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Exogenous mRNA encoding tetanus or botulinum neurotoxins expressed in *Aplysia* neurons

(cholinergic synapse/intracellular injection/light chain/transmitter release)

SUMIKO MOCHIDA*, BERNARD POULAIN*, ULRICH EISEL†, THOMAS BINZ†, HISAO KURAZONO†, HEINER NIEMANN†, AND LADISLAV TAUC*‡

*Laboratoire de Neurobiologie Cellulaire et Moléculaire, Centre National de la Recherche Scientifique, 91198 Gif-sur-Yvette Cedex, France; and †Federal Research Center for Virus Diseases of Animals, Institute for Microbiology, D-7400 Tübingen, Federal Republic of Germany

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ABSTRACT **Injection of exogenous mRNA purified from various tissue preparations into cellular translation systems such as *Xenopus* oocytes has allowed expression of complex proteins (e.g., receptors for neurotransmitters). No evidence for expression of injected exogenous mRNA, however, has been reported in terminally differentiated neurons. If achieved, it would allow the study of long-lasting changes of properties of nerve cells in their functional context. To obtain evidence of such expression, we chose two proteins that produce a detectable effect even at very low intracellular concentrations. Tetanus toxin and botulinum neurotoxin fulfill this criterion, being the most potent neurotoxins known. Both toxins block neurotransmitter release at nanomolar intracellular concentrations. These di-chain proteins, consisting of a light chain and a heavy chain, have recently been sequenced. Their active sites are located (or partly located) on the light chain. mRNAs encoding the light chain of either toxin were transcribed *in vitro* from the cloned and specifically truncated genes of *Clostridium tetani* and *Clostridium botulinum*, respectively, and injected into presynaptic cholinergic neurons of the buccal ganglia of *Aplysia californica*. Depression of neurotransmitter release appeared in <1 hr, demonstrating successful expression of foreign mRNA injected into a neuron *in situ*.**

Neurons of *Aplysia* have been used as a model to elucidate cellular mechanisms of neuronal function. Several properties make the identified cholinergic synapse in the buccal ganglion (1) an ideal preparation to test the possibility of functional expression of mRNAs encoding molecules acting on transmitter release. In particular, the cell body of the presynaptic neuron is large, 150–300 μm in diameter, and the distance from the cell body to the synaptic terminal of the presynaptic neuron is short, ≤500 μm. It was thus possible to inject big molecules such as enzymes (1) or toxins (2, 3) into the presynaptic neuron and to detect changes in the postsynaptic responses to presynaptic stimuli due to alteration of acetylcholine (AcCho) release induced by injected substances.

This cholinergic synapse is nearly as sensitive as the vertebrate neuromuscular junction (4, 5) to botulinum neurotoxins (BoNTs) (2) and to tetanus toxin (TeTx) (3), which cause botulism and tetanus in humans. TeTx ($M_r \approx 150{,}000$), produced by *Clostridium tetani*, an anaerobic spore-forming bacteria, is synthesized as a single-chain protoxin consisting of 1315 amino acids (6). The polypeptide is converted to a fully toxic bi-chain toxin by endogenous proteases (7) to yield a light chain ($M_r \approx 50{,}000$) and a heavy chain ($M_r \approx 100{,}000$), which remain covalently linked by a disulfide bridge. BoNT type A (BoNT/A), produced by *Clostridium botulinum*, resembles TeTx in regard to biosynthesis and molecular architecture and has been recently sequenced as 1296-amino acid residues (8). The action of these two toxins is considered to develop in three steps: binding of the toxin to the neuronal membrane acceptors, internalization, and intracellular action leading to blocking of neurotransmitter release (4, 5, 9). Thanks to the specific properties of the *Aplysia* preparation the internalization step could be bypassed by injecting TeTx and BoNTs into the presynaptic neuron. It was found that, when injected, the toxins equipotently inhibited neurotransmitter release at nanomolar concentrations (2, 3).

The light chains and heavy chains of the neurotoxins can be separated by reduction of the disulfide bond (7, 10). Our previous studies using these highly purified subunits (7, 10) indicate that, for TeTx, the active sites for blockade of AcCho release are located in the light chain. When injected into the presynaptic neuron at nanomolar concentrations, the light chain of TeTx inhibits neurotransmitter release as efficiently as the bi-chain TeTx (3). For BoNTs, however, the light chain was found to exert no depressive action unless the heavy chain of BoNTs was also present (2), suggesting the existence of an additional important site in the heavy chain. For both toxins the heavy chains, nontoxic by themselves (9, 11–13), are known to insure targeting to the neurons and ensuing internalization of intracellularly active moiety(ies) (2, 10, 13).

We took advantage of the high sensitivity of this cholinergic synapse to clostridial neurotoxins to see whether an exogenous mRNA could be expressed in neurons *in situ*. So far, successful expression of injected exogenous mRNAs (e.g., encoding receptors for neurotransmitters, channels, or enzymes) has been obtained either in nondifferentiated cellular translation systems, such as *Xenopus* oocytes (14–19) or in muscle fibers (20). No evidence for such expression, however, has been brought forward in terminally differentiated neurons. Here we demonstrate the functional translation, in cholinergic neurons, of mRNAs encoding light chain of TeTx or BoNT/A, which were transcribed from the cloned and artificially truncated genes of *C. tetani* and *C. botulinum*.

MATERIALS AND METHODS

Preparation of TeTx Light Chain- and BoNT/A Light Chain-Specific mRNA. The *in vitro* synthesis of a biologically stable—i.e., 5′-capped and 3′-polyadenylylated—TeTx light chain-specific mRNA was achieved as described (21). Briefly, a translation termination codon and a singular *Bam*HI restriction site were introduced by site-directed mutagenesis and linker insertion exactly into the position corresponding to the proteolytic cleavage site between the light chain and the heavy chain of TeTx. DNA encoding the light chain was cloned into a modified pSP64 vector carrying an oligo-dA·dT segment downstream from the light chain-coding

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Abbreviations: AcCho, acetylcholine; TeTx, tetanus toxin; BoNT, botulinum neurotoxin; BoNT/A, BoNT type A.

‡To whom reprint requests should be addressed.

sequences to guarantee concomitant polyadenylylation during transcription. Polyadenylylation of the mRNA was considered essential because de-adenylylated globin mRNA was unstable in microinjected *Xenopus* oocytes (22) and de-adenylylated mRNAs can be stabilized by polyadenylylation (23). The light chain-specific RNA contained 72% (A+U), a feature reported to destabilize some short-lived eukaryotic RNAs (24–26). No degradation of polyadenylylated light chain-specific mRNA was detected within 24 hr in microinjected *Xenopus* oocytes, as judged by Northern (RNA) hybridization. In contrast, injected nonpolyadenylylated mRNA was no longer detectable at this stage (data not shown). As demonstrated by *in vitro* translation in rabbit reticulocyte lysate, this mRNA yielded full-size light chain that comigrated with authentic ^{125}I-labeled LC, as purified from native TeTx (Fig. 1).

mRNA encoding the entire light chain for BoNT/A was transcribed from a deletion mutant obtained from pKN25-DNA (8) by exonuclease III treatment. Transcripts obtained from this construct were again polyadenylylated due to the presence of a 3′-located poly(dA·dT) segment. The corresponding deletion mutant, designated pBN1exo904, encoded a polypeptide that contained a Pro-Leu-Ala peptide sequence instead of the carboxyl-terminal lysine of the authentic light chain of BoNT/A.

Detection of TeTx Light Chain- and BoNT/A Light Chain-Specific mRNA Expression by Electrophysiological Recordings. Experiments were performed on the identified couple of neurons (1) that form a chloride-dependent cholinergic synapse in the buccal ganglia of *Aplysia californica* (Marinus, Long Beach, CA). Dissected buccal ganglia were pinned in a 1-ml experimental chamber and superfused continuously (10 ml/hr) with artificial sea water (460 mM NaCl/10 mM KCl/11 mM $CaCl_2$/25 mM $MgCl_2$/28 mM $MgSO_4$/10 mM Tris·HCl, pH 7.8) except for the bath application of heavy chain. Both identified pre- and postsynaptic neurons were impaled with two glass microelectrodes (3 M KCl, 1.5–4 MΩ). The presynaptic cell was current-clamped to −50 mV and stimulated once a minute. AcCho release was monitored by the amplitude of the postsynaptic response (expressed as membrane conductance, nS) evoked by an action potential elicited in the presynaptic neuron and measured in the postsynaptic cell voltage-clamped to −80 mV (Fig. 2). The size of postsynaptic responses shows variability (see Figs. 3 and 4) due to a fluctuation around a mean value of the probability of the number of quanta released per action potential (27, 28). Experiments were done at room temperature (23°C).

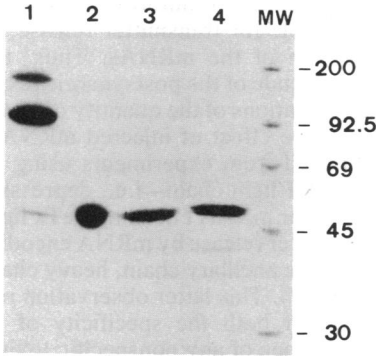

FIG. 1. *In vitro* translation of mRNA encoding the entire TeTx light chain. Products of the translation in rabbit reticulocyte lysate were analyzed on a SDS/10% polyacrylamide gel either before (lane 4) or after (lane 3) immunoprecipitation and compared with ^{125}I-labeled, partially proteolytically cleaved TeTx, as isolated from *C. tetani* (lane 1), ^{125}I-labeled light chain (lane 2), or [^{14}C]methylated molecular weight ($\times 10^{-3}$) markers (MW; Amersham).

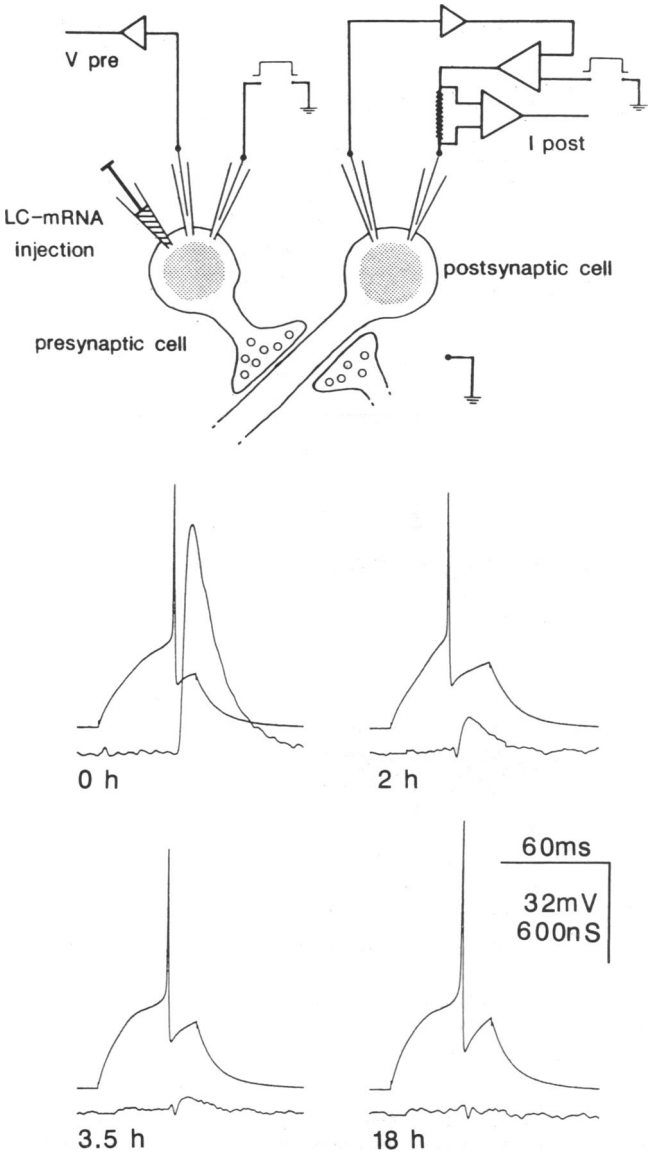

FIG. 2. Detection of light chain-specific expression in *Aplysia* neurons. (*Upper*) Both identified pre- and postsynaptic neurons were impaled with two glass micropipettes for current- and voltage-clamp measurements. (*Lower*) AcCho release was monitored by the amplitude of the postsynaptic response (expressed as membrane conductance, nS) elicited by an evoked action potential in the presynaptic neuron. Recordings show evolution of postsynaptic response (four lower traces) evoked by a presynaptic action potential (four upper traces) after injection of light chain mRNA of TeTx into the presynaptic neuron.

mRNAs of TeTx light chain and BoNT/A light chain (0.5 μg/μl) dissolved in 0.6 M NaCl solution containing 1% (wt/vol) fast green FCF dye (Sigma) were introduced into the presynaptic neuron by air-pressure injection through a third micropipette (2, 3) (Fig. 2) that was removed after injection. This micropipette also acted as a recording microelectrode to monitor its introduction into the neuron. The amount of injected material was estimated according to the intensity of color in the nerve cell. The injected volume was between 2 and 10% (<0.4 nl) of the cell-body volume, leading to a theoretical intracellular concentration of mRNAs from 20 nM to 100 nM. Injection of control buffer solutions was shown to have no effect on AcCho release. The experiments using injections of highly purified native TeTx light chain [from E. Habermann (7)] or BoNTs light chain [from J. O. Dolly (10)]

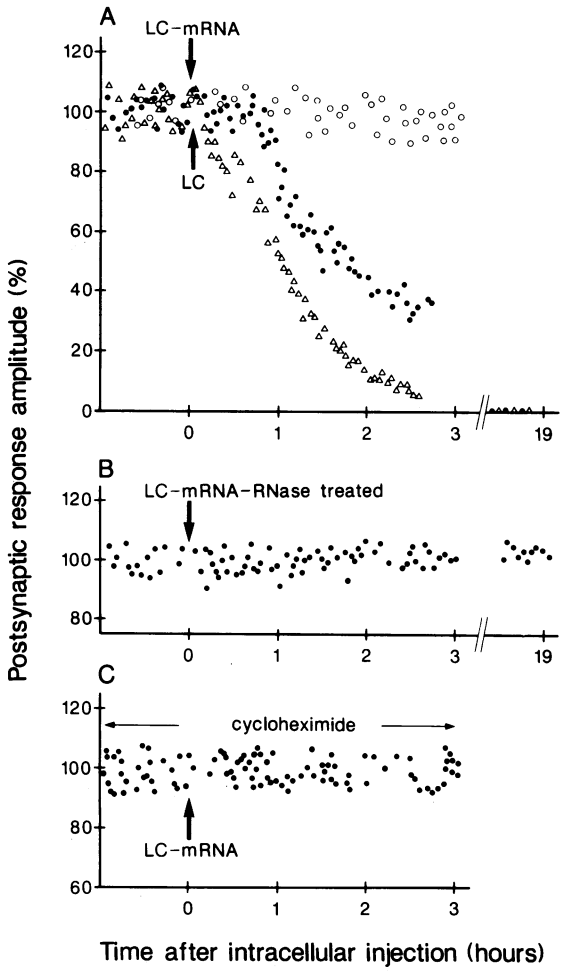

FIG. 3. Evolution of postsynaptic responses after injection of light chain mRNA or native light chain (LC) of TeTx (*A*), of inactivated light chain mRNA (*B*), and light chain mRNA in the presence of an inhibitor of protein synthesis (*C*). In all experiments the amplitude of postsynaptic responses is shown as % control before intracellular injections (arrows). (*A*) Injection of light chain mRNA (0.5 μg/μl in the injection micropipette) led to depression after delay of ≈50 min (●), whereas depression after injection of native light chain of TeTx (2 μM in the micropipette, leading to an estimated intracellular concentration of 20 nM) began after only a few minutes (△). No change in postsynaptic responses was observed in the noninjected neuron (○). (*B*) A solution of light chain mRNA (0.5 μg/μl) was treated for 1 hr with ribonuclease A (10 μg/ml) and injected into the presynaptic neuron. Failure of depression indicates that no light chain was synthesized. (*C*) Effects of cycloheximide. Injection of light chain mRNA was performed with a solution containing mRNA at 0.5 μg/μl and 100 μM cycloheximide. The inhibitor of protein synthesis at the same concentration was also present in the extracellular medium throughout the experiment.

and bath application of heavy chain of BoNTs [from J. O. Dolly (10)] have been described (2, 3).

RESULTS

Intracellular Injection of mRNA Encoding TeTx Light Chain-Induced Depression of Synaptic Transmission. After the evoked control postsynaptic responses were stabilized, mRNA of TeTx light chain was injected into the presynaptic neuron (see Fig. 2). After injection, the postsynaptic response remained unmodified for 50 ± 2 min (mean ± SD, n = 7) and then started to decrease (Fig. 3*A*). In three experiments in which high amounts of mRNA of TeTx light chain were injected, total blockade of synaptic transmission occurred within 3–4 hr. In three other less-injected neurons, the blockade of postsynaptic response was observed after overnight incubation, 18–19 hr after injection. No decrease in the amplitude and duration of the presynaptic action potential was detected (Fig. 2). The postsynaptic response to stimulation of a noninjected equivalent cholinergic presynaptic neuron afferent to the same postsynaptic cell (1) was not modified (Fig. 3*A*).

Injections of Inactivated mRNA or of mRNA with Inhibitor of Protein Synthesis Fail to Depress Transmitter Release. To ascertain that the decrease in AcCho release resulted from the expression of active light chain, we injected into the presynaptic neuron mRNA of TeTx light chain pretreated with ribonuclease A. After ribonuclease A treatment, no RNA could be detected on agarose gels. Ribonuclease A was removed before microinjection by phenol/chloroform extraction and subsequent ethanol precipitation of the aqueous phase. No alteration of AcCho release was observed even 19 hr after injection (n = 2) (Fig. 3*B*). When neurons were preincubated for at least 1 hr with cycloheximide (100 μM), which is known to inhibit protein synthesis by blocking the peptidyltransferase reaction, the subsequent coinjection of mRNA of TeTx light chain and cycloheximide (100 μM) did not modify neuronal transmission over 3 hr (n = 3) (Fig. 3*C*).

Expression of mRNA Encoding BoNT/A Light Chain. Contrary to what was seen after injection of mRNA encoding the light chain of TeTx, no changes in responses were observed after injection of mRNA of BoNT/A light chain even after several hours (n = 3). Only when heavy chain of BoNT/A was added to the bath did the responses start to decrease (Fig. 4*A*). This was similar to results observed when, instead of mRNA of BoNT light chain, the native light chain of BoNT/A was injected (Fig. 4*B*, refs. 2 and 9).

To preload the presynaptic neuron with heavy chain, heavy chain of BoNT/A was added in the bath before injecting the mRNA of BoNT/A light chain. In this case the time course of the depression of AcCho release was similar to that seen after injection of the mRNA of TeTx light chain (see Figs. 3*A* and 4*C*). The depression started after a latency of 37 ± 2 min (n = 5) (Fig. 4*C*), in contrast to the few minutes delay obtained by injection of light chain derived from native BoNT (Fig. 4*D*, refs. 2 and 9).

DISCUSSION

Several arguments confirm that the observed depression of AcCho release from the *Aplysia* neuron resulted from translation of the injected mRNAs, as derived from TeTx light chain- and BoNT/A light chain-specific recombinant DNA: (*i*) Products of *in vitro* translation of the mRNAs were homogenous and comigrated with light chains isolated from native toxins. (*ii*) The size or duration of presynaptic action potentials, responsible for transmitter release, was not depressed by injection of the mRNAs. Thus, the recorded changes of the amplitude of the postsynaptic response reflect proportional modifications of the quantity of AcCho released per impulse. (*iii*) The effect of injected mRNAs correlated with results obtained from experiments using native TeTx light chain and BoNT light chain—i.e., depression of neurotransmitter release for mRNA encoding TeTx light chain and no effect on transmitter release by mRNA encoding BoNT/A light chain, unless the ancillary chain, heavy chain of BoNT, was added to the bath. This latter observation represents an additional control of both the specificity of the injected mRNA and the absence of any nonspecific toxin action. (*iv*) The onset of toxin action from injected mRNAs was greater than that from injected native toxins: ≈50 min for mRNA of TeTx light chain and ≈37 min for mRNA of BoNT/A light chain versus ≈7 min for native toxins (2, 3). This indicates that for light chain mRNA injection, the excess delay corresponded, in large part, to the time required for the synthesis and accumulation of translated light chain of TeTx or

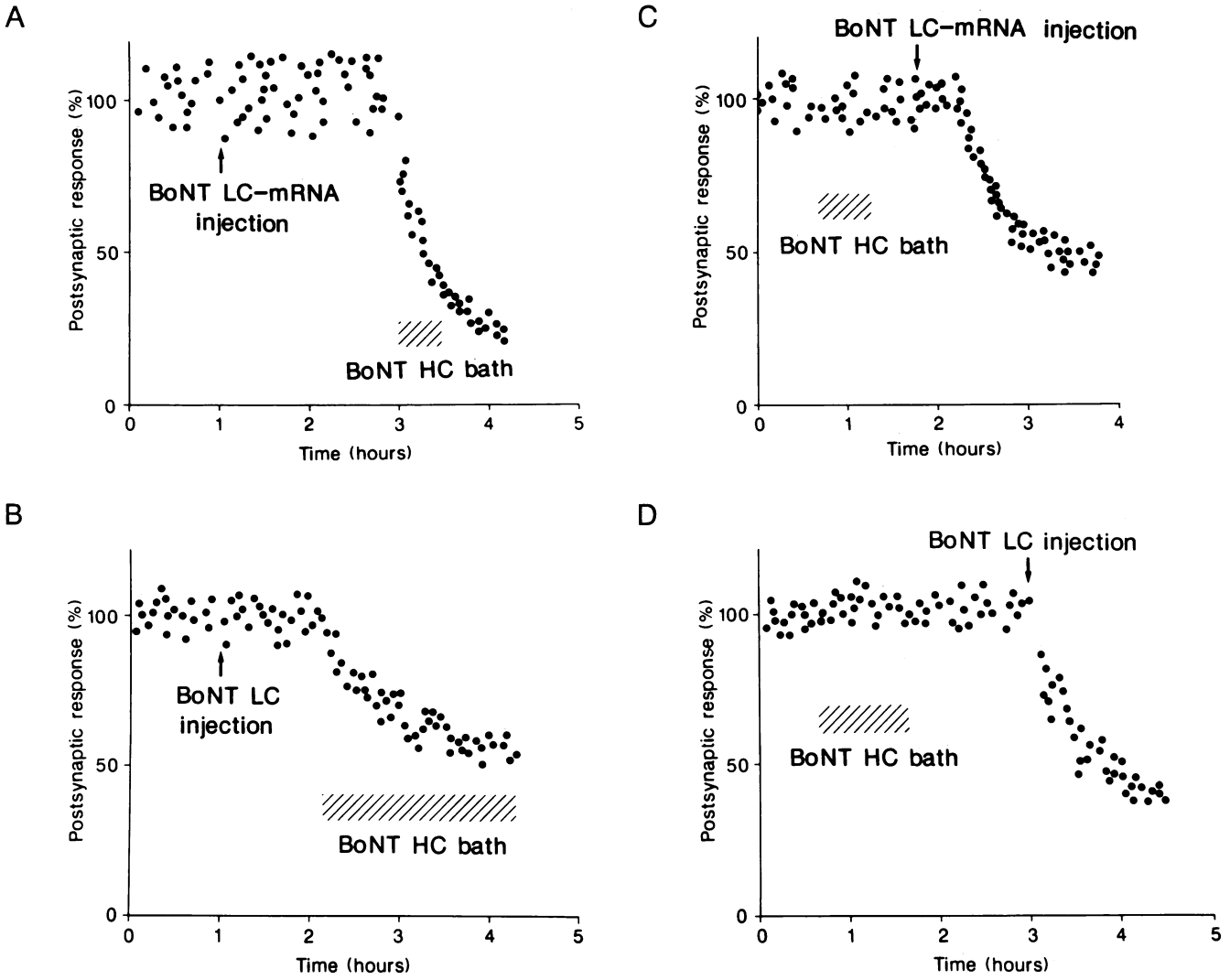

FIG. 4. Injection of light chain mRNA of BoNT/A and native light chain of BoNT/A into presynaptic neuron (*A* and *B*) and into preloaded presynaptic neuron with heavy chain of BoNT/A (*C* and *D*). No change in postsynaptic responses was induced by injection of light chain mRNA (0.5 μg/μl) (*A*) or light chain of BoNT/A (200 nM in the injection micropipette leading to intracellular concentration at 2 nM) (*B*). Only when heavy chain of BoNT/A was added into the bath (hatched bars: *A*, 50 nM; *B*, 20 nM) did depression of postsynaptic responses start. Absence of depression before heavy chain of BoNT/A added indicates that the injected substances are not toxic by themselves. (*C* and *D*) Heavy chains were added in the bath (hatched bars: *C*, 50 nM; *D*, 10 nM) and washed out before injections (arrows). Depression started at ≈40 min after injection of light chain mRNA (*C*); however, responses decreased immediately after injection of light chain of BoNT (*D*).

BoNT/A in the injected neuron. (*v*) No influence on transmitter release was observed when the injected mRNA was either inactivated by ribonuclease A or when the protein synthesis was inhibited by cycloheximide.

A tentative evaluation of translation efficacy can be made from comparing mRNA- and toxin-induced decrease of AcCho release. In Fig. 3*A*, the two depression curves resulting from injection of light chain of TeTx and light chain mRNA are nearly parallel, suggesting that at the presynaptic neuron terminal the amount of synthesized light chain from the mRNA and the amount of injected light chain isolated from native TeTx were similar. As the estimated theoretical concentrations reached in the cell body were 20 nM for the light chain of TeTx and 50 nM for the light chain mRNA, which may be translated several times, we conclude that only a fraction of the injected mRNAs was functionally expressed. For BoNT, the amount of synthesized light chain of BoNT/A from mRNA can also be estimated by the similarity of depression curves resulting from the light chain mRNA and light chain of BoNT/A injection (see Fig. 4). Again, only a fraction of this mRNA appears to have been expressed. Also, the presence of rare codons in clostridial genes [73% (A+T)] (6, 8) could decrease translation efficacy from a relative lack of corresponding tRNAs in *Aplysia*. If so, expression may possibly be increased by replacing the rare codons, as has been shown for expression of the TeTx C fragment in *Escherichia coli*, where the rare codons were replaced by those from the highly expressed *E. coli* genes (29, 30).

The fundamental conclusion of this work is that exogenous mRNAs are accepted by a nerve cell *in situ*, which has all the subcellular machinery necessary for their successful translation. Thus, it now seems possible to express exogenous mRNAs or their mutants or other biologically active molecules (such as enzymes that synthesize foreign neurotransmitters) in neurons in their normal context and to study any functional changes induced by such interference.

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