

†Present address: Miles, Inc., Institute for Metabolic Disorders, 400 Morgan Lane, Building 24, West Haven, CT 06516.

‡To whom reprint requests should be sent at the present address: Laboratory of Molecular Neurobiology, Institute for Molecular Biology and Genetics, Seoul National University, Seoul 151-742, Korea.

§The sequence reported in this paper has been deposited in the GenBank data base (accession no. U03125).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.
RESULTS

Cloning of the Aplysia Synaptotagmin Homolog and Identification of the Endogenous Gene Product. We isolated Aplysia synaptotagmin cDNA clones using low stringency hybridization to a marine ray (D. ommata) synaptotagmin A-specific probe (24). The Aplysia synaptotagmin open reading frame encodes a polypeptide of 426 residues with a calculated molecular mass of 47,455 Da (Fig. 1). Aplysia synaptotagmin shares the putative domain structure of all other synaptotagmin isoforms: two cytoplasmic repeats of the C2 domain, a single transmembrane spanning region, and an intravesicular amino terminus (3, 25). Aplysia synaptotagmin shares sequence identity of 55.6% with synaptotagmin A from D. ommata and 56.1% with rat synaptotagmin 1.

From 300,000 clones screened with the synaptotagmin A clone from D. ommata, the five positively hybridizing clones (end-capped) were found to be effective at a dose of 30 μM without being toxic. For bath application of antisense oligonucleotides, a 10× solution of oligonucleotides suspended in L15 medium (Sigma) was added to the culture medium immediately prior to plating of the sensory neuron and was replenished daily. In other experiments, oligonucleotides were injected into sensory cells 3–12 hr after plating by pressure application to microelectrodes containing 300 μM oligonucleotides in 0.5 M potassium acetate/10 mM Tris, pH 7.2. The final intracellular concentration of oligonucleotides was estimated to be 10–30 μM.

Immunocytochemistry and Immunoblotting. Cultured Aplysia neurons were prepared for immunocytochemistry as described (19). Antibody specificity was determined by immunoblotting as described (19). In competition experiments, affinity-purified anti-synaptotagmin antibodies were preincubated with a 10-fold molar excess of maltose binding protein–synaptotagmin fusion protein. Immunoblots of pedal ganglia neurons microinjected with pNEX8-p65 HA were incubated with mouse monoclonal anti-HA antibody 12CA5 (Berkeley Antibody, Richmond, CA) diluted 1:2500 followed by horseradish peroxidase-conjugated goat anti-mouse antibody (Pierce) diluted 1:5000. Immunoblots were stripped according to the manufacturer's instructions (Amersham).

Aplysia Culture, Dissection, and Electrophysiology. Sensory cells (one or two) isolated from the pleural ganglia of adult (80–100 g) animals were plated with L7 motor cells from the abdominal ganglia of juvenile (1.5–4 g) animals as described (20, 21). In overexpression studies, electrophysiological recordings were as described (21, 22). In some antisense experiments, the sensory neuron was also impaled with a microelectrode to record the action potential triggered by a brief injection of depolarizing current (5–10 msec, 2–6 nA).

The buccal ganglia were dissected from 70- to 100-g animals and desheathed, and either cell B4 or B5 was randomly chosen to be injected with plasmid. Synaptic potentials were recorded as described in Kaang et al. (17) and Gardner and Kandel (23).

Because of an upward drift in the excitatory postsynaptic potential (EPSP) amplitude with time, the effect of overexpression was normalized by the change in the uninjected presynaptic cell: % change = [(D2 × C1)/(D1 × C2) – 1] × 100, where D1 = the initial EPSP amplitude of the injected cell, D2 = the final EPSP amplitude of the injected cell, C1 = the initial EPSP amplitude of the uninjected cell, and C2 = the final EPSP amplitude of the uninjected cell.

The statistical significance of antisense treatment in sensory-motor cocultures and of overexpression in the buccal ganglia was tested using an unpaired Student’s t test. The statistical significance of overexpression in sensory-motor cocultures was tested using a one-way analysis of variance followed by a Newman–Keuls range of means test. All results are given as means ± standard error.

Fig. 1. Amino acid sequence of Aplysia synaptotagmin. 

Aplysia synaptotagmin and were identical. Only one gene was detected on Aplysia genomic Southern blots probed at low and high stringency with the Aplysia synaptotagmin cDNA, and only one transcript was detected on Northern blots (data not shown). These findings suggest that only one synaptotagmin isoform exists in Aplysia. However, in rat and mouse there is <40% sequence identity among the multiple synaptotagmin isoforms (3). It remains possible, therefore, that other synaptotagmin isoforms exist in Aplysia that were not detected.

Polyclonal antibodies were generated in rabbits against a synaptotagmin fusion protein. On Western blots of Aplysia central nervous system, the affinity-purified antisera recognized a set of at least four proteins between 55 and 75 kDa as well as two smaller breakdown products, both of which increased in intensity in the absence of protease inhibitors (Fig. 2A). To test the specificity of this antibody, we performed immunoblots of Aplysia neurons overexpressing a HA epitope-tagged Aplysia synaptotagmin construct and compared staining with our antibody to that of a monoclonal anti-HA antibody. As shown in Fig. 2B, lane 1, the anti-HA antibody recognized a very sharp band at ~65 kDa. The same immunoblot reprobed with our anti-synaptotagmin antibody produced a broad set of bands around 55–75 kDa as well as two smaller breakdown products (lane 2). This strongly suggests that our antibody either recognizes several Aplysia synaptotagmin isoforms or cross-reacts with proteins sharing common epitopes, such as raphlin-3A, which also contains C2 domains (26).

Immunohistochemical assays with anti-Aplysia synaptotagmin antibody showed intense staining in the neuropil in cryostat sections of dissected ganglia (data not shown) and punctate staining in the neurites of cultured Aplysia neurons (see Fig. 5B). The staining pattern was indistinguishable from that observed using an affinity-purified antibody against Aplysia synaptobrevin, another synaptic vesicle protein (data not shown). This suggested to us that our anti-synaptotagmin antibody

Fig. 2. Immunoblots of Aplysia synaptotagmin. (A) Aplysia central nervous system (CNS) probed with affinity-purified polyclonal anti-Aplysia synaptotagmin antibody (lane 1) or with polyclonal anti-Aplysia synaptotagmin antibody depleted of specific antibodies by preincubation with maltose binding protein-Aplysia synaptotagmin fusion protein (lane 2). (B) Pedal ganglia injected with plasmids encoding HA epitope-tagged Aplysia synaptotagmin (pNEX8-p65 HA), probed with monoclonal anti-HA antibody 12CA5 (lane 1) and then stripped and reprobed with polyclonal anti-Aplysia synaptotagmin antibody (lane 2).
antibody most likely recognized multiple synaptotagmin isoforms or closely related synaptic proteins.

**Overexpression of Synaptotagmin cDNA in Presynaptic Neurons Decreases Release.** To analyze the function of synaptotagmin in vesicle release, we first transiently overexpressed the cDNA by injecting pNEXδ-p65 into identified cholinergic presynaptic neurons in the buccal ganglion. As a control for injection and overexpression, pNEXδ-lacZ was injected into some presynaptic cells. Baseline recordings were made 1–2 hr after plasmid injection, when the protein product of the injected plasmid was weakly expressed, if at all, and was present only in the perinuclear region. Recordings were repeated 8–12 hr later to allow time for further expression and transport of the protein out to the synapse. The synaptic strength of control-injected and un.injected cells gradually increased over the 8–12 hr between recordings, possibly triggered by the acute injury of dissection (27). Normalizing for this upward drift (see Materials and Methods), we found a 32% decrease in the postsynaptic response in cells that were injected with the synaptotagmin plasmid compared to those injected with control plasmid or buffer alone ($P < 0.05$; Fig. 3C).

To rule out the possibility that the decreased EPSP resulted from an altered response to the acute injury of dissection, we overexpressed synaptotagmin in cultured glutamnergic sensory neurons, in which the synaptic strength of control synapses does not change significantly over a 24-hr period. EPSPs were recorded from the motor neuron in response to a series of four action potentials evoked in the sensory neuron once every 20 sec. This provided a measure of baseline synaptic strength as well as of the degree of homosynaptic depression (28, 29). Sensory cells were then microinjected with pNEXδ-p65 or pNEXδ-lacZ. Fifteen to 20 hr later, the stimulus series was repeated and the cells were processed for indirect immuno-

**Antisense Oligonucleotides Enhance Neurotransmitter Release.** Through the application of antisense oligonucleotides, we attempted to reduce the amount of endogenous synaptotagmin in cultured cells. When *Aplysia* neurons are cultured, the cell body and initial axon segment are pulled out of the ganglion, leaving the synaptic terminals behind. Antisense oligonucleotides applied immediately might therefore block synthesis of the synaptic proteins required for the new synapse formed in vitro.

We used a 17-bp antisense oligonucleotide complementary to a sequence that is conserved among the *Aplysia* homolog and isoforms 1 and 2 of rat synaptotagmin. We also performed a set of experiments using antisense oligonucleotides complementary to a sequence spanning the initiation codon. Oligonucleotides were either added to the culture medium or injected into the presynaptic cell.

We examined the effects of the oligonucleotides on synaptic transmission by recording from the synapse on day 4 or day 5. The amplitude of the postsynaptic potential was 1.5–1.75 times greater in the antisense treated synapses than in synapses treated with control oligonucleotides ($P < 0.01$; Fig. 5) whether the antisense was applied to the culture medium or

**Fig. 3.** Transient overexpression of *Aplysia* synaptotagmin in dissected buccal ganglia. (A) Examples of the EPSPs recorded in B7 after stimulating one of two presynaptic cells, B4 or B5, before (recording 1) and after (recording 2) expression of plasmids encoding *Aplysia* synaptotagmin (ApSyt) in one of the presynaptic cells; control recording is from the un injected presynaptic cell. (B) Examples of EPSPs recorded in motor neuron L7 after stimulation of the sensory neuron before (1st recording) or 15–20 hr after (2nd recording) injection of plasmids encoding synaptotagmin (ApSyt) or lacZ into the sensory cell. Impaled cells were injected with plasmid but did not express the encoded protein. (C and D) Effects of transient overexpression of synaptotagmin (ApSyt) or lacZ on synaptic transmission in buccal ganglia (C) and sensory neurons (D). The height of each bar corresponds to the mean percentage change ± SEM in the EPSP amplitude between the 1st and 2nd recordings (in C, the EPSP has been normalized as described in the text). A one-way analysis of variance followed by a comparison of the mean (Newman–Keuls multiple range test) indicates that overexpression of synaptotagmin in B4 or B5 (C) significantly decreased EPSP amplitude relative to impalement ($P < 0.01$) and expression of lacZ ($P < 0.01$). In contrast, expression of lacZ did not significantly alter EPSP amplitude relative to impalement. In sensory-motor cultures (D), overexpression of synaptotagmin significantly decreased EPSP amplitude relative to impalement ($P < 0.01$) and to expression of lacZ ($P < 0.05$). In contrast, expression of lacZ did not significantly alter EPSP amplitude relative to impalement.
DISCUSSION

Does Synaptotagmin Facilitate or Inhibit Release? Our studies confirm previous work showing that synaptotagmin plays an important role in synaptic transmission. From this earlier work, however, it was difficult to determine whether synaptotagmin acts to facilitate or inhibit release.

In three genetically modified animals, C. elegans, Drosophila, and mouse, loss of synaptotagmin largely abolished evoked neurotransmitter release (10–13). However, recordings from the Drosophila neuromuscular junction of third instar larvae of synaptotagmin hypomorphs revealed that while evoked release was diminished, spontaneous release was increased (10, 11, 30). These findings are consistent with synaptotagmin acting to inhibit vesicle fusion. In its complete absence, vesicles would constantly undergo spontaneous fusion, thus depleting the pool of vesicles available for synchronous release in response to the calcium signal (31). That synaptotagmin may inhibit release is further supported by the findings of Sollner et al. (15), showing that synaptotagmin forms a complex with synaptobrevin, syntaxin, and SNAP-25 and is dispersed from this complex by α-SNAP prior to formation of a 20S complex containing the fusion factor NSF.

In mice bearing mutations of synaptotagmin 1, however, spontaneous release was unaffected, the rapid phase of evoked release was eliminated, and there were no obvious alterations in active zone morphology (13). In contrast to the findings in Drosophila, the findings in mice are more consistent with synaptotagmin acting to promote vesicle fusion. The discrepancy between the two mutant animals may reflect differences between vertebrates and invertebrates. More likely, however, it may reflect differences in the physiological function of the particular isoform eliminated in each mutant animal.

Synaptotagmin Acts as an Inhibitor of Neurotransmitter Release in Aplysia. We have attempted to increase and decrease synaptotagmin expression and to assess the effects of such changes on synaptic function. In the Aplysia culture system, changes in EPSP amplitude are likely to reflect changes in transmitter release because there is neither the complication of multiple synaptic inputs nor that of nervous system development. In addition, because the cells are electrically silent in the absence of extracellular stimulation, we can interpret changes in the size of the initial EPSP as changes in exocytosis as opposed to endocytosis of synaptic vesicles following release.

When we overexpressed synaptotagmin, immunostaining indicated a large increase in protein concentration throughout the cell. This resulted in a 26% decrease in EPSP amplitude in cultured cells and a 32% decrease in EPSP amplitude in dissected ganglia. There are several explanations for why such a large increase in protein expression should result in a relatively small change in release. (i) Synaptotagmin may act with one or more cofactors that are present at limited concentrations. (ii) Newly expressed synaptotagmin may not be efficiently incorporated into vesicles residing at the active zone. (iii) The small size of the vesicles may permit only a limited number of proteins to be present on their surface and this maximum may normally be reached. If any of these explanations apply, one would predict that decreasing the concentration of synaptotagmin would produce a much greater effect.

Indeed, when we used antisense oligonucleotides to block synaptotagmin synthesis during synapse formation, we saw a 50–75% increase in EPSP amplitude. This occurred despite the fact that we were unable to determine whether antisense treatment reduced synaptotagmin concentration since our antibody appeared to cross-react with other synaptotagmin isoforms or proteins with common epitopes. It is possible that even a small decrease in synaptotagmin expression caused a large change in release. This could occur if synaptotagmin
acted to inhibit release and if there were normally more than one copy per vesicle. Only when calcium bound and inactivated all copies would fusion proceed. Then, if antisense treatment decreased the copy number, the probability of all copies being inactivated would increase nonlinearly, leading to an increase in the total number of vesicles released.

Synaptotagmin may inhibit synaptic vesicle release in a number of ways, all of which are consistent with our data. (i) It may prevent docking of synaptic vesicles at the plasma membrane. (ii) It may act as a physical barrier to fusion of docked, fusion-competent vesicles (31). (iii) Synaptotagmin may inhibit release by preventing the formation of a fusogenic complex. Thus, Sollner et al. (15) have proposed that docked synaptic vesicles contain a complex of synaptotagmin, synaptobrevin, syntaxin, and SNAP-25 and that synaptotagmin must be replaced by α-SNAP prior to fusion of the vesicle with the plasma membrane. It may be possible to test this particular model in our system by overexpressing α-SNAP and synaptotagmin in the same presynaptic cell.

We thank Richard Scheller for the D. ommata synaptotagmin A clone; Oligo Etc. for technical support; and the Howard Hughes Medical Institute and a National Institute of Mental Health Center Grant, P50 MHS0733-02.