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# Differential effect of α-latrotoxin on exocytosis from small synaptic vesicles and from large dense-core vesicles containing calcitonin gene-related peptide at the frog neuromuscular junction

(acetylcholine/quantal release/neurosecretion)

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**ABSTRACT** **The regulatory peptide called calcitonin gene-related peptide (CGRP) was detected by immunofluorescence in frog motor neurons and motor nerve terminals. In motor nerve terminals, CGRP-like immunoreactivity was found to be segregated within large dense-core vesicles. To determine whether exocytosis from acetylcholine-containing small synaptic vesicles and from CGRP-containing large dense-core vesicles can be independently stimulated, nerve–muscle preparations were exposed to α-latrotoxin. This toxin induced complete depletion of acetylcholine-containing small synaptic vesicles but did not induce a parallel depletion of CGRP-like immunoreactivity and of large dense-core vesicles. These effects were independent of the presence of extracellular $Ca^{2+}$ and occurred both at room temperature and at low temperature (1–3°C). These findings suggest that exocytosis from the two vesicle populations is mediated by distinct biochemical mechanisms, which might be differentially regulated by physiological stimuli.**

Cosecretion of classical neurotransmitters and regulatory peptides has been shown to occur at a variety of synapses (1, 2). These two classes of molecules appear to be stored in and released from two different types of secretory organelles. Small synaptic vesicles (SSVs), 40–60 nm in diameter, are thought to contain only classical neurotransmitters, whereas peptides appear to be confined within a different population of larger vesicles, 80–120 nm in diameter, with a dense core [large dense-core vesicles (LDCVs)] (1–3).

Pharmacological evidence has suggested that the release of classical neurotransmitters and of regulatory peptides can be differentially stimulated and/or regulated (2, 4–6), suggesting that, within a given nerve ending, exocytosis from SSVs and from LDCVs can be dissociated.

The frog motor nerve ending is densely populated by SSVs, which contain quanta of acetylcholine (AcCho). In addition, it contains a minor complement of LDCVs, which are scattered throughout the entire terminal length. LDCVs represent about 1% of the total vesicle population, and their number has been found to decrease after electrical stimulation (7). The content and the physiological function of LDCVs have been so far totally unknown. Recently, the presence of calcitonin gene-related peptide (CGRP) (8, 9) in at least a subpopulation of motor neurons and motor end plates of mammals has been reported (10, 11). It also has been shown that this peptide increases cAMP levels in the muscle fiber (12) and modulates some parameters of muscle function, including rate of synthesis (13–15), state of phosphorylation (16), and rate of desensitization (as reviewed in ref. 16) of AcCho receptors (11–16).

In this study we show that also frog motor neurons express CGRP and that CGRP is contained in the LDCVs of motor nerve terminals. Furthermore, we show that α-latrotoxin (α-LTX), the purified active protein of black widow spider venom (17), has a differential effect on exocytosis from AcCho-containing SSVs and from CGRP-containing LDCVs.

## MATERIALS AND METHODS

**Animals.** Frogs (*Rana pipiens*) of 5-cm body lengths (Connecticut Valley Biological Supply, South Hampton, MA) were used.

**Antiserum Directed Against CGRP.** An antiserum directed against CGRP was prepared and characterized as described (18, 19).

**Light Microscopy Immunocytochemistry.** For the immunostaining of spinal cord sections (10 μm thick), frogs were anaesthetized with MS 222 (Sandoz Pharmaceutical) and transcardially perfused first with cold 0.1 M sodium phosphate buffer (pH 7.2) and then with 4% formaldehyde (freshly prepared from paraformaldehyde) in 0.1 M phosphate buffer. The cervical spinal cord was subsequently dissected out and further processed for frozen sectioning as described (20).

For immunostaining of neuromuscular junctions, cutaneous pectoris nerve–muscle preparations were dissected from frogs anesthetized as above. They were subsequently fixed with cold 4% formaldehyde/0.1 M phosphate buffer after incubation in the presence or absence of α-LTX.

Immunofluorescence staining for CGRP was performed by an indirect immunorhodamine procedure (rabbit anti-CGRP antibodies followed by rhodamine-conjugated goat anti-rabbit IgGs) on Triton-permeabilized specimens as described (20). Anti-CGRP antiserum was used at a dilution of 1:600 (vol/vol). After the immunostaining procedure, neuromuscular preparations were briefly incubated with fluorescein-conjugated α-bungarotoxin (α-BTX) (≈5 μg/ml; gift of R. Zanoni and M. Vittadello, Fidia Research Laboratory, Abano Terme, Italy). Finally, single muscle fibers were teased apart under a dissecting microscope and mounted on glass-slides in 95% (vol/vol) glycerol.

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Abbreviations: CGRP, calcitonin gene-related peptide; SSV, small synaptic vesicle; LDCV, large dense-core vesicle; AcCho, acetylcholine; α-LTX, α-latrotoxin; α-BTX, α-bungarotoxin.

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The specificity of the immunostain for CGRP was verified by experiments in which the anti-CGRP antiserum was preadsorbed with synthetic CGRP [Sigma; 0.1 nmol/ml of diluted antiserum (19)].

**Electron Microscopy Immunocytochemistry.** Nerve–muscle preparations were fixed by treatment with 0.25 glutaraldehyde/2% formaldehyde/0.1 M phosphate buffer, pH 7.2, for 60 min at room temperature. Small tissue blocks rich in motor end plates were washed in 0.1 M phosphate buffer for 60 min and then infiltrated in 2.3 M sucrose and frozen in Freon 22. Ultrathin frozen sections were prepared with an Ultracut Microtome equipped with an FC4 attachment (Reichert, Vienna) and were immunolabeled with anti-CGRP antiserum followed by protein A-colloidal gold conjugates as described (21). CGRP antiserum was used at a dilution of 1:2000 (vol/vol).

**Ultrastructure and Morphometric Analysis.** Nerve–muscle preparations to be processed for standard electron microscopy were fixed with 2% glutaraldehyde in 0.1 M phosphate buffer at room temperature and post-fixed with 2% $OsO_4$ in 0.1 M phosphate buffer. Small specimens rich in motor end plates were subsequently embedded in Epon 812, thinly sectioned, and counterstained with uranyl acetate and lead citrate. The morphometric analysis was performed on micrographs (×36,000) of cross-sectioned terminals. Data were expressed as means ± SEM. The statistical significance was determined by Student's *t* test.

**α-LTX Incubations.** Pairs of cutaneous pectoris nerve–muscle preparations were used for control and stimulation experiments. They were soaked in 2 ml of modified Ringer's solution containing 116 mM NaCl, 2.1 mM KCl, 1 mM $NaH_2PO_4$, 2 mM $Na_2HPO_4$, 4 mM $MgCl_2$, and 1.8 mM $CaCl_2$ or $Ca^{2+}$-free 1 mM EGTA.

α-LTX prepared as described (17) was added to final concentrations of either 1 μg/ml (60-min incubation at room temperature) or 2 μg/ml (120-min incubation at 1–3°C). To ascertain that the overall secretory process induced by the toxin corresponded to the one previously described, standard electrophysiological techniques were used to record miniature end-plate potentials intracellularly from at least a neuromuscular junction in each experiment (22).

## RESULTS

Staining of longitudinal and cross sections of the frog spinal cord with immunorhodamine revealed the presence of a variable, moderate-to-intense, punctate CGRP-like immunoreactivity in the perikarya and proximal dendrites of motor neurons (Fig. 1*A*). In addition, immunoreactive fibers were visible throughout the gray matter of the spinal cord. In particular, a prominent system of fibers (primarily longitudinally aligned) was visible in the dorsal spinal cord (not shown) in agreement with the known presence of CGRP in sensory fibers of a large number of vertebrate species (9, 10).

Presence of abundant CGRP-like immunoreactivity was also demonstrated by immunofluorescence at motor end plates (Fig. 1*B*). Nerve–muscle preparations were counterstained with fluorescein-conjugated α-BTX, which binds to nicotinic AcCho receptors and therefore is a cytochemical probe to reveal the location and the branching pattern of frog neuromuscular synapses (Fig. 1*C*). As shown by the precise correspondence between the two stains at low-power observation (Fig. 1 *B* and *C*), CGRP-like immunoreactivity appeared to be present throughout the entire nerve-ending arborization. At higher power observation, however, the two patterns of staining were different. CGRP immunostaining occurred in the form of puncta that were randomly distributed throughout the whole motor end plate (Fig. 1*D*), whereas α-BTX stain had a striped appearance (Fig. 1*E*). Most likely, CGRP-positive puncta represent individual LDCVs (see below), while α-BTX-positive stripes represent shoulders of postsynaptic infoldings.

In neuromuscular preparations, a low level of CGRP immunoreactivity (represented by scattered puncta) was also visible on preterminal motor axons (for example, see Fig. 3). In addition, a dense network of CGRP-positive varicose fibers was observed around blood vessels (not shown).

The subcellular distribution of CGRP-like immunoreactivity within the motor nerve terminal was determined by electron microscopy with colloidal gold immunolabeling of

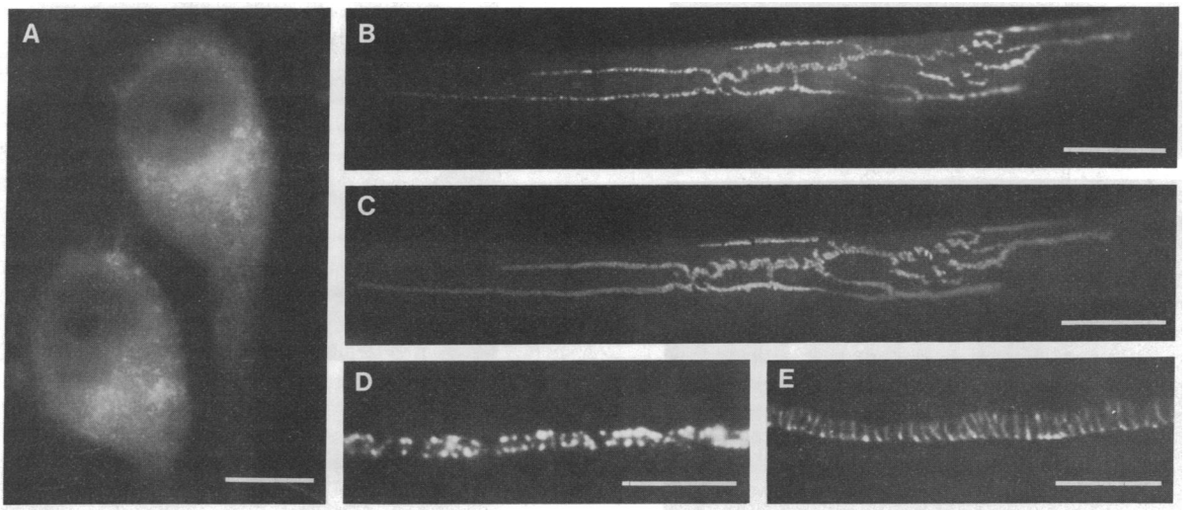


FIG. 1. Immunocytochemical identification of CGRP-like immunoreactivity in frog motor neurons and motor end plates. (*A*) Fluorescence micrograph of a frozen section of the cervical spinal cord stained by immunorhodamine. Puncta of immunoreactivity are present throughout the cytoplasm of the perikaryon and proximal dendrites. (*B* and *C*) Fluorescence micrographs showing the junction of an isolated muscle fiber double-stained to reveal the distribution of CGRP-like immunoreactivity by immunorhodamine (*B*) and of AcCho receptors by fluorescein-conjugated α-BTX (*C*). The similarity between the two fluorescent stains indicates that all branches of the nerve ending contain CGRP immunoreactivity. (*D* and *E*) Details of terminal axon branches from two different junctions showing at high magnification the pattern of fluorescence produced by CGRP immunostain and by fluorescein-conjugated α-BTX. CGRP-like immunoreactivity appears in the form of bright puncta, which probably represent individual LDCVs (*D*). Fluorescein-conjugated α-BTX produces a striped pattern of the ending. Stripes probably represent shoulders of postsynaptic infoldings (*E*). (Bars: *A*, 37 μm; *B* and *C*, 50 μm; *D* and *E*, 10 μm.)

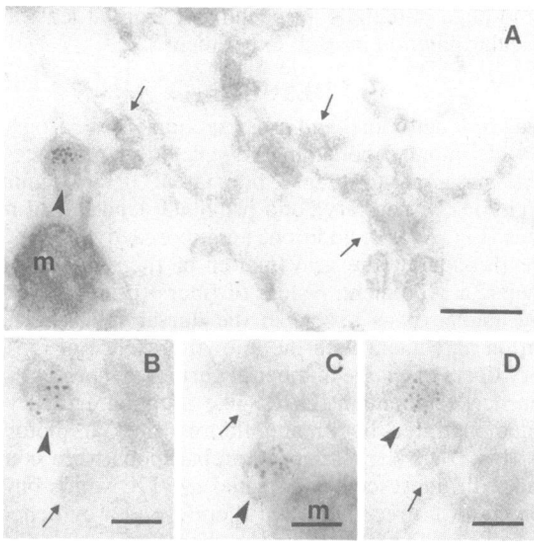


FIG. 2. Gallery of electron micrographs showing details of frog motor nerve endings from ultrathin frozen sections labeled for CGRP-like immunoreactivity by a colloidal gold immunostaining technique. Gold particles are selectively localized on the core of LDCVs (arrowheads) while SSVs (small arrows) are unlabeled. m, Mitochondria. (Bars: *A*, 130 μm; *B* and *C*, 74 μm; *D*, 93 μm.)

ultrathin frozen sections. Immunogold particles were found to be selectively associated with the core of LDCVs, whereas other structures of the synapse, including SSVs, were completely unlabeled (Fig. 2).

To determine whether exocytosis from AcCho-containing SSVs and from CGRP-containing LDCVs could be dissociated, nerve–muscle preparations were treated with α-LTX. This agent, when applied to neuromuscular junctions, causes a massive increase in the rate of occurrence of miniature end-plate potentials and ultimately may deplete the nerve terminals of both SSVs and quanta of AcCho (17, 22). The depletion occurs either when low doses of α-LTX are applied in $Ca^{2+}$-free medium (provided that other divalent cations are present) or when high doses of α-LTX are applied with or without the presence of extracellular $Ca^{2+}$ (22, 23). The depletion is caused (*i*) by the massive stimulation of SSV exocytosis and (*ii*) by the blockage of the recycling mechanism that normally operates to maintain a constant SSV population during physiological stimulation.

Nerve–muscle preparations were incubated for 1 hr at room temperature in the presence or absence of high doses of α-LTX (1 μg/ml) in both $Ca^{2+}$-free and $Ca^{2+}$-containing solutions. Intracellular recordings showed that, as expected, the miniature end-plate potential rate of occurrence rapidly increased to high levels and decreased to virtually zero within 1 hr. Nerve–muscle preparations were then fixed and processed for CGRP immunofluorescence. Intense, punctate, CGRP-like immunoreactivity was still present after the toxin treatment irrespective of the presence (Fig. 3*A*) or absence (Fig. 3*B*) of extracellular $Ca^{2+}$. The only noticeable differences from control preparations were that nerve terminal branches appeared to be swollen and puncta appeared to be somehow more disperse, probably as a result of their dilution

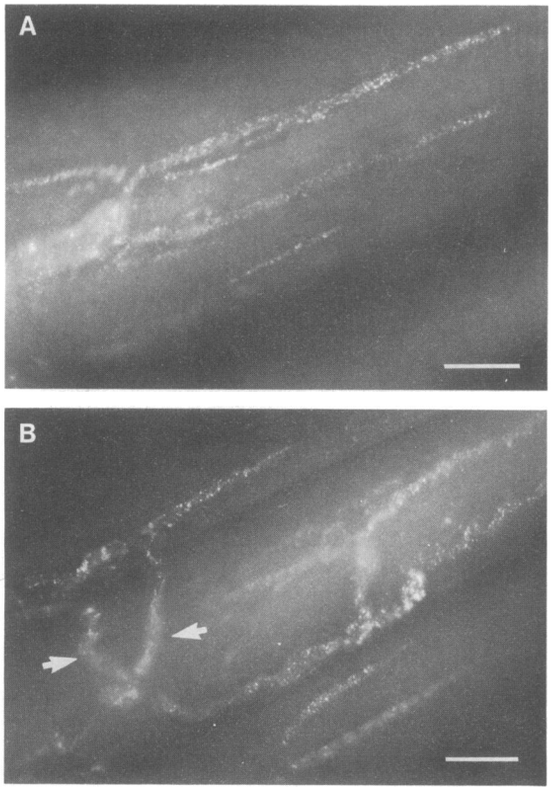


FIG. 3. Fluorescence micrographs showing the distribution of CGRP-like immunoreactivity at frog motor end plates after incubation with α-LTX (1 μg/ml) at room temperature in the absence (*A*) or presence (*B*) of extracellular $Ca^{2+}$. Note the swelling of the branches of the ending in comparison with the control terminals (Fig. 1*A*). Arrows indicate a branching of unmyelinated preterminal axons. (Bars: *A*, 271 μm; *B*, 27 μm.)

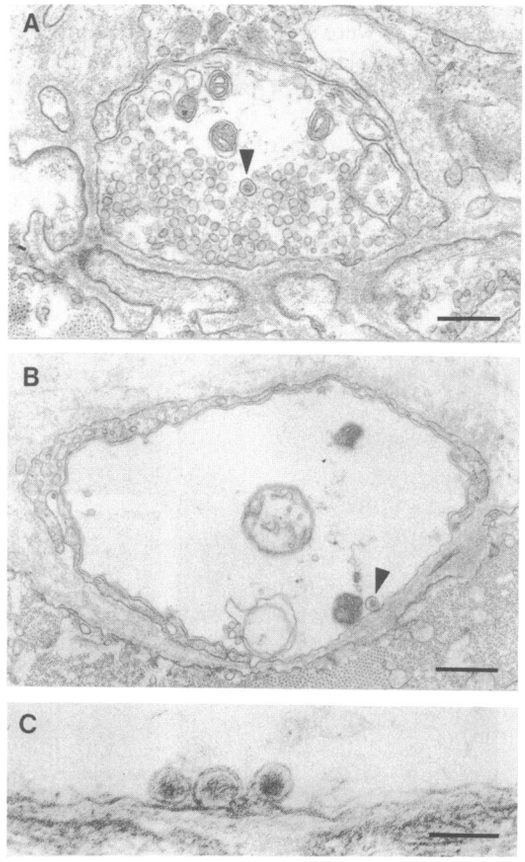


FIG. 4. Electron micrographs of cross-sectioned nerve terminals incubated with and without α-LTX in $Ca^{2+}$-free solution at room temperature. (*A*) Control terminal densely populated by SSVs. (*B* and *C*) α-LTX-treated terminals. The nerve endings are swollen and completely depleted of SSVs, but LDCVs are still present. Arrowheads in *A* and *B* indicate LDCVs. Identical results were obtained in the presence of extracellular $Ca^{2+}$. (Bars: *A*, 340 μm; *B*, 529 μm; *C*, 178 μm.)

Table 1. Number of LDCVs per cross section

| Sample | Temp. | LDCV, mean ± SEM | *n* |
|---|---|---|---|
| Control α-LTX | RT | 0.83 ± 0.12 | 47 |
| $Ca^{2+}$-free | | 0.62 ± 0.14 | 56 |
| 1.8 mM $Ca^{2+}$ | | 0.61 ± 0.11 | 49 |
| Control α-LTX | 1–3°C | 0.73 ± 0.19 | 22 |
| $Ca^{2+}$-free | | 0.66 ± 0.15 | 32 |
| 1.8 mM $Ca^{2+}$ | | 0.82 ± 0.17 | 34 |

Temp., temperature; RT, room temperature; *n*, number of terminal cross sections examined.

in the increased (22) nerve terminal volume. Electron micrographs from the toxin-treated muscles corroborated the finding obtained by immunofluorescence. Nerve terminals treated with the toxin with or without extracellular $Ca^{2+}$ (Fig. 4) were swollen and completely depleted of SSVs as described (17, 22, 23). In spite of such a depletion, LDCVs were still present. They often were located close to the presynaptic plasmalemma (Fig. 4 *B* and *C*), possibly as a result of a disassembly (see Fig. 4*B*) of the nerve terminal cytomatrix. A morphometric analysis performed on cross-sectioned terminals confirmed that the complete depletion of SSVs was not accompanied by a significant decline in the number of LDCVs (Table 1).

Steady-state concentration of LDCVs can be maintained in nerve terminals when a number of these organelles, equal to that lost by exocytosis, are replaced from the perikaryon by axonal transport (3). Thus, the observed lack of depletion of LDCVs might have stemmed from a continuous replacement of LDCVs through fast axonal transport (24).

Since low temperature appears to be an effective inhibitor of fast axonal transport (25), we performed similar experiments with α-LTX at 1–3°C. Previous studies had shown that the toxin was effective also at this temperature. In particular, 2 μg of the toxin per ml applied at 1–3°C in both the absence and presence of extracellular $Ca^{2+}$ raised the miniature end-plate potential rate of occurrence to several hundred per sec and caused a profound depletion of vesicles after 2 hr (26). Therefore, we fixed some nerve–muscle preparations after such treatments and analyzed them for the presence both of CGRP-like immunoreactivity and of LDCVs.

In the presence and absence of extracellular $Ca^{2+}$, the pattern and intensity of immunostained CGRP were similar to those obtained after exposure to the toxin at room temperature under corresponding conditions (Fig. 5). Furthermore, in agreement with immunofluorescence findings, a morphometric analysis carried out on electron micrographs of nerve terminals revealed that, in spite of the profound (≈75%) depletion of SSVs (26), there was no significant change in the number of LDCVs (Table 1).

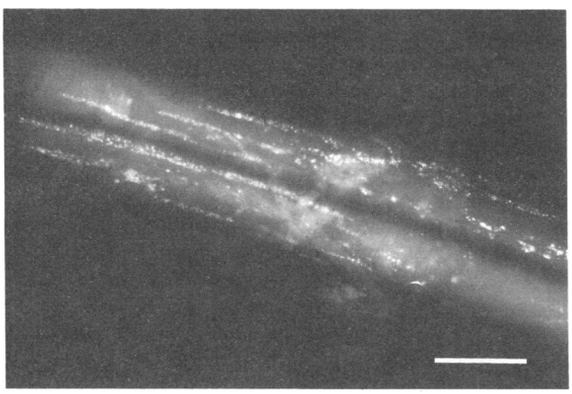

FIG. 5. Fluorescence micrographs showing the distribution of CGRP-like immunoreactivity at frog motor end plates after incubation with α-LTX (2 μg/ml) at low temperature (1–3°C) in the presence of extracellular $Ca^{2+}$. Similar results were obtained in the absence of extracellular $Ca^{2+}$. (Bar: 56 μm.)

## DISCUSSION

Our study shows that frog motor neurons express CGRP-like immunoreactivity and that this immunoreactivity in motor nerve terminals is confined within LDCVs. The frog neuromuscular junction is the most thoroughly characterized model for the study of quantal release of classical neurotransmitters via SSVs (26–28). The detection of CGRP-containing LDCVs at these terminals opens the possibility of obtaining important new information on the relation between secretion of peptides and classical neurotransmitters from the same nerve ending. We show here that a striking dissociation between exocytosis from SSVs and exocytosis from LDCVs can be obtained by stimulation with α-LTX.

α-LTX is not a physiological stimulus, but its depleting effect on the nerve terminal store of SSVs has been useful in understanding some important features of neuromuscular transmission (22). We used here such a property as a tool to study whether exocytosis from SSVs and LDCVs at motor nerve endings is mediated by different molecular mechanisms.

The precise mechanism by which α-LTX triggers exocytosis from SSVs is unclear. The toxin action is highly dependent upon the presence of multivalent cations in the bathing solution (29), and the toxin has been shown to open nonclosing cation channels in the excitable membrane that allow an influx of a variety of multivalent cations including $Ca^{2+}$ (30, 31). However, the toxin stimulates exocytosis of SSVs irrespective of the presence or absence of extracellular $Ca^{2+}$, and when high doses of the toxin are used, it depletes the terminal population of SSVs under both conditions (23, 32). We considered the possibility that the exocytotic response of LDCVs might be more strictly dependent upon extracellular $Ca^{2+}$ (33), and for this reason we performed stimulation experiments in the absence and presence of extracellular $Ca^{2+}$. Under both conditions, the depletion of SSVs was neither accompanied by a parallel reduction of CGRP-like immunoreactivity, as seen at the light microscope, nor accompanied by a depletion of LDCVs, as seen at the electron microscope. Low-temperature experiments ruled out steady-state concentrations of LDCVs, maintained by continuous replacement through axonal transport of LDCVs lost by exocytosis, as an explanation of the results.

Our interpretation of these findings is that different mechanisms are probably involved in the process leading to the release of a quantum of AcCho and of a packet of CGRP. Proteins exposed to or present at the cytoplasmic surface of the vesicle membrane are those that are thought to mediate interactions between the cytoskeleton and synaptic vesicles. These interactions are likely to play an important role in regulating the kinetics and the topology of the exocytotic process. Therefore, it is of interest in connection with our present results that the membranes of SSVs and of LDCVs differ, at least partially, in their protein composition (34–36). In particular, synapsin I, a protein that is thought to provide a regulatory link between vesicles and the actin-based cytoskeleton of the terminals (37, 38), has been found to be preferentially associated with the cytoplasmic surface of SSVs (34).

Not only do SSV membranes and LDCV membranes differ in their protein composition, but also both membranes differ from the presynaptic axolemma (35, 37, 39). One cannot exclude the possibility that the rapid massive incorporation of SSV membranes into the axolemma produced by the toxin might rapidly make the latter unsuitable for LDCV fusion, in spite of its ability to continue to fuse with SSVs until their

depletion. Had this been the case in our experiments, a slight decline in the level of CGRP-like immunoreactivity and in the number of LDCVs might have occurred soon after the start of the toxin action. Such a decline might have gone undetected by our experimental approaches. Even if this had been the case, our results still indicate that the molecular mechanisms involved in SSV and LDCV exocytosis are different.

The dissociation of SSV and LDCV exocytosis produced by α-LTX is independent of extracellular $Ca^{2+}$. It is possible, however, that under physiological conditions a differential control of the two release processes [for example, by different frequencies of nerve stimulation (4, 5)] is achieved through a different sensitivity to $Ca^{2+}$ signals and/or through a regional heterogeneity in the concentration of different types of $Ca^{2+}$ channels in the presynaptic membrane. In this regard, it is of interest that drugs that are known to block selectively $Ca^{2+}$ channels of the L type have been found to inhibit the release of a neuropeptide from sensory neurons but not of a classical neurotransmitter from sympathetic neurons (40–42).

In conclusion, our study supports the hypothesis that the mechanisms of exocytosis for SSVs and LDCVs at the motor end plate (and possibly at other synapses) are at least partially different. This is consistent with the different physiological significance of the two release processes. AcCho-mediated signaling involves the delivery of concentrated pulses of AcCho at highly specialized sites. In contrast, the modulatory and/or trophic function of CGRP appears compatible with a temporal and topological (43) dispersion of the secretory event.

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1. Hökfelt, T., Johansson, O. & Goldstein, M. (1984) *Science* **225**, 1326–1334.
2. Hökfelt, T., Millhorn, D., Seroogy, K., Tsuruo, Y., Ceccarelli, S., Lindh, B., Meister, B., Melander, T., Schalling, M., Bartfai, T. & Terenius, L. (1987) *Experientia* **43**, 768–780.
3. Klein, R. L., Lagercrantz, H. & Zimmermann, H. (1982) *Neurotransmitter Vesicles* (Academic, London).
4. Andersson, P. O., Bloom, S. R., Edwards, A. V. & Jaerhult, J. (1982) *J. Physiol. (London)* **322**, 469–483.
5. Lundberg, J. & Hökfelt, T. (1983) *Trends Neurosci.* **6**, 325–333.
6. Ip, N. Y. & Zigmond, R. E. (1984) *Nature (London)* **311**, 472–474.
7. Lynch, K. (1980) *Brain Res.* **194**, 249–254.
8. Amara, S. G., Jonas, V., Rosenfeld, M. G., Ong, E. S. & Evans, R. M. (1982) *Nature (London)* **298**, 240–244.
9. Rosenfeld, M. G., Mermod, J. J., Amara, S. G., Sawchenko, P. E., Rivier, J., Vale, W. W. & Evans, R. M. (1983) *Nature (London)* **304**, 129–135.
10. Gibson, S. J., Polak, J. M., Bloom, S. R., Sabate, I. M., Mulderry, P. K., Ghatei, M. A., McGregor, G. P., Morrison, J. F. B., Kelly, J. S., Evans, R. M. & Rosenfeld, M. G. (1984) *J. Neurosci.* **4**, 3101–3111.
11. Takami, K., Kawai, Y., Uchida, S., Tohyama, M., Shiotanj, Y., Yoshida, H., Emson, P. C., Girgis, S., Hillyard, C. J. & MacIntyre, I. (1985) *Neurosci. Lett.* **60**, 227–230.
12. Laufer, R. & Changeux, J. P. (1987) *EMBO J.* **6**, 901–906.
13. New, H. V. & Mudge, A. W. (1986) *Nature (London)* **323**, 809–811.
14. Fontaine, B., Klarsfeld, A., Hökfelt, T. & Changeux, J. (1986) *Neurosci. Lett.* **71**, 59–65.
15. Fontaine, B., Klarsfeld, A. & Changeux, J. P. (1987) *J. Cell Biol.* **105**, 1337–1342.
16. Miles, K. & Huganir, R. L. (1988) *Mol. Neurobiol.*, in press.
17. Frontali, N., Ceccarelli, B., Gorio, A., Mauro, A., Siekevitz, P., Tzeng, M.-C. & Hurlbut, W. P. (1976) *J. Cell Biol.* **68**, 462–479.
18. Mulderry, P. K., Ghatei, M. A., Rodrigo, J., Allen, J. M., Rosenfeld, M. G., Polak, J. M. & Bloom, S. R. (1985) *Neuroscience* **14**, 947–954.
19. Rodrigo, J., Polak, J. M., Terenghi, G., Cervantes, C., Ghatei, M. A., Mulderry, P. K. & Bloom, S. R. (1985) *Histochemistry* **82**, 67–74.
20. De Camilli, P., Cameron, R. & Greengard, P. (1983) *J. Cell Biol.* **96**, 1337–1354.
21. Keller, G. A., Tokuyasu, K. T., Dutton, A. H. & Singer, S. J. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 5744–5747.
22. Hurlbut, W. P. & Ceccarelli, B. (1979) in *Advances in Cytopharmacology*, eds. Ceccarelli, B. & Clementi, F. (Raven, New York), Vol. 3, pp. 87–115.
23. Ceccarelli, B. & Hurlbut, W. P. (1980) *J. Cell Biol.* **87**, 297–303.
24. Schnapp, B. J., Vale, R. D., Sheetz, M. P. & Reese, T. S. (1987) *Ann. N.Y. Acad. Sci.* **493**, 908–918.
25. Edström, A. & Hanson, M. (1973) *Brain Res.* **58**, 345–354.
26. Ceccarelli, B., Hurlbut, W. P. & Iezzi, N. (1988) *J. Physiol. (London)* **402**, 195–217.
27. Segal, J. R., Ceccarelli, B., Fesce, R. & Hurlbut, W. P. (1985) *Biophys. J.* **47**, 183–202.
28. Haimann, C., Torri-Tarelli, F., Fesce, R. & Ceccarelli, B. (1985) *J. Cell Biol.* **101**, 1953–1965.
29. Misler, S. & Falke, L. C. (1987) *Am. J. Physiol.* **253**, C469–C476.
30. Meldolesi, J., Scheer, H., Madeddu, L. & Wanke, E. (1986) *Trends Pharmacol. Sci.* **35**, 213–216.
31. Wanke, E., Ferroni, A., Gattanini, P. & Meldolesi, J. (1986) *Biochem. Biophys. Res. Commun.* **134**, 320–325.
32. Fesce, R., Segal, J. R., Ceccarelli, B. & Hurlbut, W. P. (1986) *J. Gen. Physiol.* **88**, 59–81.
33. Mason, R. T., Peterfreund, R. A., Sawchenko, P. E., Corrigan, A. Z., Rivier, J. E. & Vale, W. W. (1984) *Nature (London)* **308**, 653–655.
34. Navone, F., Greengard, P. & De Camilli, P. (1984) *Science* **226**, 1209–1211.
35. Navone, F., Jahn, R., Di Gioia, G., Stukenbrok, H., Greengard, P. & De Camilli, P. (1986) *J. Cell Biol.* **103**, 2511–2527.
36. De Camilli, P. & Navone, F. (1987) *Ann. N.Y. Acad. Sci.* **493**, 461–479.
37. De Camilli, P. & Greengard, P. (1986) *Biochem. Pharmacol.* **35**, 4349–4357.
38. Bähler, M. & Greengard, P. (1987) *Nature (London)* **326**, 704–706.
39. Valtorta, F., Villa, A., Jahn, R., De Camilli, P., Greengard, P. & Ceccarelli, B. (1988) *Neuroscience* **24**, 593–603.
40. Perney, T. M., Hirning, L. D., Leeman, S. E. & Miller, R. J. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 6656–6659.
41. Rane, S. G., Holz, G. G., IV, & Dunlap, K. (1987) *Pflügers Arch.* **409**, 361–366.
42. Hirning, L. D., Fox, A. P., McCleskey, E. W., Olivera, B. M., Thayer, S. A., Miller, R. J. & Tsien, R. W. (1988) *Science* **239**, 57–60.
43. Zhu, P. C., Thureson-Klein, A. & Klein, R. L. (1986) *Neuroscience* **19**, 43–54.