Antibodies Against Cysteine String Proteins Inhibit Evoked Neurotransmitter Release at Xenopus Neuromuscular Junctions

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Poage, Robert E., Stephen D. Meriney, Cameron B. Gundersen, and Joy A. Umbach. Antibodies against cysteine string proteins inhibit evoked neurotransmitter release at Xenopus neuromuscular junctions. J. Neurophysiol. 82: 50–59, 1999. Cysteine string proteins (CSPs) are evolutionarily conserved proteins that are associated with synaptic vesicles and other regulated secretory organelles. To investigate the role of CSPs in vertebrate neuromuscular transmission, we introduced anti-CSP antibodies into the cell bodies of Xenopus spinal motor neurons that form synapses with embryonic muscle cells in culture. These antibodies produced a rapid (within 3–6 min), and in most cases complete, inhibition of stimulus-dependent neurotransmitter secretion. However, spontaneous neurotransmitter release was stable (both in frequency and amplitude) throughout the period of antibody exposure. Several control experiments validated the specificity of the anti-CSP antibody effects. First, the anti-CSP antibody actions were not mimicked either by antibodies against another synaptic vesicle protein SV2, or by nonspecific immunoglobins. Second, heat treatment of the anti-CSP antibodies eliminated their effect on evoked secretion. Third, immunoblot experiments showed that the anti-CSP and anti-SV2 antibodies were highly selective for their respective antigens in these Xenopus cultures. We conclude from these results that CSPs are vital constituents of the pathway for regulated neurotransmitter release in vertebrates. Moreover, the selective inhibition of evoked, but not spontaneous transmitter release by anti-CSP antibodies indicates that there is a fundamental difference in the machinery that mediates these secretory processes.

INTRODUCTION

Appreciable progress has been made in identifying and characterizing proteins that participate in regulated secretion at nerve endings (Augustine et al. 1996; Scheller 1995; Sudhof 1995). For instance, compelling evidence indicates that proteins such as synaptotagmin, syntaxin, vesicle-associated membrane protein (VAMP)/synaptobrevin and synaptosomal-associated protein of 25 kDa (SNAP-25) play vital, although incompletely defined roles in the “synaptic vesicle cycle” (the cycle of exocytosis, endocytosis, and recycling of vesicles that occurs locally at nerve terminals) (Augustine et al. 1996; Scheller 1995; Sudhof 1995). Two strategies have figured prominently in efforts to resolve more clearly the function of these and other membrane-trafficking proteins in rapid synaptic transmission. One strategy involves the analysis of organisms with mutations in the gene(s) encoding these proteins; the second approach uses specific reagents (recombinant proteins, peptides, or antibodies) to alter selectively the function of individual components of the secretory machinery (Augustine et al. 1996). Here we document the use of affinity purified antibodies against cysteine string proteins (CSPs) as perturbants of neurotransmitter release at Xenopus neuromuscular junctions in vitro.

CSPs are synaptic vesicle proteins (Mastrogiacomo et al. 1994b) that have been shown to be ubiquitously distributed at nerve endings of the central and peripheral nervous system of fruit flies (Zinsmaier et al. 1990) and rats (Kohan et al. 1995). CSPs are also associated with regulated secretory organelles in a variety of nonneuronal cells (e.g., Braun and Scheller 1995; Chamberlain et al. 1996; Jacobsson and Meister 1996; Kohan et al. 1995; Papier et al. 1997). However, the precise role of CSPs in regulated secretion has not been established (Buchner and Gundersen 1997). To date, most of the information concerning CSP function has emerged from the analysis of csp mutant alleles of Drosophila. These mutant alleles exhibit complex phenotypic changes that include temperature-sensitive paralysis and premature death (Zinsmaier et al. 1994). The cellular basis of the temperature-sensitive paralysis of csp mutant Drosophila is the complete failure of action potentials to elicit the quantal release of neurotransmitter (Umbach et al. 1994). Additional physiological and pharmacological evidence (Umbach et al. 1994; Umbach and Gundersen 1997; Ranjan et al. 1998) along with Ca-imaging results (Umbach et al. 1998) are compatible with the hypothesis that presynaptic Ca channels fail to open (or conduct) in these mutants at elevated temperatures. However, alternative proposals (such as a physical separation of synaptic vesicles from presynaptic Ca channels) have also been advanced to explain the secretory disturbance in csp mutant Drosophila (Heckmann et al. 1997; Ranjan et al. 1998). Thus further work is needed to clarify the molecular basis of the temperature-sensitive paralysis in these organisms that lack the csp gene.

As an independent approach to assess CSP function, we used anti-CSP antibodies as potential perturbants of CSP-mediated events in Xenopus nerve-muscle cultures. This strategy complements prior work done with csp mutant Drosophila and avoids some of the complications of interpreting results from mutant organisms (as discussed in Augustine et al. 1996). Thus the Xenopus system allows one to administer reagents presynaptically and monitor their impact on secretory events that are detected postsynaptically (Alder et al. 1992; Rettig et al. 1997). We observed that intracellular application of anti-CSP antibodies into the presynaptic cell specifically inhibited the stimulus-dependent secretion of neurotransmitter without affecting spontaneous transmitter release. These results indicate that...
CSPs are vital constituents of the evoked secretory pathway at vertebrate fast synapses, and establish the *Xenopus* co-culture system as a useful means to study the function of CSPs.

**METHODS**

**Nerve-muscle cultures**

Cultures of spinal neurons and muscle cells were prepared essentially as described by Tabti and Poo (1991) and Yaziejian et al. (1997). Briefly, stage 19–22 *Xenopus laevis* embryos (Nieuwkoop and Faber 1967) were dejellied and rinsed in sterile 10% normal frog Ringer (NFR) solution (NFR is, in mM: 116 NaCl, 2 KCl, 1.8 CaCl2, 1 MgCl2, and 10 HEPES, pH 7.3), and the spinal cord and myotomes were removed and dissociated in a divalent-cation–free solution (in mM: 116 NaCl, 2 KCl, 0.4 EDTA, and 10 HEPES, pH 7.3). After 60–90 min at room temperature (20–22°C), disaggregated cells were plated on plastic culture dishes and maintained (at 20–22°C), disaggregated cells were plated on plastic culture dishes and maintained (at 20–22°C) in medium composed of 48% NFR with 40% ISOves (this and other medium constituents were from Sigma, St. Louis, MO) and 12% H2O supplemented with 25 ng/ml brain-derived neurotrophic factor (Regeneron, Tarrytown, NY), insulin (0.1 mg/ml), transferrin (0.6 mg/ml), sodium selenite (0.7 mg/ml), and 50 units/ml penicillin-streptomycin. Motor neurons form functional synaptic contacts with muscle cells within 24 h (Kidokoro and Yeh 1982), and cultures were used 1–3 days after plating.

**Electrophysiological analysis of synaptic transmission**

Conventional whole cell recording configurations were used to measure transmembrane currents or voltages from neuronal somata or muscle cells (at 20–22°C). Patch pipettes (2–4 MΩ) were filled in a two-step procedure: for muscle cells, the tip was first dipped in solution A (in mM: 100 KCl, 1 CaCl2, 1 MgCl2, 11 EGTA, and 20 HEPES, pH 7.3). The tips of pipettes for neurons were backfilled with solution B (in mM: 110 KCl, 1 NaCl, 1 MgCl2, and 10 HEPES, pH 7.3). Muscle pipettes were backfilled with solution A plus 5 mM of the local anesthetic QX-314 (Alomone, Jerusalem, Israel) to block muscle contraction. Neuronal pipettes were backfilled with solution B plus 4 mM MgATP and 0.3 mM GTP along with any perturbant. The bath solution was NFR.

Action potentials were elicited (using pulses of 0.5 ms duration and 0.5–2 nA) in the neuron during whole cell recording in the current-clamp mode. These action potentials normally evoked the quantal release of transmitter from the presynaptic element, and this released transmitter was detected postsynaptically with a second patch pipette on the muscle cell (nerve-muscle pairs were chosen where the axon showed minimal branching and the nerve terminal was <100 μm from the cell body). Excitatory postsynaptic currents (EPSCs) were recorded using a holding potential of −80 mV. We discarded experiments in which any of the following parameters changed: 1) access resistance; if the muscle patch pipette exhibited sustained changes of access resistance, data were not analyzed; 2) action potential waveform; if the neuronal action potential failed or if the threshold for generating an action potential increased more than threefold, the recording was terminated; 3) changes of spontaneous transmitter release: if there was a trend (over several minutes) toward a large (>4-fold) increase in the frequency of spontaneous release events (miniature EPSCs, or mEPSCs), the data were not used, because this was often a prelude to cell death.

EPSCs were elicited at low frequency (0.06 Hz), because there is greater variability of EPSC amplitude at higher stimulation frequencies. Spontaneous mEPSCs were recorded continuously between EPSCs. Data were stored and analyzed using pCLAMP 6.0 and Axograph 3.0 (Axon Instruments).

**Antibody preparation and characterization**

Antibodies were raised against a recombinant construct of rat CSP from which the cysteine-string domain was deleted. Vertebrate CSPs are highly conserved (Mastrogiacomo et al. 1998), and we have found that antibodies against rat CSP efficiently cross-react with CSPs of other species (Coppola and Gundersen 1996; Mastrogiacomo et al. 1998). In addition, removal of the cysteine string precludes the formation of thiol-linked multimers of the recombinant protein (Gundersen, unpublished observations). Thus rat CSP cDNA in Bluescript (see Mastrogiacomo and Gundersen 1995) was treated with *Sph* I. This restriction endonuclease excises a 96 nucleotide fragment from rat CSP cDNA that leads to the deletion of amino acid residues from Lys107 through Lys 139. This region encompasses the entire cysteine string of rat CSP. After ligation, this “C-free” construct was used as a template for polymerase chain reaction (PCR) (sense primer: CAT-string of rat CSP. After ligation, this “C-free” construct was used as a template for polymerase chain reaction (PCR) (sense primer: CAT-
ATGGCTGACCAAGGG; antisense primer: GGATCCTAGTGTG-AACCCGTCGG). This PCR product was ligated into the Eco RV site of Bluescript that had been treated with *Taq* polymerase to generate a T overhang. This insert was excised with *Nde* I and *Bam* HI and ligated into pET15b (Novagen, Madison, WI). The resulting construct encodes a His6-tagged version of C-free rat CSP under control of the lac promoter. This plasmid was transformed into BL-21 cells, and after induction, recombinant protein was purified using Talon resin (Clontech, Palo Alto, CA) according to the supplier’s protocols. This His-tagged, C-free rat CSP was used to induce antibody production in rabbit (performed by Babco, Richmond, CA). Antibodies specific for rat CSP were purified from immune serum using Aminolink resin (Pierce, Rockford, IL) to which we coupled the recombinant C-free rat CSP were purified from immune serum using Aminolink resin (Pierce, Rockford, IL) to which we coupled the recombinant C-free protein that had been treated with thrombin to remove the His-tag. These antibodies exhibited high specificity for detection of rat CSP on immunoblot, as judged by the same criteria in Mastrogiacomo et al. (1994a). In addition, owing to the high degree of sequence conservation among vertebrate CSPs (see Mastrogiacomo et al. 1998), these antibodies efficiently cross reacted with brain CSP (XCSP) as shown by Mastrogiacomo et al. (1998). We verified that these antibodies recognized XCSP in the *Xenopus* nerve-muscle cultures by solubilizing cells on individual culture plates using 10 mM 3-((3-cholamidopropyl)dimethylammonio)-2-hydropyr-1-propanesulfonate (CHAPS) in 0.1 M NaCl, 50 mM Tris, and 1 mM EDTA (pH 7.5) and extracting protein using an organic solvent extraction procedure (Wessell and Flügge 1984). The recovered protein was dissolved at 30°C in sample buffer (Laemmli 1970) with 5% SDS and 10 mM dithiothreitol and alkylated with 22 mM iodoacetamide for 1–2 h in the dark. Samples were resolved on a 12.5% SDS-polyacrylamide gel for immunoblot analysis as described before (Mastrogiacomo et al. 1994a,b) except that we used enhanced chemiluminescence detection (Amersham-Pharmacia, Arlington Heights, IL).

Various control antibodies were prepared for these studies. First, we used ammonium sulfate precipitation (Harlow and Lane 1988) to recover immunoglobulin (IgGs) from rabbit immune serum that had been depleted of CSP-specific antibodies by affinity chromatography (see above). At least 500 times more of these “flow-through” IgGs were required to produce an equivalent immunoblot signal to that obtained using 0.5 μg/ml of the affinity purified anti-CSP IgG (data not shown). Thus these flow-through IgGs (that did not bind to the CSP affinity matrix) were used to control for nonspecific effects of IgGs in these experiments. Second, we used monoclonal antibodies against the SV2 protein of synaptic vesicles (Buckley and Kelly 1985) (these antibodies were a kind gift from Dr. E. Schweitzer, UCLA) that recognize a cytosolic epitope of this protein. Third, we heated treated (10 min at 70°C) the anti-CSP antibodies. In all cases, antibodies were dialyzed against 0.1 M KCl, 10 mM HEPES, 0.1 mM EDTA (pH 7.5) and adjusted to a concentration of 2 mg/ml before use in these experiments. Antibodies were diluted to 0.02 mg/ml in the intracellular patch pipette solution.

RESULTS

Characteristics of evoked and spontaneous neurotransmitter release at *Xenopus* nerve-muscle contacts

In our initial experiments, we monitored evoked and spontaneous transmitter release in preparations that were not exposed to any antibody. As illustrated in Fig. 1A, EPSCs can be elicited by action potentials (Fig. 1C) initiated in the neuronal cell body. Although the amplitude of individual EPSCs fluctuated (as seen in Fig. 1, A and D) during a recording session, the mean EPSC amplitude in this experiment (Fig. 2A, ○) did not deviate appreciably from the value in the first minute. In all experiments, we monitored somatically evoked action poten-

![Figure 2](https://via.placeholder.com/150)
can be seen in the mEPSC records of Fig. 1B, that mEPSC amplitude varies greatly in these nerve-muscle preparations. The highly skewed nature of the mEPSC amplitude distribution (data not shown, but see Kidokoro et al. 1980; Song et al. 1997) precludes us from undertaking conventional quantal analysis (because for any given evoked response it is not known which size quanta contribute to that release event). As an alternative approach to normalize data for comparison among different experiments, we used the same normalization strategy alluded to above for EPSCs. Thus Fig. 8 includes comparisons of mean mEPSC frequency and amplitude at the beginning and end of each experiment. This enables us to determine whether there are any consistent, time-dependent changes of these parameters of secretion. For these control experiments (Fig. 8), neither mEPSC amplitude nor frequency showed any significant change over time, as judged by ANOVA. These findings are similar to those of Alder and colleagues (1992), who also found that spontaneous transmitter release was relatively stable in this preparation.

**Anti-CSP IgG reduces evoked, but not spontaneous transmitter release**

To investigate the effect of affinity purified anti-CSP antibodies on transmitter release, we included 0.02 mg/ml of these antibodies in the motor neuron patch pipette and monitored EPSCs, mEPSCs, and action potentials as in the control experiments of Figs. 1 and 2. In the example of Fig. 3, EPSC amplitude was initially stable, but within 2 min a systematic decline of EPSC amplitude was observed, and by 9 min action potentials (shown in Fig. 3C) no longer elicited postsynaptic responses (Fig. 3, A and D). This gradual decline of EPSC amplitude is consistent with the hypothesis that the anti-CSP antibodies are migrating from their site of introduction at the cell body to sites of transmitter release where they interfere with a process that is vital for evoked secretion of transmitter. Indeed, Popov and Poo (1992) showed that macromolecules comparable in size to IgGs can transit from the neuronal soma to the nerve terminal within a few minutes. However, because action potential generation is unimpaired for the duration of this experiment (Fig. 3C), it is unlikely that this antibody effect is mediated via a general impairment of membrane excitability. Indeed, because the anti-CSP antibodies were introduced in the cell body, this is where changes in action potentials should have been most prominent. The absence of an effect on action potentials indicates that there is an alternative target of these antibodies.

In contrast to the decline of EPSC amplitude in Fig. 3A, there was no significant change in the frequency, mean amplitude, or time course of mEPSCs throughout this experiment (Fig. 3, B, E, and F). In other words, at the same time that stimulus-evoked responses could no longer be elicited, spontaneous quantal release events persisted (Fig. 3). These results show that presynaptic introduction of anti-CSP antibodies has no effect on the postsynaptic sensitivity to transmitter. In addition, these data indicate that CSP antibodies have a selective effect to inhibit evoked neurotransmitter secretion, but they do not simultaneously affect the secretory process that underlies mEPSCs.

Representative EPSC and mEPSC data for five additional experiments using anti-CSP antibodies are presented in Fig. 4. The results in Fig. 4A were chosen to illustrate the variability in the time course of the decline of EPSCs in these experiments. However, in all cases, EPSC amplitude is reduced to an extremely low level, or abolished, within 5–20 min. As in Fig. 3, we infer that part of the explanation for the different kinetics of EPSC blockade is that there are differences in the rate of transit of anti-CSP IgGs from the neuronal pipette to the nerve terminal. Nevertheless, in each instance, the effect of these IgGs is to inhibit evoked transmitter release (Fig. 4A).
In preparations exposed to anti-CSP IgGs, mEPSCs were detected throughout the experiment (Fig. 4, B and C). The summary records (Fig. 4, B and C) show that mEPSCs are still present at times when EPSCs are either reduced to extremely low amplitudes or abolished. This conclusion holds even in the example (Fig. 4C, □) where mEPSC frequency exhibited a statistically significant ($P < 0.05$ by ANOVA) decline with time. However, this decline of mEPSC frequency was not a consistent finding as documented in the other examples in Fig. 4C, and in the summary results of Fig. 8. These results further illustrate the divergent effects of anti-CSP IgGs on EPSCs and mEPSCs.

Antibody control experiments

To control for the specificity of the effect of anti-CSP IgG on EPSCs, we first tested the action of IgG from the immune serum that had been depleted of CSP-specific IgG by affinity chromatography. These flow-through IgGs control for potential nonspecific effects of IgG in these experiments. Inclusion of this IgG fraction at 0.02 mg/ml into the motor neuron soma patch pipette showed no time-dependent effect to alter either evoked (EPSCs) or spontaneous (mEPSCs) transmitter release (Fig. 5). In contrast to the anti-CSP antibodies, there was no trend toward a decline of EPSC amplitude (Fig. 5, A and C) in this example, or in the additional seven trials using these IgGs (Fig. 8). A similar conclusion holds for mEPSC amplitude and frequency (Figs. 5 and 8). Thus there does not appear to be any nonspecific effect of this IgG fraction on evoked or spontaneous transmitter release.

To control for the possibility that IgG binding to synaptic vesicles produces steric hindrance that inhibits secretion, we tested the effects of IgG directed against a cytoplasmic portion of the protein, SV$_2$. This anti-SV$_2$ IgG was chosen as a control, because SV$_2$ is a well-established synaptic vesicle protein (Buckley and Kelly 1985; Sudhof 1995), but there is no evidence that SV$_2$ is involved in any vital function related to the regulation of vesicle fusion. A typical recording made using anti-SV$_2$ shows no consistent change of either EPSCs or mEPSCs (Fig. 6). Summary data from a total of eight experiments using SV$_2$ IgG are shown in Fig. 8. These data demonstrate that there are no consistent effects on EPSCs or mEPSCs of this IgG at the concentration used in these experiments.

As a final control for the specificity of anti-CSP IgGs on evoked secretion, we tested heat-treated antibodies. Anti-CSP IgG was heated to 70°C for 10 min before inclusion in the presynaptic patch pipette. After this treatment, anti-CSP IgG had no effect on EPSC amplitude (Fig. 7). As with untreated controls, or preparations exposed to flow-through IgG, or SV$_2$ IgG, there was no consistent effect on either EPSCs or mEPSCs (Figs. 7 and 8). Thus the impact of anti-CSP IgG on EPSCs (Figs. 3 and 4) appears to be specific and can be eliminated by heat treatment that denatures these antibodies.

In Fig. 8, we summarize the impact of different empiric treatments on EPSC amplitude and mEPSC amplitude and frequency in these investigations. These results were obtained by normalizing the mean amplitude of the final five EPSCs of the recording to the mean amplitude of the first five EPSCs of the recording. We selected for inclusion in this figure all experiments that satisfied our criteria of stability (see METHODS). As is evident from these data (Fig. 8), EPSC amplitude does not change significantly relative to control (no antibody) in three of the experimental conditions (Flow, Heat, and SV$_2$). However, there is a significant difference ($P < 0.002$ using a 1-way ANOVA with Tukey’s post hoc test) between control and CSP IgG. This method of data analysis confirms the potent and selective inhibition of evoked secretion by anti-CSP IgG. At the same time, none of these treatments had any significant effect on mEPSC amplitude or frequency relative to control with no antibody (Fig. 8; significance was tested as for EPSCs using 1-way ANOVA). These results highlight the fact that the anti-CSP IgGs preferentially affect EPSCs, but not mEPSCs.
Immunoblot identification of CSP and SV$_2$ in Xenopus nerve-muscle cultures

An important consideration for this work is the specificity of antibody binding to CSP and SV$_2$ in these cultures. As shown in the immunoblots of Fig. 9, both the CSP and SV$_2$ antibodies identified single proteins of $\sim$34 and 100 kDa, respectively. These masses are compatible with the mass of CSP in *Xenopus* (Mastrogiacomo et al. 1998) and with the mass of SV$_2$ originally detected in electric fish (Buckley and Kelly 1985).

These data are consistent with the conclusion that these antibodies selectively interact with the appropriate antigens in these experiments.

**DISCUSSION**

The current investigations reveal that affinity purified antibodies against CSPs selectively abolish stimulus-dependent neurotransmitter release without affecting spontaneous transmitter release in *Xenopus* nerve-muscle cultures. Various con-
trol antibody preparations do not mimic this anti-CSP antibody effect. These results indicate that CSPs are vital components of the regulated secretory pathway in vertebrates, and they provide useful insights into the role of CSPs in secretory membrane trafficking at nerve terminals.

An important motivation for undertaking the current experiments was that most of the information concerning CSP function had emerged from studies of csp mutant Drosophila (Heckmann et al. 1997; Ranjan et al. 1998; Umbach et al. 1994, 1998; Umbach and Gundersen 1997; Zinsmaier et al. 1994). Although these studies of the phenotype of csp mutant organisms have considerably advanced our understanding of the role of CSPs, there were certain constraints on the conclusions that could be drawn from these investigations. For example, all of the functional studies of csp mutant Drosophila have focused on a minor subset of these organisms that escaped the developmental lethality of mutating the csp gene. Indeed, Zinsmaier and colleagues (1994) reported that the survival of their csp mutant alleles during development ranged between ~1 and 15%. Thus the full spectrum of phenotypic changes of these csp mutants remains to be established. As an independent approach to investigate CSP function, we chose an antibody-perturbation strategy using Xenopus nerve-muscle cultures.

Antibodies have been widely used as probes of regulated secretion (Alder et al. 1992; Ali et al. 1989; Elferink et al. 1993; Kenigsberg and Trifaro 1985; Mikoshiba et al. 1995; Mochida et al. 1995; Perrin et al. 1987; Pieribone et al. 1995; Schweizer et al. 1989; Skehel et al. 1995; Walent et al. 1992). We address three important considerations that are relevant to the design and interpretation of such experiments (also see comments in the preceding cited papers and Augustine et al. 1996). First, one needs highly selective antibodies. Second, one needs to control for the possibility that antibody binding produces steric occlusion of events (such as vesicle docking at the plasma membrane), rather than interfering more specifically with a function mediated by the antigen. And third, one needs to control for nonspecific consequences of antibody cross-linking of target antigens.

The first issue concerns antibody selectivity. The anti-CSP antibodies used here were affinity purified, and, based on immunoblot analysis, they were highly selective for CSP in Xenopus nerve-muscle cultures. These results are comparable with findings made using other preparations of affinity purified anti-CSP antibodies (Kohan et al. 1995; Mastrogiacomo et al. 1994a,b; Mastrogiacomo and Gundersen 1995), and they are identical to results reported for the immunoblot detection of CSP in adult Xenopus brain (Mastrogiacomo et al. 1998). Moreover, the differential action of the anti-CSP antibodies on evoked versus spontaneous transmitter release further supports the argument for the selectivity of these antibodies. We conclude that the effects of the anti-CSP antibodies are mediated via antibody binding to CSP.

Two arguments diminish the likelihood that steric hindrance
accounts for the effects of the anti-CSP antibodies. First, if steric hindrance were involved, antibodies against other synaptic vesicle proteins should also have inhibited transmitter release. Instead, we found that monoclonal antibodies targeted to a cytosolic domain of the synaptic vesicle protein SV2 (Buckley and Kelly 1985) had no effect on secretion. Our observations are reminiscent of results of Elferink and co-workers (1993), who reported that SV2 antibodies did not affect secretion in PC-12 cells. Indeed, Elferink and colleagues (1993) found that antibodies against two other synaptic vesicle proteins (synaptobrevin and synaptophysin) also had no impact on secretion in the PC-12 system. These data indicate that antibody binding to secretory vesicle antigens does not universally impair transmitter release, as would be expected if steric hindrance mediated the antibody effect.

The second argument against steric hindrance is that we observed a selective inhibition of evoked, but not spontaneous transmitter release. Had steric hindrance been an issue, we would have expected to record a decline of both spontaneous and evoked secretion. Because this is not what we observed, we conclude provisionally that the blockade of evoked transmitter release is not mediated via steric hindrance. The only caveat here is that there have been recent indications that the machinery underlying regulated and constitutive secretion may involve distinctive macromolecular components at nerve terminals. For instance, Deitcher and colleagues (1998) reported the complete loss of stimulus-dependent transmitter release in mutant alleles of Drosophila that lacked neuronal synaptobrevin (n-syb). In these n-syb mutants, spontaneous transmitter secretion persisted (albeit at a rate lower than observed in wild-type controls). This led to the conclusion that these spontaneous release events were supported by a protein complex that was distinct from the complex required for stimulus-dependent secretion (Deitcher et al. 1998). Independently, work using clostridial neurotoxins has yielded a similar conclusion for the role of synaptobrevin in secretory events at crayfish nerve endings (Hua et al. 1998). Interestingly, we had also observed that spontaneous transmitter release continued unabated in csp mutant Drosophila, even when evoked transmitter secretion was abolished (Umbach et al. 1994). Thus synaptic transmission in csp mutant Drosophila exhibits interesting parallels to the effects of anti-CSP antibodies in Xenopus nerve-muscle cultures. Together, these data point to a crucial role of CSPs in stimulus-secretion coupling at both vertebrate and invertebrate nerve terminals. What remains unresolved at this time is the mechanism of the selective sparing of spontaneous secretion in these widely divergent experimental paradigms (Xenopus cultures and mutant Drosophila larvae). Among the explanations for our observations in the current experiments are as follows: separate populations of vesicles that mediate constitutive or evoked secretion, differential sensitivity of the release machineries to anti-CSP antibodies (as would occur if the antibodies interfered with a CSP-Ca channel link), or the presence of a form of CSP that is not recognized or affected by these antibodies. Regardless of which explanation is correct, it is evident that the selective occlusion by anti-CSP antibodies of evoked transmitter release makes these reagents useful probes that should help us to understand the underlying differences between the evoked and constitutive secretory pathways.

The third consideration for the interpretation of our findings is the issue of antigen cross-linking. Here the arguments are very similar to those raised for steric hindrance. Thus the failure of anti-SV2 antibodies to block secretion argues that even if cross-linking occurs between vesicular antigens, it does not obstruct transmitter release. An obvious concern here is that monoclonal anti-SV2 antibodies are less likely to cross-link antigens than the polyclonal anti-CSP antibodies. In spite of this, the selective impact of anti-CSP antibodies on evoked, but not spontaneous secretion is also incompatible with the idea that cross-linking generally immobilizes the synaptic vesicle pool or distorts the vesicle surface. In this same context, an independent approach that is commonly used to exclude antigen cross-linking is to assess the effect of monovalent Fab fragments of antibodies (e.g., see Alder et al. 1992; Perrin et al. 1987). In our hands, preparation of Fab fragments led to decline in the threshold for CSP detection on immunoblots and a concomitant loss of the antibody effect on evoked transmitter secretion (data not shown). Thus, although we cannot unequivocally exclude antigen cross-linking as contributing to our results, the foregoing arguments vitiate this explanation.

Another perspective on this work emerges from a comparison of our results with those of Alder and colleagues (1992), who administered anti-synaptophysin antibodies presynaptically in this same preparation. The prominent difference is that...
anti-synaptophysin antibodies depressed both spontaneous and evoked secretion (Alder et al. 1992). Because of this effect, Alder and colleagues (1992) could not exclude the possibility of steric hindrance as an underlying explanation for their results. This contrasts with the selective inhibition of evoked secretion by anti-CSP antibodies and further emphasizes the potential value of resolving the molecular basis of the differential effects of these antibodies on the constitutive and evoked secretory pathways.

Another striking feature of our results was the relative rapidity with which the anti-CSP antibodies blocked transmitter release. Popov and Poo (1992) previously demonstrated that molecules as large as antibodies were transported within minutes to nerve terminals in this preparation. Thus, from our results showing that the blockade of EPSCs could occur within minutes, we infer that anti-CSP antibodies act swiftly to occlude evoked secretion once they reach the nerve terminal. This time course of antibody action is incompatible with a depletion of synaptic vesicles or any similarly slow process and suggests instead that these antibodies interfere directly with an event that is vital for evoked transmitter release. Given past studies that implicate CSPs in the modulation of presynaptic calcium channels (Gundersen and Umbach 1992; Leveque et al. 1998; Ranjan et al. 1998; Umbach et al. 1994, 1995, 1998; Umbach and Gundersen 1997), it is likely that anti-CSP antibodies interfere with this modulatory interaction and selectively block evoked transmitter release. However, CSPs have also been postulated to contribute to other steps in the regulated secretory pathway (Buchner and Gundersen 1997), and additional work will be needed to exclude an antibody effect on these steps.

It is in this context that the Xenopus nerve-muscle system is particularly advantageous, because it was recently shown that one can record both presynaptic calcium channel currents and rapid calcium transients in this preparation (DiGregorio and Vergara 1997; Yazejian et al. 1997). Thus an important future direction for this work will be to assess the impact of anti-CSP antibodies on the behavior of presynaptic calcium channels, because this will provide mechanistic insight into the inhibition of evoked secretion presented here.

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