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Role of the C2B domain of synaptotagmin in vesicular release and recycling as determined by specific antibody injection into the squid giant synapse preterminal

(inositol high-polyphosphate series/vesicular fusion/vesicular recycling)

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ABSTRACT **Synaptotagmin (Syt) is an inositol high-polyphosphate series [IHPS inositol 1,3,4,5-tetrakisphosphate (IP_4), inositol 1,3,4,5,6-pentakisphosphate, and inositol 1,2,3,4,5,6-hexakisphosphate] binding synaptic vesicle protein. A polyclonal antibody against the C2B domain (anti-Syt-C2B), an IHPS binding site, was produced. The specificity of this antibody to the C2B domain was determined by comparing its ability to inhibit IP_4 binding to the C2B domain with that to inhibit the Ca^{2+}/phospholipid binding to the C2A domain. Injection of the anti-Syt-C2B IgG into the squid giant presynapse did not block synaptic release. Coinjection of IP_4 and anti-Syt-C2B IgG failed to block transmitter release, while IP_4 itself was a powerful synaptic release blocker. Repetitive stimulation to presynaptic fiber injected with anti-Syt-C2B IgG demonstrated a rapid decline of the postsynaptic response amplitude probably due to its block of synaptic vesicle recycling. Electron microscopy of the anti-Syt-C2B-injected presynapse showed a 90% reduction of the numbers of synaptic vesicles. These results, taken together, indicate that the Syt molecule is central, in synaptic vesicle fusion by Ca^{2+} and its regulation by IHPS, as well as in the recycling of synaptic vesicles.**

Synaptotagmins (Syt) are abundant synaptic vesicle proteins (2) that function as a Ca^{2+} sensor in synaptic vesicle exocytosis (1, 3–5). The structural feature of Syt is two copies of highly conserved repeats, known as the C2A and C2B domains, which are homologous to the C2 regulatory region of protein kinase C in the cytoplasmic domain (2). The importance of the C2 domain of synaptotagmin in Ca^{2+}-regulated exocytosis has been shown by several injection experiments (6, 7). In the companion paper we address the physiological role of the C2A domain of Syt in transmitter release and show that it functions as a Ca^{2+} sensor (8). However, the role of the C2B domain of Syt in transmitter release is not clear in spite of the recent *in vitro* biochemical work concerning inositol high-polyphosphate series (IHPS) binding (9, 10) and clathrin assembly protein (AP2) binding (11).

In the present study a specific antibody against the C2B fraction of Syt (anti-Syt-C2B) was used to examine the role of this domain in transmitter release. Injection of the antibody at the squid (*Loligo pealii*) giant preterminal did not affect synaptic transmission; however, coinjection of anti-Syt-C2B and inositol 1,3,4,5-tetrakisphosphate (IP_4), a member of IHPS and a blocker of transmitter release (1), failed to inhibit transmitter release. In addition, repetitive stimulation of anti-Syt-C2B IgG-injected preterminal demonstrated a second role for Syt: the C2B domain appears to be important in recycling of released synaptic vesicle membrane, which is severely retarded following antibody injection.

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MATERIALS AND METHODS

Construction, Expression, and Purification of Fusion Proteins. Construction, expression, and purification of glutathione *S*-transferase (GST) fusion proteins were as described by Fukuda *et al.* (10). GST-Syt-C2A and GST-Syt-C2B contain amino acids 145–273 and 274–403, respectively, of squid Syt (8).

Expression of Squid Syt in COS-7 Cells. cDNA fragments of Syt encoding open reading frames [1–1650 bp of B3 clone (8)] were inserted into the *Not* I site of pEF-BOS (12) expression vector (referred to as pEF-Syt). COS-7 cells were transfected with pEF-Syt by the DEAE-dextran method. Cells were harvested 48 hr after transfection.

Production of Polyclonal Antibody against C2 Domains. New Zealand White rabbits were immunized with purified GST-Syt-C2A and GST-Syt-C2B using Freund's adjuvant system. After the third booster injection, antisera were collected. After absorption by GST-Syt-C2B (or C2A) to remove the cross-reactive component, mainly anti-GST, the Syt-C2A (or C2B) domain-specific antibody was affinity purified by Affi-Gel 10 immobilized with each antigen according to the manufacturer's notes. Conjugation of IgG with rhodamine was performed according to instructions from Molecular Probes.

Immunoblot analysis was carried out with these antibodies as follows. Proteins were transferred to a polyvinylidene difluoride membrane (Millipore), blocked with 0.5% skim milk in phosphate-buffered saline/0.05% Tween 20, incubated with anti-Syt-C2A (or C2B) polyclonal antibody, and incubated with peroxidase-labeled goat IgG (Fab) against rabbit IgG. Immunoreactive bands were visualized by the enhanced chemiluminescence detection system (ECL kit; Amersham).

[^{3}H]IP_4 Binding Assay. GST fusion proteins (1 μg) were incubated with 9.6 nM [^{3}H]IP_4 (New England Nuclear) in 50 μl of 50 mM Hepes (pH 7.2) for 10 min at 4°C. The sample was then mixed with 1 μl of 50 mg of γ-globulin per ml and 51 μl of a solution containing 30% (wt/vol) PEG-6000 and 50 mM Hepes, and placed on ice for 5 min. The precipitate obtained by centrifugation at 10,000 × *g* for 5 min was solubilized in 500 μl of Solvable (DuPont/NEN), and radioactivity was mea-

Abbreviations: IHPS, inositol high-polyphosphate series; IP_4, inositol 1,3,4,5-tetrakisphosphate; GST, glutathione *S*-transferase; Syt, synaptotagmin(s).

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sured in Aquasol 2 (DuPont/NEN) with a liquid scintillation counter (10).

In inhibition experiments, GST-Syt-C2B (1 μg) was incubated with 2.5 μg of purified anti-Syt-C2A, anti-Syt-C2B, or rabbit IgG in 49 μl of 50 mM Hepes (pH 7.2). After incubation for 60 min at 4°C, [^{3}H]IP_4 was added at a final concentration of 9.6 nM and the IP_4 binding assay was carried out as described above. Protein concentrations were determined by the Bio-Rad protein assay kit with bovine serum albumin used as a reference.

Electrophysiology and Morphology. The electrophysiological and electron microscopic techniques utilized in this paper are the same as described in the companion paper (8). The anti-Syt-C2B IgG was pressure injected into the preaxon, and its diffusion into the preterminal digit was monitored by fluorescence imaging of the rhodaminated dextan coinjected. As no effect on transmitter release was encountered following the anti-Syt-C2B antibody injection, voltage clamp experiments were not performed in this study. The repetitive stimulation of the preterminals was always implemented after the antibody had diffused from the point of injection to the end of the presynaptic digit. In all cases, sufficient time was allowed to elapse following their arrival to that site (10 min) to ensure that antibody concentration was homogeneously distributed along the terminal length before activating the stimulus train. Repetitive stimulation was implemented, as in the companion paper, at 200 stimuli per second (8).

Electron micrographs were obtained from five different synapses utilizing the same steps described in the companion paper (8). The results from the two groups where anti-Syt-C2B IgG ($n = 3$) and anti-Syt-C2B where IgG plus IP_4 ($n = 2$) were injected showed comparable results for each group concerning the size and distribution of the active zones as well as concerning the quantitative changes in vesicular cluster area and vesicular density.

RESULTS

Characterization of Antibodies Against C2 Domains of Squid Syt. In our previous *in vitro* biochemical studies, the C2A and C2B domains of Syt showed different properties in terms of Ca^{2+}/phospholipid and IHPS binding (10). To further examine the functional difference of two C2 domains in transmitter release, two antibodies against the C2A or C2B domain of squid Syt were produced (referred to as anti-Syt-C2A or anti-Syt-C2B). These antibodies recognized squid Syt expressed in COS-7 cells with apparent molecular weight (M_r) 48,000, which is almost identical to the predicted M_r (47,654) deduced from the cDNA (8) (Fig. 1*A*, lane 1). However, in the squid optic lobe, a slightly heterogeneous band with relative M_r 65,000 was detected by use of the same antibodies (Fig. 1*A*, lane 2). This suggests that squid optic lobe Syt undergoes a posttranslational modification other than N-glycosylation (8). The heterogeneity was also reported by Bommert *et al.* (7) and may be due to the difference of posttranslational modification.

The specificity of these antibodies to each C2 domain was checked by exploiting two previously described biochemical properties of the C2 domain (10): IP_4 binding to the C2B domain and Ca^{2+}-dependent phospholipid binding to the C2A domain. We confirmed that the C2B domain of squid Syt functions as an IP_4 binding domain (Fig. 1*B*): GST-Syt-C2B can bind IP_4, while GST-Syt-C2A cannot. Anti-Syt-C2B efficiently inhibited the IP_4 binding to the GST-Syt-C2B (70% reduction, Fig. 1*B*) but cannot inhibit the Ca^{2+}-dependent phospholipid binding to the GST-Syt-C2A (8). On the other hand, anti-Syt-C2A almost completely inhibited the Ca^{2+}-dependent phospholipid binding (8) and also inhibited the IP_4 binding (45% reduction, Fig. 1*B*), though the effect was weaker than that of anti-Syt-C2B. Control rabbit IgG had no significant effect on both biochemical properties.

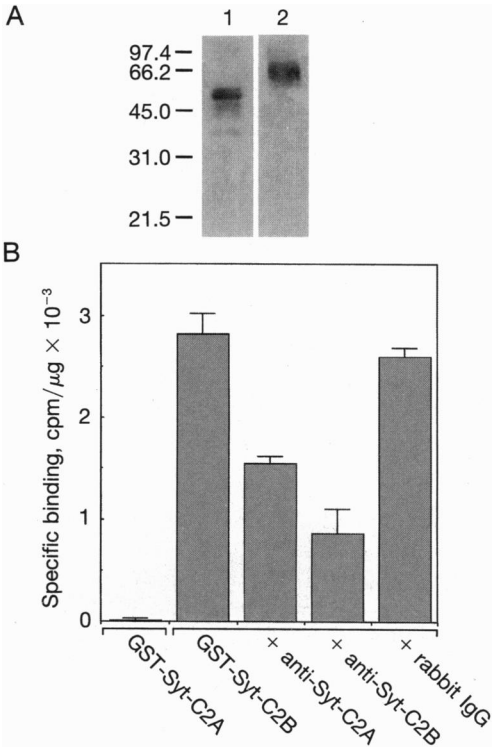

FIG. 1. Immunoblot analysis and inhibition of IP_4 binding to GST-Syt-C2B by antibodies. (*A*) About 20 μg of protein was subjected to 10% SDS/PAGE followed by immunoblot analysis using anti-Syt-C2A (lane 1) and anti-Syt-C2B (lane 2). Lane 1, total homogenate of COS-7 cells transfected with pEF-Syt; lane 2, P2 plus P3 membrane fractions of squid optic lobe. Positions of M_r markers ($\times 10^{-3}$) are indicated. (*B*) Inhibition of IP_4 binding to GST-Syt-C2B by anti-Syt-C2A and anti-Syt-C2B. GST-Syt-C2B showed significant IP_4 binding properties, but GST-Syt-C2A did not bind (10). Anti-Syt-C2A and anti-Syt-C2B were effective in inhibition of IP_4 binding to GST-Syt-C2B, but rabbit IgG had no effect. Inhibition by anti-Syt-C2B is larger than that of anti-Syt-C2A. Data are means ± SD of three measurements.

Electrophysiology. The effect of C2B antibody injection was tested in six different giant synapses utilizing simultaneous pre- and postsynaptic recording. In four of these, the anti-Syt-C2B IgG was injected on its own, and in two it was coinjected with IP_4 at concentrations of 125 and 300 μM. As IP_4 can become unstable with time, it was injected alone at 125 μM in another preparation (Fig. 2*B*), to ensure that this compound was capable of blocking synaptic release (1).

The results of the injection of the anti-Syt-C2B IgG antibody were electrophysiologically neutral. No change in the amplitude or duration of the presynaptic potential or of the latency or amplitude of the postsynaptic response was observed (Fig. 2*A*) in any of the six preparations. To ensure that these results were not due to incomplete binding of the antibody to the C2B domain, the testing of injected synapses was continued, in two cases, for up to 1 hr after the fluorescence had reached the end of the preterminal.

As confocal images of antibody binding to the C2B domain could not be obtained, due to difficulties in labeling the small amount of antibody available, other experiments were performed to determine whether the intracellular presence of the antibody modified the blocking effect of IP_4 on transmitter release. To this effect, two experiments were done in which the C2B antibody was coinjected with IP_4. As it is known that the IP_4 does not interact with the C2B antibody, the coinjection could result in synaptic transmission block by IP_4. If, on the other hand, the antibody were to prevent IP_4 binding to the C2B domain, as shown in our biochemical assay above, no such

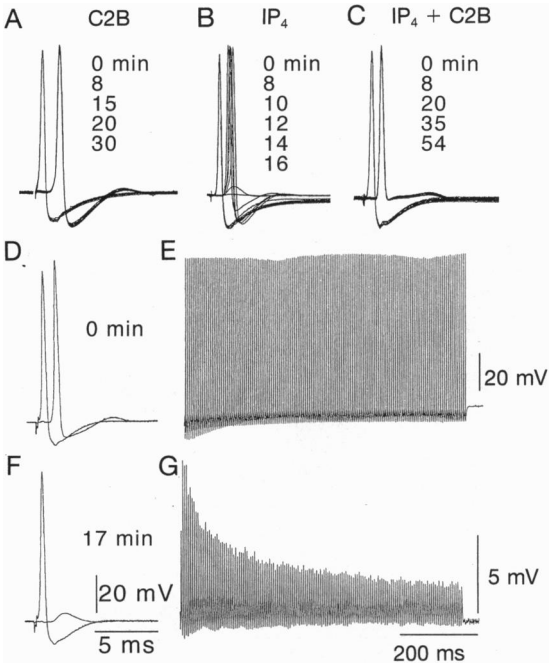

FIG. 2. Electrophysiological effect of anti-Syt-C2B IgG injection into the preterminal. (*A*) Intraterminal injection of anti-Syt-C2B IgG produced no change in synaptic transmission. The records show superposition of five different responses taken at the times indicated following the injection. (*B*) Similar protocol as in *A*, but after IP_4 injection, which blocked synaptic transmission within 16 min. This gradual decrease in transmission is illustrated by superimposing six records taken after injection at the times indicated. (*C*) C2B antibody and IP_4 coinjected have no blocking action even after 54 min following injection. (*D–G*) Effect of tetanic stimulation of synaptic transmission following anti-Syt-C2B IgG antibody injection. (*D*) Recordings immediately prior to tetanic stimulation. (*E*) Postsynaptic response to a 200-Hz stimulus for 750 ms. (*F*) Seventeen min later, synaptic response is markedly reduced. (*G*) Tetanic stimulation produced a rapid amplitude decrease of the postsynaptic response. Notice the difference in gain between *E* and *G*.

block should take place. Indeed, as shown in Fig. 2*C*, coinjection of the anti-Syt-C2B IgG prevented the inhibitory effect of IP_4 on synaptic release. From the above it can be concluded that the C2B domain of Syt does not appear to be directly related to the release of synaptic transmitter but is essential for the regulatory block of synaptic release by IHPS.

Effect of Anti-Syt-C2B IgG Injection on Transmitter Depletion. In the previous set of experiments synaptic transmission was tested by activating the presynaptic terminal once every 2 or 3 min. At that rate of stimulation, synaptic transmitter was basically not modified by the anti-Syt-C2B IgG injection given our testing period (1 hr). By contrast, when presynaptic repetitive stimulation was performed at frequencies of 200 per s for 750 ms, every 30 s, a reduction in transmitter release occurred within 20 min.

In addition, the type of postsynaptic amplitude reduction differed from that following preterminal injection of IHPS compounds (1) or of the C2A antibody (8). Thus, the block in this case was accompanied by a rapid reduction of the size of the postsynaptic responses upon repetitive stimulation, as opposed to the very gradual one observed in the two cases cited above.

In the initial phase of this experiment, repetitive stimulation produced no reduction of transmitter release (Fig. 2*E*). As the stimulus trains were continued a rapid decline of the postsynaptic response amplitude was observed, within the duration of a single train (Fig. 2*F*) and became progressively enhanced with time. The general character of this reduction is illustrated in Fig. 2*G* at 17 min after the tetanic stimulation regime was started. Note that the initial eight stimuli (the first 40 ms) produce a facilitation of synaptic release followed by a rapid reduction of the postsynaptic potential with subsequent stimuli. This pattern of response was repeated until synaptic block was complete.

At this point, two likely mechanisms for this tetanic depression could be envisioned. (*i*) The anti-Syt-C2B antibody prevented docking of synaptic vesicles reducing the probability of vesicular fusion. In this case, the number of vesicles in the vicinity of the active zone should be increased (as with presynaptic injection of anti-Syt-C2A antibody), since vesicles not being released should accumulate. (*ii*) Vesicular membrane was not being recycled, in which case a reduction in the number of vesicles should be observed at the active zone and throughout the cytosol. This would be the case if the C2B domain were to be involved in vesicular membrane uptake following release.

Morphological Studies. As illustrated in Fig. 3*A*, electron microscopy of the anti-Syt-C2B antibody-injected preterminals, following repetitive stimulation, demonstrated a marked reduction of synaptic vesicles. Note that in this cross section of the terminal, where five active zones (Fig. 3*A*, arrows) are shown next to each other, a reduction and, in some of the profiles, even total absence of vesicles was observed. A portion of that image is shown in Fig. 3*B* at higher magnification illustrating two examples of active zones. The overall morphology of these vesicles and their spatial relation to the active zone seem to be normal, compared with uninjected synapses (8) or with similar areas following anti-Syt-C2A antibody injection.

A morphometric analysis of such active zones showed a mean vesicular cluster area of $0.07 \pm 0.04\ \mu m^2$ ($n = 20$) with a mean number of vesicles in the plane of section of 15.46 ± 9.8 ($n = 402$) and an average vesicle density per unit area (μm^2) of 210 ± 32. Compared with the morphometric finding in the companion paper (8), these results indicate a reduction in vesicular cluster area and of numbers of synaptic vesicles of close to 90%.

By contrast, synapses coinjected with anti-Syt-C2B antibody and IP_4 showed, in the absence of tetanic stimulation, no significant structural difference (Fig. 3 *C* and *D*) when compared with control synapses (8). In this case, the morphometric analysis showed $0.4 \pm 0.086\ \mu m^2$ ($n = 20$) with a mean number of vesicles, in the plane of section, of 98.8 ± 22.7 ($n = 1976$) and an average vesicle density per unit area (μm^2) of 259 ± 39.6. However, following repetitive stimulation the addition of IP_4 to the anti-Syt-C2B IgG did not protect the synapse from depletion.

DISCUSSION

The experimental results described here indicate that the C2B domain of Syt has two different roles in synaptic transmitter release. Thus, as demonstrated previously (1), in the presence of IHPS members (IP_4, inositol 1,3,4,5,6-pentakisphosphate, and inositol 1,2,3,4,5,6-hexakisphosphate) binding to the C2B domain of Syt reduces synaptic release without affecting presynaptic Ca^{2+} entry or the conductance to either sodium or potassium responsible for the presynaptic action potential. The finding that presynaptic injection of the anti-Syt-C2B antibody does not produce, *per se*, any noticeable change in synaptic function is in accordance with our previous conclusion that the C2B domain serves mostly to inhibit release, as dictated by IHPS concentration at the preterminal (1). The absence of an increase in transmitter release, given IHPS receptor block, suggests that the concentration of the IHPS compounds may not be sufficiently high to be a limiting factor, under our experimental conditions. On the other hand, the fact that this antibody blocks the inhibitory effect of IP_4 presynaptic injection demonstrates that the IHPS compounds control synaptic

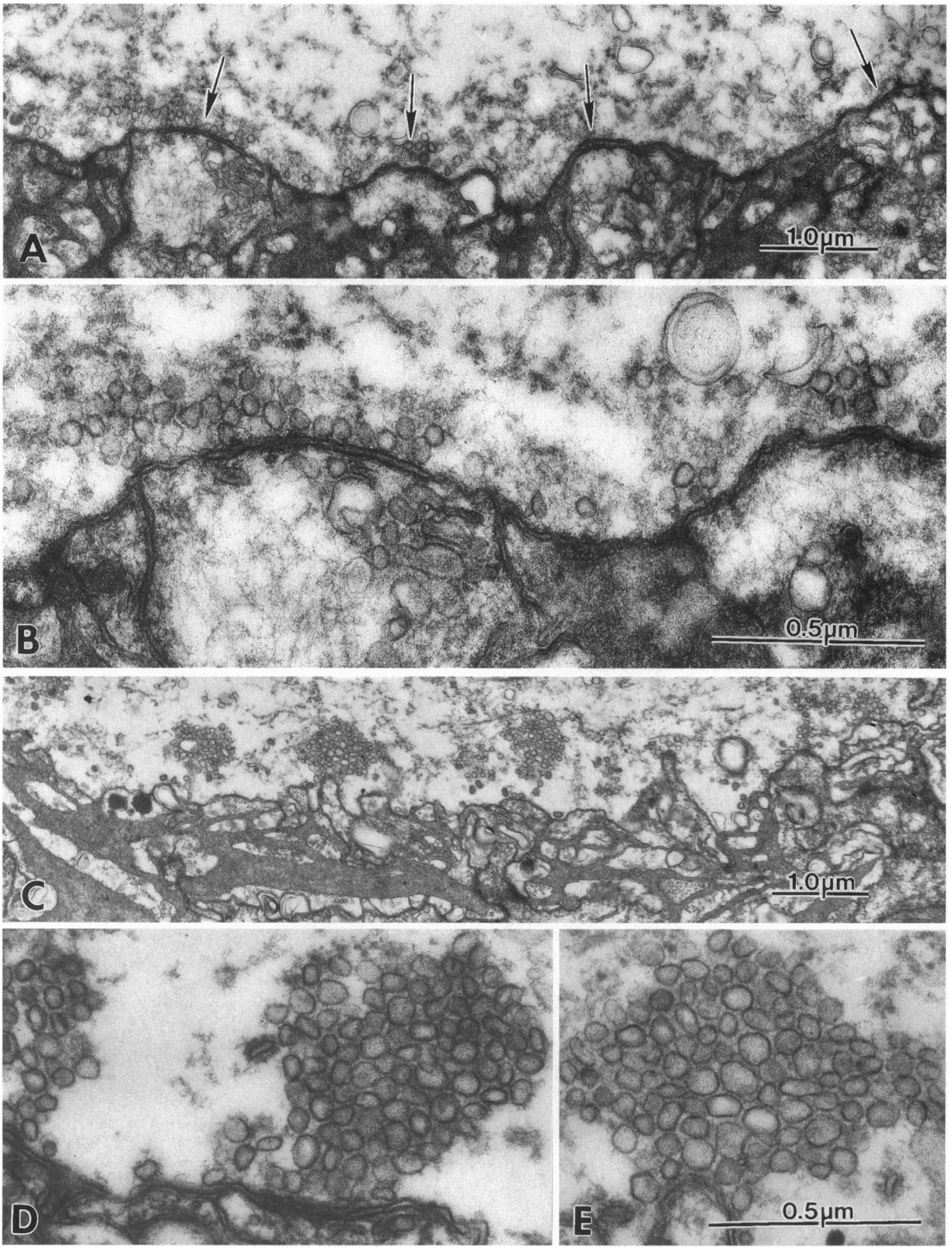

FIG. 3. Electron micrographs of a presynaptic terminal cross section following anti-Syt-C2B IgG (*A* and *B*) and anti-Syt-C2B IgG plus IP_4 injections (*C–E*). (*A*) Low-magnification image showing active zones between the pre- and postsynaptic elements at the junction. The arrows in the presynaptic terminal point to the active zones, two of which (to the right) are devoid of vesicles. (*B*) Higher magnification of a portion of the micrograph in *A*. These two synaptic profiles were among those demonstrating a higher number of vesicles. *C* and *D–E* are similar to *A* and *B*, respectively, in a synapse injected with anti-Syt-C2B IgG and IP_4, but not stimulated tetanically.

release by binding to the C2B domain of Syt in accordance with the biochemical binding studies described above.

Our results also indicate that although C2B antibody does not block synaptic transmission directly, it can interfere with vesicular recycling. Note also that the presence of IP_4 in the terminal does not interfere with the vesicular reuptake block produced by the C2B antibody injection. The electrophysiological results demonstrating tetanic depression of the

postsynaptic response in synapses injected with anti-Syt-C2B antibody indicate that this domain may have a second role as part of the regulatory machinery involved in vesicular recycling. These results are consistent with a previous *in vitro* study (11) indicating that the clathrin assembly protein (AP2), probably involved in synaptic vesicle endocytosis, is exclusively bound to the C2B domain of Syt.

In considering the results presented here, and in the companion paper (8), we conclude that Syt is involved in several types of vesicular transaction. Concerning synaptic release it seems evident, although by no means universally accepted (3), that the C2A domain of Syt is part of the machinery regulating the vesicular fusion triggered by Ca^{2+} influx and, thus, ultimately responsible for vesicular exocytosis. We also conclude that this fusion step is regulated by the IHPS binding to the C2B domain of Syt. The role of Syt does not end there, however, as the C2B domain seems to be also responsible for some aspects of vesicular membrane reuptake.

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