Interaction of ^{125}I-Labeled Botulinum Neurotoxins with Nerve Terminals. II. Autoradiographic Evidence for Its Uptake into Motor Nerves by Acceptor-mediated Endocytosis

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Abstract. Using pharmacological (Simpson, L. L., 1980, *J. Pharmacol. Exp. Ther.* 212:16–21) and autoradiographic techniques (Black, J. D., and J. O. Dolly, 1986, *J. Cell Biol.*, 103:521–534), it has been shown that botulinum neurotoxin (BoNT) is translocated across the motor nerve terminal membrane to reach a postulated intraterminal target. In the present study, the nature of this uptake process was investigated using electron microscopic autoradiography. It was found that internalization is acceptor-mediated and that binding to specific cell surface acceptors involves the heavier chain of the toxin. In addition, uptake was shown to be energy and temperature-dependent and to be accelerated by nerve stimulation, a treatment which also shortens the time course of the toxin-induced neuroparalysis. These results, together with the observation that silver grains were often associated with endocytic structures within the nerve terminal, suggested that acceptor-mediated endocytosis is responsible for toxin uptake. This proposal is supported further by the fact that lysosomotropic agents, which are known to interfere with the endocytic pathway, retard the onset of BoNT-induced neuroparalysis and also affect the distribution of silver grains at nerve terminals treated with ^{125}I-BoNT. Possible recycling of BoNT acceptors (an important aspect of acceptor-mediated endocytosis of toxins) at motor nerve terminals was indicated by comparing the extent of labeling in the presence and absence of metabolic inhibitors. On the basis of these collective results, it is concluded that BoNT is internalized by acceptor-mediated endocytosis and, hence, the data support the proposal that this toxin inhibits release of acetylcholine by interaction with an intracellular target.

THE mechanism of action of botulinum neurotoxin (BoNT)[1] has been shown, using pharmacological (44) and autoradiographic (1, 11, 12) techniques, to involve at least one additional step after binding to specific cell surface acceptors, i.e., internalization. For a large protein, such as the BoNT molecule, to cross the hydrophobic lipid bilayer enclosing the motor nerve terminal, a high energy barrier must be overcome. Several possible means of achieving this have been discussed (45): (*a*) Toxin could enter the cytosol directly by means of an active mechanism inherent in the molecule, (*b*) uptake might be accomplished by an active process involving the cell membrane such as endocytosis, or (*c*) a combination of both these possibilities. Based on indirect evidence (21, 44, 46, 47), the process of acceptor-mediated endocytosis has been implicated in the entry of BoNT into the cytosol of target cells. Animal cells use this process to take up nutritional and regulatory factors from the extracellular fluid (16, 33); the internalization process is effectively coupled to binding and has a half-time of <10 min at 37°C. It involves initial binding of the ligand to mobile acceptors which are distributed randomly on the cell surface, with subsequent clustering of the ligand–acceptor complexes in specialized regions of the plasma membrane known as coated pits (37). The pits then invaginate to form coated vesicles which, as they move deeper into the cytoplasm, shed their clathrin (35) coats (15–60 s) (36) and fuse with one another to form larger, smooth-surfaced vesicles called endosomes. The latter are often surrounded by small tubular or round vesicular profiles that can form anastomosing networks (15, 34). An important characteristic of these structures is their low internal pH (51). This is thought to induce dissociation of ligands from their acceptors; hence, this vesicle-plus-tubule complex has been named compartment of uncoupling of receptors and ligands, or CURL (15). The ligands accumulate in the endosomal lumen, while the acceptors are concentrated in the membrane of an attached tubular structure. The vesicular part may deliver dissociated ligands to lysosomes for degradation or direct them to some

1. *Abbreviation used in this paper*: BoNT, botulinum neurotoxin.

other destination (for review, see reference 30). The membraneous tubular structures, on the other hand, are thought to recycle acceptors to the plasma membrane (8).

A number of invasive agents, such as enveloped viruses and bacterial toxins, which introduce their genomes or toxic subunits into the cytosol, appear to use the process of acceptor-mediated endocytosis to reach their intracellular targets. In the virus systems (toga viruses, orthomyxo viruses, and rhabdo viruses), the low pH of the endosomes induces a conformational change in the viral spike glycoproteins; this results in the fusion of the viral membrane with the limiting membrane of the endosome and release of the nucleocaspid into the cytoplasm (25). Similarly, in the case of bacterial toxins (e.g., diphtheria toxin), the low pH in the endosome induces a conformational change in the molecule that facilitates the passage of an active fragment across the vesicular membrane (2, 13).

The relevance of such findings to the mechanism of access of BoNT to its intracellular target lies in the similarities in overall structure and action of the BoNT molecule and other bacterial or plant toxins. The "nicked" BoNT molecule consists of two heterologous polypeptides linked by a disulfide bond (7). When the disulfide bond is reduced, the heavy (M_r ~100,000) and light (M_r ~50,000) chains can be separated using denaturing agents (e.g., urea or guanidine) (23). Several bacterial (e.g., cholera, diphtheria, and tetanus toxins) and plant (abrin and ricin) toxins as well as some glycoprotein hormones, which appear to act intracellularly, also consist of two heterologous components (30, 53). They seem to have a common strategy of access to their targets; one of the polypeptides binds to an acceptor on the external surface of the cell, while the other expresses its biological activity intracellularly. For BoNT types A and B, it has been shown that binding to acceptors on rat cerebrocortical synaptosomes is mediated by the heavier chain (22, 55). Interestingly, tetanus toxin which, like BoNT, is thought to bind to acceptors in the central nervous system by its larger subunit (27, 52), is retarded from binding at the neuromuscular junction by fragment C of the toxin (part of the heavier subunit) (48).

In this study, the proposal that BoNT, like other molecules of similar structure and mode of action, is taken up by acceptor-mediated endocytosis was investigated using electron microscopic autoradiography. BoNT is shown to bind to cell surface acceptors on motor neurons (1) through its heavier chain, resulting in uptake by an energy- and temperature-dependent process. This internalization is enhanced by nerve stimulation and modified by drugs (lysosomotropic agents) known to affect acceptor-mediated endocytosis (31) and, also, to delay the onset of BoNT-induced blockade of neurotransmission, maintaining the toxin at an antitoxin-sensitive site (44, 46, 47).

Materials and Methods

Toxins

Preparations (unlabeled and ^{125}I-labeled) of BoNT type A (1) and of its isolated larger chain (55) were kindly provided by Dr. R. Williams of this laboratory. The polypeptides of BoNT were separated by QAE-Sephadex anion-exchange chromatography in the presence of dithiothreitol and urea (23). The heavy chain was then dialyzed against 0.02 M sodium phosphate buffer (pH 7.2) for 48 h before use in competition experiments with ^{125}I-BoNT. Tetanus toxin, isolated from *Clostridium tetanii* (52), was kindly provided by Dr. P. D. Walker (Wellcome Labs, Beckenham, Kent, England).

Tissue Labeling

All studies were carried out in vitro. Mouse hemidiaphragms were incubated with ^{125}I-BoNT under different conditions and then washed at 4°C, before being processed for electron-microscopic autoradiography as described in the preceding paper (1). Nonspecific binding was determined in control samples labeled in the presence of excess nonradioactive BoNT. The energy-dependence of the internalization process was investigated using tissue that was pretreated for 20–30 min at 22°C with Na azide (15 mM) or dinitrophenol (0.5 mM) before labeling with ^{125}I-BoNT. To study the temperature-dependence of toxin internalization, samples were equilibrated in Krebs-Ringer's solution at 4° and 22°C before incubation with radiolabeled BoNT in the latter buffer for 2 h at each of these temperatures. The effects of the larger polypeptide of BoNT on the binding and internalization of ^{125}I-BoNT were studied by incubating mouse hemidiaphragms in Krebs-Ringer's solution containing 10 nM ^{125}I-BoNT and a 50-fold excess of the larger chain, in the presence and absence of 15 mM Na azide. Likewise, the action of tetanus toxin was observed in autoradiograms of tissue pretreated for 1 h at 22°C with 0.9 or 1.0 μM tetanus toxin in Krebs-Ringer's solution, followed by incubation with 10 or 11 nM ^{125}I-BoNT for 2 h.

To examine the internalization step of toxin-induced neuroparalysis, samples of mouse diaphragm were labeled at 22°C for 90 min with ^{125}I-BoNT in the absence of Ca^{++} (+2 mM EGTA) or in the presence of the lysosomotropic agents: methylamine (6 mM), ammonium chloride (6 mM), or chloroquine (50 μM or 2 mM). The effect of nerve stimulation on toxin uptake was studied by incubating two mouse hemidiaphragms (dissected with the phrenic nerve intact) in Krebs-Ringer's solution/0.5% bovine serum albumin (BSA) (gassed continuously with a fine stream of 95% O_2/5% CO_2) containing 10 nM ^{125}I-BoNT; one of these was stimulated electrically at 0.25 Hz throughout the incubation period, until complete blockade of neurotransmission was observed (150 min at 22°C).

Analysis of Autoradiographic Data

Electron-microscopic autoradiograms were analyzed quantitatively as follows: photographs were taken of at least 20–30 endplates from each preparation, at a magnification of 3,600; prints were then prepared at a magnification of 19,600 and the nerve terminal membrane lengths were determined (1). The grains detectable on the plasma membrane and within the terminal cytoplasm (where applicable) were counted and expressed per unit length of membrane measured. To quantitate the extent of internalization of radioactivity under different conditions, silver grains on or within 60–70 nm of the membrane (see reference 1, section on resolution of the autoradiographic technique used) and grains in the cytoplasm were counted separately, and expressed as a percentage of the total associated with the nerve terminals.

Results

Energy- and Temperature-dependence of the Internalization of Radioactivity at Motor Nerve Terminals Labeled with ^{125}I-BoNT

Autoradiograms of murine motor nerve terminals treated with ^{125}I-BoNT in vitro showed a saturable deposition of silver grains on the terminal plasma membrane and also within the cytoplasm (1). When hemidiaphragms were pretreated with the inhibitor of mitochondrial oxidative phosphorylation Na azide (15 mM) and then incubated with the radiolabeled toxin, silver grains were observed only on the nerve terminal plasma membrane (Fig. 1 *a*). Thus, internalization of radioactivity was prevented totally (90% of the grains were found on the membrane itself and the remainder, which could arise from expected scatter, within 150 nm of the latter). When samples of tissue were incubated for 90 min with low toxin concentrations (e.g., 1.5 nM), the extent of labeling was the same at azide-treated and untreated nerve terminals (Table I). However, at higher toxin concentrations (e.g., 15 or 35 nM), Na azide-treated terminals showed only 90%

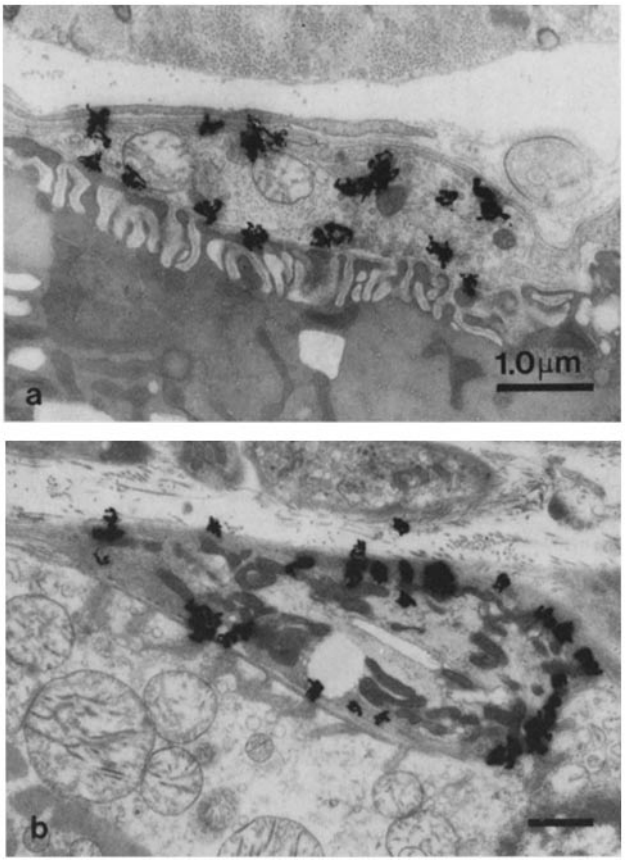

Figure 1. Effect of metabolic inhibitors on the uptake of ^{125}I-BoNT into the murine nerve terminal. Nerve–muscle preparations were preincubated for 20–30 min at 22°C with Krebs-Ringer's solution containing 15 mM Na azide or 0.5 mM dinitrophenol. The tissues were then incubated with ^{125}I-BoNT (11 nM) in the appropriate preincubation medium for 90 min (at 22°C) and washed extensively in the same solutions (six changes, 5 min each) before processing for electron microscopic autoradiography. Slides were developed after 3-wk exposure at 4°C. Silver grains in the resultant autoradiograms show the location of acceptors labeled with toxin in the presence of azide (*a*) or dinitrophenol (*b*).

Table I. Effect of Na Azide on Labeling of Motor Nerve Terminals by ^{125}I-BoNT under Different Conditions

Toxin concentration	Extent of labeling* in Na azide-treated samples relative to controls
nM, 90 min	%
1.5	104
15.0	90
35.0	90
Incubation with 15 nM ^{125}I-BoNT	
min	
20	92
90	90
150	80

* This was quantified by counting the number of grains in autoradiograms of mouse hemidiaphragms labeled with ^{125}I-BoNT. Using ~30 endplates from each sample, the total number of grains therein (70–160) was determined. The total length of nerve terminal plasma membrane from tissue treated with Na azide was measured by digitization and the number of grains per micrometer was calculated; this is expressed relative to that measured similarly for control specimens where grains both on the plasma membrane and in the cytoplasm were included.

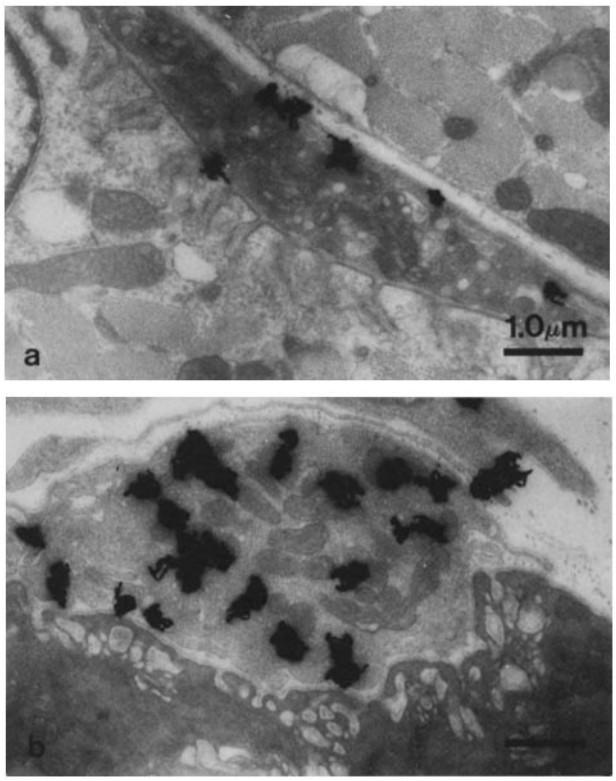

Figure 2. Temperature dependence of the binding and internalization of ^{125}I-BoNT at the motor nerve terminal. Pieces of diaphragm were pre-equilibrated with Krebs-Ringer's solution at 4°C and then incubated for 2 h in 0.5 ml Krebs-Ringer's solution containing 11 nM ^{125}I-BoNT. The tissue was then washed, fixed in 2% glutaraldehyde for 75 min (at 4°C), and processed for electron microscopic autoradiography. Control preparations were treated similarly but at room temperature. Slides were developed after 3-wk exposure. (*a*) Nerve terminal treated with ^{125}I-BoNT at 4°C. Note the reduced number of silver grains associated with the synaptic bouton (30% relative to controls, see Table II) and the absence of internalized radioactivity. (*b*) Control sample treated with ^{125}I-BoNT at 22°C showing membrane-bound and internalized silver grains in a ratio of ~60:40.

of the labeling observed in untreated samples. To determine whether the length of incubation also contributed to these different extents of labeling seen, nerve–muscle preparations were incubated at 22°C with 15 nM ^{125}I-BoNT for various periods in the presence and absence of Na azide. Even after 20 min, a reduction of 8% was observed in Na azide-treated samples; this increased to 10 and 20% after 90 and 150 min, respectively (Table I). Clearly, the length of incubation accentuated the difference in the extent of labeling under the two conditions. Dinitrophenol (0.5 mM), another inhibitor of energy production, also prevented uptake of radioactivity into the nerve terminal (Fig. 1 *b*). Silver grains were detectable on all unmyelinated areas of the nerve terminal plasma membrane in treated samples, as previously observed in tissue preincubated with azide (1). Since both azide and dinitrophenol inhibit the production of ATP within the cell, the internalization of ^{125}I-labeled toxin appears to be an energy-requiring process.

The effect of low temperature on this energy-dependent uptake process was studied by exposing mouse hemidi-

Table II. Effects of Various Agents on the Binding and Internalization of ^{125}I-BoNT at the Murine Motor Nerve Terminal

Treatment	Relative number of grains per micrometer plasma membrane*	Distribution of grains with respect to the plasma membrane‡ % of total	
		On	*Within*
	%		
11 nM ^{125}I-BoNT			
22°C	100	60	40
4°C	30	100	0
10 nM ^{125}I-BoNT			
Control	100	65	35
BoNT large subunit (0.5 μM)	0	–	–
Na azide (15 mM)	100	100	0
Na azide (15 mM) + large subunit of BoNT (0.5 μM)	0	–	–
11 nM ^{125}I-BoNT			
Control	100	61	39
Tetanus toxin (0.9 μM)	31	61	39
10 nM ^{125}I-BoNT			
Control	100	56	44
Tetanus toxin (1.0 μM)	41	59	41
10 nM ^{125}I-BoNT			
Control	100	61	39
Nerve stimulation	100	41	59
15 nM ^{125}I-BoNT			
Control	100	57	43
$-Ca^{++}$ (+2 mM EGTA)	106	54	46
20 nM ^{125}I-BoNT			
Control	ND	59	41
Chloroquine (50 μM)	ND	50	50
Methylamine (6 mM)	ND	73	27
Ammonium chloride (6 mM)	ND	74	26

* Using electron microscopic autoradiograms, the total number of grains (0–200) observed on the nerve terminal membrane and enclosed within it were counted in a minimum of 30 endplates from each sample. The calculated number of grains per unit length of membrane (see legend to Table I) for treated samples was expressed relative to that of control specimens in each experiment.
‡ The extent of internalization was quantified by counting separately the number of grains on the plasma membrane (i.e., within 70 nm) and within the terminal in at least 30 endplates from each sample.
ND, not determined.

aphragms to ^{125}I-BoNT at 4°C for 2 h. Under these conditions, the toxin still interacted with the nerve terminal membrane as silver grains could be detected in the resultant autoradiograms (Fig. 2 *a*). However, the extent of labeling was only 30% (determined by quantitative analysis of the results as described in Materials and Methods; see Table II) of that seen in controls incubated at room temperature (Fig. 2 *b*). The internalization of toxin was inhibited completely at 4°C, silver grains being found on the plasma membrane (or very close to it) but not inside it.

Effect of the Larger Polypeptide on the Binding and Internalization of ^{125}I-BoNT

The heavy chain (M_r 97,000), which retained only 0.1% of the toxicity of the native protein (55), was tested for its ability to compete with the binding of ^{125}I-BoNT. A 50-fold excess (0.5 μM) (lower concentrations were not tested) was effective in totally preventing the binding of ^{125}I-BoNT both in the absence (Fig. 3) and presence of Na azide. Its inhibition of the internalization of radioactivity in samples labeled with ^{125}I-BoNT in the absence of Na azide (Table II) emphasizes that toxin binding is a prerequisite for translocation into the nerve terminal. This complete inhibition of binding and internalization of toxin by the larger chain suggests that (*a*) the latter is involved directly in saturable binding to acceptors on the nerve terminal membrane and (*b*) it is responsible indirectly (and possibly also directly) for the uptake of radioactivity into the cytoplasm.

Action of Tetanus Toxin on the Binding and Internalization of ^{125}I-BoNT

The overall pharmacological effects of BoNT and tetanus toxin (both *Clostridial* neurotoxins) are qualitatively very similar, although BoNT is 1,000 times more potent at susceptible peripheral synapses (18) and some differences have been detected in their detailed action (14). A fivefold excess of tetanus toxin had no effect on the binding or uptake of ^{125}I-BoNT at motor nerve terminals (data not shown); however, a larger (80–100-fold) excess inhibited the binding by 60–70% (Table II). The distribution of ^{125}I-BoNT labeling of the motor nerve terminal in the presence of tetanus toxin was the same as that observed in control samples; for example, tetanus toxin did not interact solely with sites located away from the active zones or along the axonal plasma membrane. The ratio of membrane-bound to internalized silver grains was also unaffected by tetanus toxin, indicating that it does not interact only with a population of sites responsible for mediating toxin uptake into the cytosol. Collectively, the results suggest that tetanus toxin interacts with some of the acceptors for BoNT, either directly or indirectly; however, the significance of this is questionable as treatment of nerve–muscle preparations with the binding fragment C (at a concentration that antagonizes the action of tetanus toxin) does not prevent the blockade of transmission induced by 0.01 mM BoNT A (48).

Nerve Stimulation Increases the Uptake but not the Binding of ^{125}I-BoNT at the Motor Nerve Terminal

In this experiment, both test and control preparations were set up identically—each hemidiaphragm was attached to an electrode and immersed in a bath of Krebs-Ringer's solution/0.5% BSA containing ^{125}I-BoNT (10 nM) and gassed continuously with 95% O_2/5% CO_2 at 22°C, but only one of these was electrically stimulated. The twitch response in this preparation was abolished after 150 min (Fig. 4 *a*). After 4-mo exposure of sections cut from test and control blocks, silver grains were seen at nerve endings and on unmyelinated axons in both preparations (Fig. 4 *b*). Grains at the nerve terminal appeared on the plasma membrane and in the cytoplasm, indicating that uptake of toxin had occurred but, interestingly, the ratio of membrane-bound to internalized grains was different in unstimulated and stimulated specimens. In the former, 61% of the grains were located on the plasma membrane and 39% within the terminal (Table II). This accords very closely with the ratio obtained in the initial localization experiments (62% : 38%; see reference 1). In

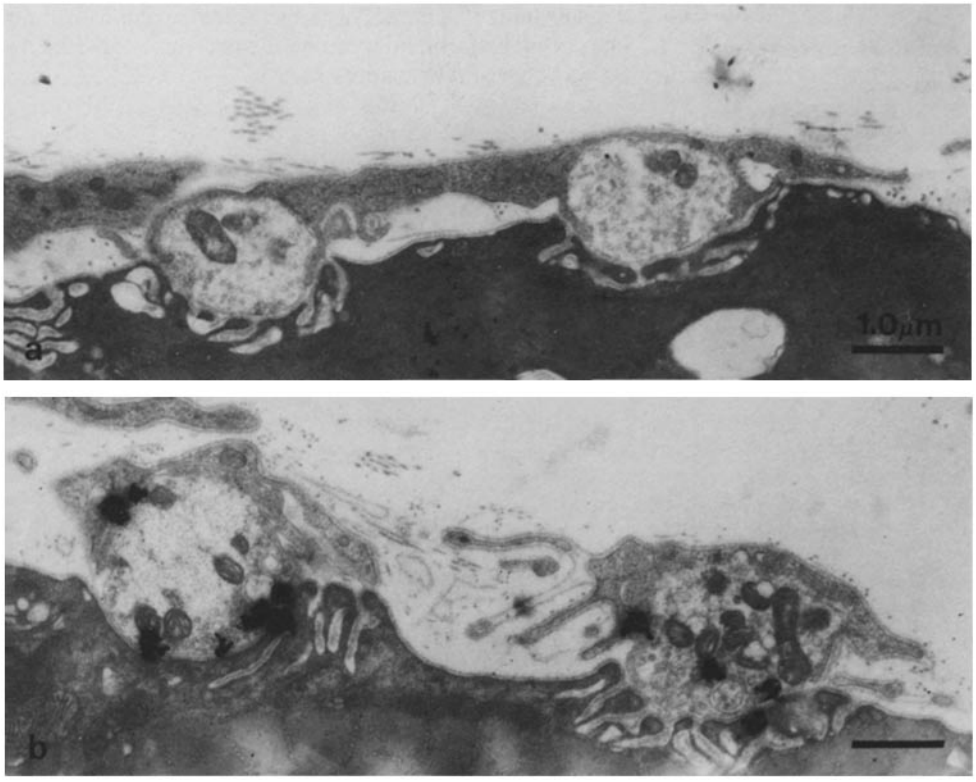

Figure 3. Inhibition of ^{125}I-BoNT binding to nerve terminals by the larger polypeptide of BoNT. Mouse diaphragm was incubated with 0.5 ml Krebs-Ringer's solution containing ^{125}I-BoNT (10 nM) and a 50-fold excess of its larger subunit (*a*). After 90 min at 22°C, the samples were washed and fixed in 2% glutaraldehyde. Control sections were treated similarly but in the absence of the large subunit of the toxin (*b*). Autoradiograms were prepared as before.

the stimulated preparation, however, 41% of the silver grains were on the membrane and 59% in the nerve terminal cytoplasm; a 50% increase in the relative uptake of radioactivity, therefore, became apparent upon stimulation although the total number of grains per micrometer of membrane remained unchanged (Table II).

Effect of Ca^{++} Deprivation and of Lysosomotropic Agents on the Uptake of ^{125}I-BoNT into the Nerve Terminal

All of the data presented, together with pharmacological findings (45–47), suggest that acceptor-mediated endocytosis is responsible for toxin internalization. To obtain further evidence for this, the effects of incubation conditions and drugs known to alter this process were studied. Ca^{++} deprivation has been shown, in a number of cases, to prevent certain steps in the endocytic pathway (31). Likewise, lysosomotropic agents such as chloroquine, ammonium chloride, and methylamine interfere with this process by raising the intravacuolar pH of lysosomes and other acidic compartments, thus preventing ligand–acceptor dissociation and/or lysosomal degradation (9, 10, 24). These agents are known to inhibit the action of diphtheria toxin (24), modeccin (41), certain viruses (19), and many other substances that act intracellularly. Most importantly, Ca^{++} deprivation or lysosomotropic agents are known to delay the onset of BoNT-induced blockade of neurotransmission in phrenic nerve–hemidiaphragm preparations (44, 46, 47) and to maintain the toxin at an unidentified site, where it is sensitive to antitoxin antibodies.

Ca^{++} Deprivation

Nerve–muscle preparations were exposed to ^{125}I-BoNT in Krebs-Ringer's solution or the latter lacking Ca^{++} and containing 2 mM EGTA; quantitative analysis of the binding of labeled toxin to the nerve terminal was then carried out at the ultrastructural level. The number of silver grains observed at endplates was unaltered by removal of Ca^{++} (Table II). This suggests that the protective effect of Ca^{++} deprivation on the time course of toxin-induced neuroparalysis cannot be attributed to inhibition of the binding step. Surprisingly, the internalization step was also unaffected in preparations incubated in Ca^{++}-free medium, the proportion of cytoplasmic silver grains being the same in test and control preparations (Table II; Fig. 5, *a* and *b*).

Effect of Lysosomotropic Agents

Mouse diaphragm preparations were treated with chloroquine, ammonium chloride, or methylamine at concentrations known to interfere with the toxin's neuroparalytic effects (46, 47), followed by the addition of ^{125}I-BoNT. Qualitative analysis of the resultant autoradiograms suggested that binding of toxin to the terminal membrane was unaffected by these agents. Although internalization of labeled toxin was not prevented fully by any of these drugs, changes in the ratio of membrane-associated to internalized grains were apparent (Table II). In chloroquine-treated samples, there were slightly more (20%) internalized grains relative to control untreated preparations; the grains were quite often associated with vacuole-like structures. In the presence of a higher concentration (2 mM) of chloroquine (a level at which the drug itself causes neuromuscular blockade; [46]), extensive vacuolation was detected in the cytoplasm of the nerve endings (data not shown). Under the latter conditions, it could be said (within the limitations in resolution of the autoradiographic technique) that the internalized silver grains were associated almost exclusively with vesicular structures; areas devoid of vacuoles were unlabeled.

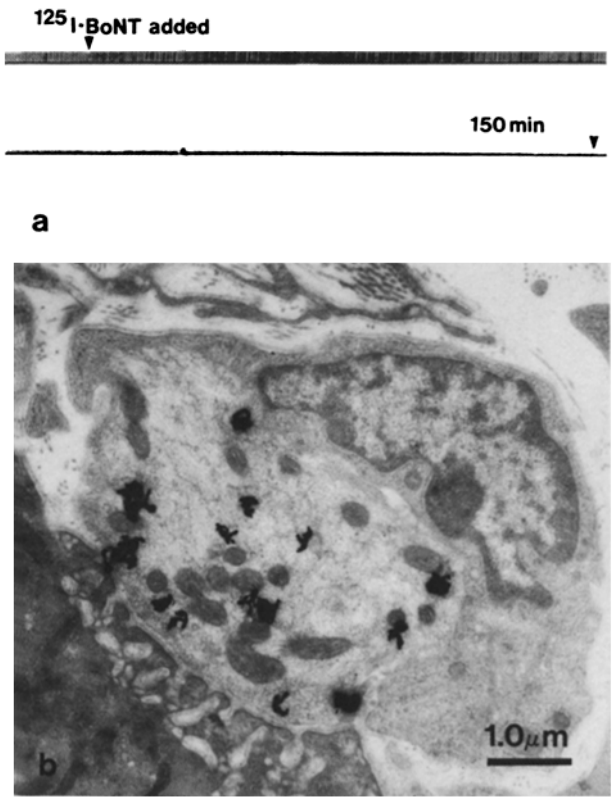

Figure 4. Measurement of the effect of nerve stimulation on the uptake of ^{125}I-BoNT into the nerve terminal. A mouse hemidiaphragm was attached to an electrode and immersed in 8 ml Krebs-Ringer's solution/0.5% BSA, gassed gently with 95% O_2/5%CO_2 at 22°C. It was stimulated electrically at 0.25 Hz for 15 min before the addition of ^{125}I-BoNT to a final concentration of 10 nM. When complete blockade of neurotransmission was observed, the hemidiaphragm was washed thoroughly with Krebs-Ringer's solution/BSA at 4°C, fixed, and processed for electron microscopic autoradiography. A control preparation was treated similarly but was not stimulated. Slides were exposed for 4 mo at 4°C. (*a*) Trace showing muscle contraction in response to nerve stimulation (see reference 44) in the presence of ^{125}I-BoNT. (*b*) Autoradiogram showing the effect of stimulation on the distribution of ^{125}I-BoNT molecules at the motor nerve terminal. Silver grains are seen on the nerve terminal membrane and in the cytoplasm but the proportion of internalized radioactivity is greater than in controls (by 50%, see Table II).

In the presence of ammonium chloride, however, the proportion of membrane-associated to internalized silver grains was 74%:26%; thus, relative to control preparations, uptake of radioactivity was reduced by ~35% (Table II). Under these conditions, vacuolation was extensive in the terminal cytoplasm and the majority of internalized silver grains were associated with vesicular structures. Similar results were obtained in samples treated with methylamine (Table II); the extent of uptake was reduced by ~33% relative to controls (only 27% of the grains associated with the nerve terminal were in the cytoplasm). As seen in ammonium-chloride–treated samples, many internalized grains were associated with endocytic vesicles. This inhibitory action of the short chain amines (ammonium chloride and methylamine) on the internalization of BoNT accords with their known ability to prevent the endocytic uptake of α_2-macroglobulin and epidermal growth factor into target cells (28); however, the incomplete inhibition with the toxin may indicate limited uptake of toxin by an additional route that would be less likely to cause neuroparalysis.

Discussion

Autoradiograms of motor nerve endings treated with ^{125}I-BoNT show that toxin molecules, or fragments thereof, are internalized; silver grains can be observed both on the membrane and within the terminal (1). The importance of this uptake is emphasized by a number of studies in which it was shown that (*a*) there is a latent period between irreversible binding of toxin and onset of paralysis (4); (*b*) paralysis occurs more rapidly when nerves are stimulated frequently (21), a treatment that also increases toxin uptake (Table II); (*c*) antitoxin antibodies can inactivate toxin bound to the nerve membrane but cannot prevent paralysis subsequent to this stage of the intoxication process (43). Collectively, these findings are in accordance with the possibility that toxin internalization is a prerequisite for its blockade of neurotransmitter release. Nevertheless, it must be realized that, until the neurotoxic effects of BoNT can be demonstrated in a cell-free system, it cannot be stated conclusively that the target site for its action is located in the cytosol.

Using electron microscopic autoradiography, it has been shown directly for the first time that ^{125}I-BoNT type A is taken up into the nerve terminal by an acceptor-mediated process. Treatment of samples with an excess of the native neurotoxin (11, 12), or of its larger subunit, prevented the binding of radiolabeled BoNT and no grains were then detected within the nerve terminal cytoplasm. Thus bulk pinocytosis, which is nonsaturable (49), can be ruled out as the main mechanism of toxin entry. It would seem, therefore, that the binding step, demonstrated here to be mediated by the heavier chain of BoNT, is essential for translocation. This is also the case with other bacterial and plant toxins which act intracellularly. For example, the binding of diphtheria toxin to its acceptor is necessary for expression of its cytotoxicity; insensitive cell lines lack the acceptor (3). Likewise, binding to carbohydrates containing galactose residues is required for the action of abrin and ricin as cells can be partially protected by the addition of galactose to the incubation medium (32).

The findings described in this paper also suggest that endocytosis is involved in the acceptor-mediated uptake of BoNT at motor nerve terminals. Firstly, as characteristic of this process (33), the internalization step for BoNT is energy- and temperature-dependent. Thus, it is unlikely that the toxin enters the cell by a mechanism dependent solely on the molecule itself; the requirement of an intact plasma membrane points to the involvement of a normally occurring cellular process. In addition, the structures used in acceptor-mediated endocytosis, i.e., coated pits, coated vesicles, endosomes, and prelysosomes (33), are all present in nerve endings (Fig. 6). Moreover, silver grains in autoradiograms of nerve endings labeled with ^{125}I-BoNT were often associated with the membrane of endocytic structures ([1]; Fig. 6 *d*), lending further support to the above proposal. Another important characteristic of endocytosis at the nerve terminal is portrayed in the uptake of BoNT; nerve stimulation results in accelerated endocytosis (5) and this could explain the increased uptake (by 50%) of toxin molecules

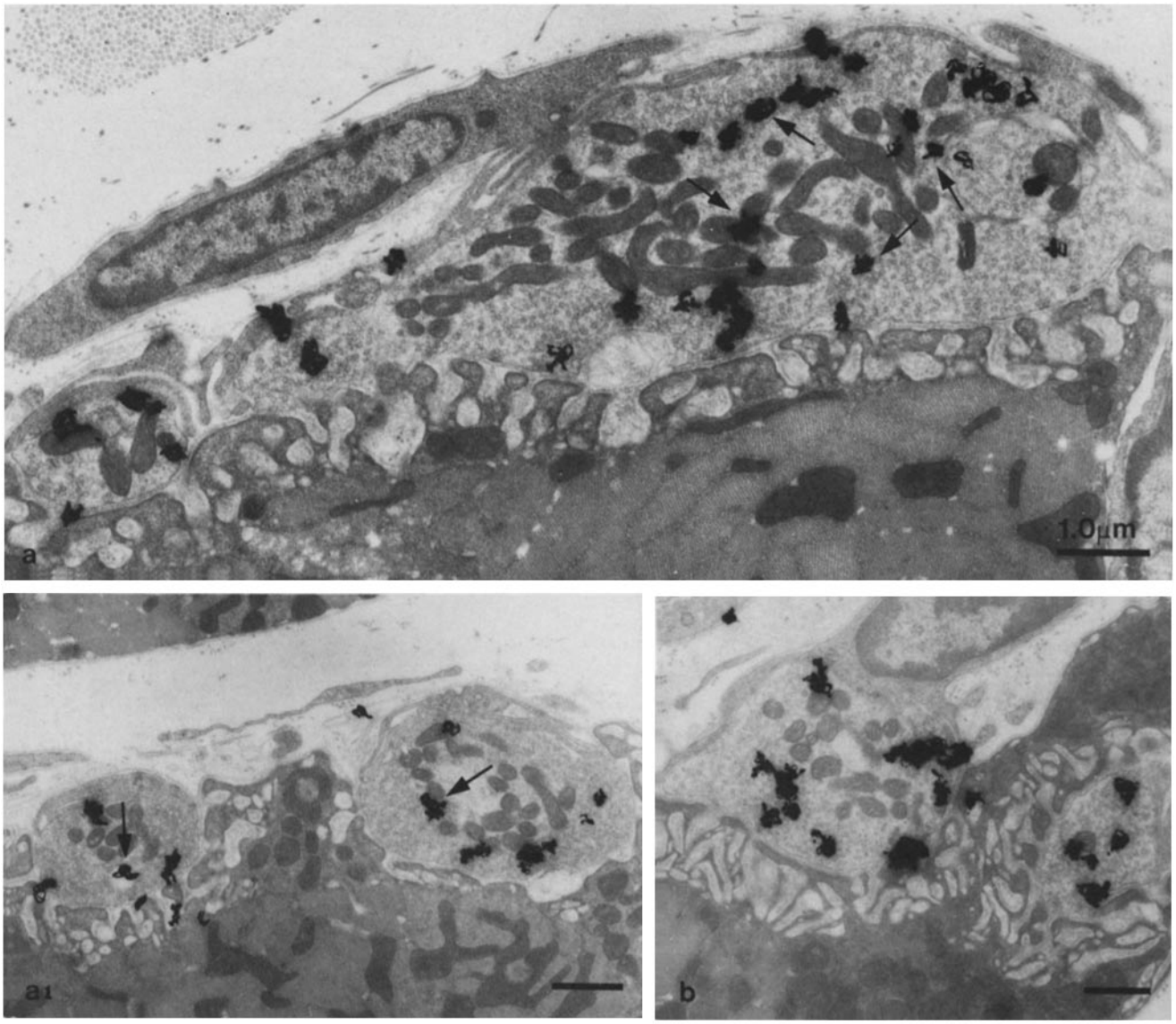

Figure 5. Effect of Ca^{++} deprivation on the neural internalization of ^{125}I-BoNT. (*a* and a_1) A piece of mouse diaphragm was preincubated in Ca^{++}-free Krebs-Ringer's solution containing 2 mM EGTA for 15 min at 22°C. This was followed by incubation in 0.5 ml of the same buffer containing 15 nM ^{125}I-BoNT for 90 min at 22°C. Extensive washing was carried out using Ca^{++}-free Krebs-Ringer's solution before fixation in 2% glutaraldehyde and preparation of the tissue for electron microscopic autoradiography. *Arrows*, intraterminal silver grains. (*b*) Control samples were treated in the same way except that normal Ringer's solution was used.

bound to the presynaptic membrane after such treatment. Furthermore, this accords with the evidence that intoxication by BoNT occurs more rapidly on stimulating the nerve (21). These collective findings emphasize the likelihood of an intracellular target for BoNT, as well as support the involvement of acceptor-mediated endocytosis. In this process, the acceptors serve to selectively concentrate ligands present in the extracellular fluid (even if they occur in very low amounts) on the plasma membrane so that efficient uptake takes place compared with bulk fluid pinocytosis (8). Hence, the presence of specific acceptors for BoNT at the nerve terminal, at relatively high content (1), could enable adequate amounts of the toxin to be internalized, even with the very low concentrations (10^{-11}M) known to be effective in blocking neuromuscular transmission (44).

Lysosomotropic agents, known to affect the acidic compartments (i.e., endosomes, lysosomes) of the endocytic pathway, antagonize the actions of numerous protein toxins (16, 24, 31). Interestingly, in cases such as that of cholera toxin where endocytosis is apparently not involved, these drugs (e.g., chloroquine) do not affect toxin action (see reference 31). Further evidence for an involvement of endocytic vesicles of low pH in the internalization process is given by the report that, under artificial conditions in which the extracellular medium is made acidic, both diphtheria toxin (38, 39) and certain viruses (e.g., Semliki Forest virus [19, 25] and influenza virus [26]) enter target cells directly from the cell surface. It is envisaged that under normal conditions, these toxins and viruses gain access to their cytoplasmic targets (without degradation in lysosomes) by abandoning the endocytic route at the endosome, which has a low pH but few or no degradation enzymes. Such avoidance of lysosomal

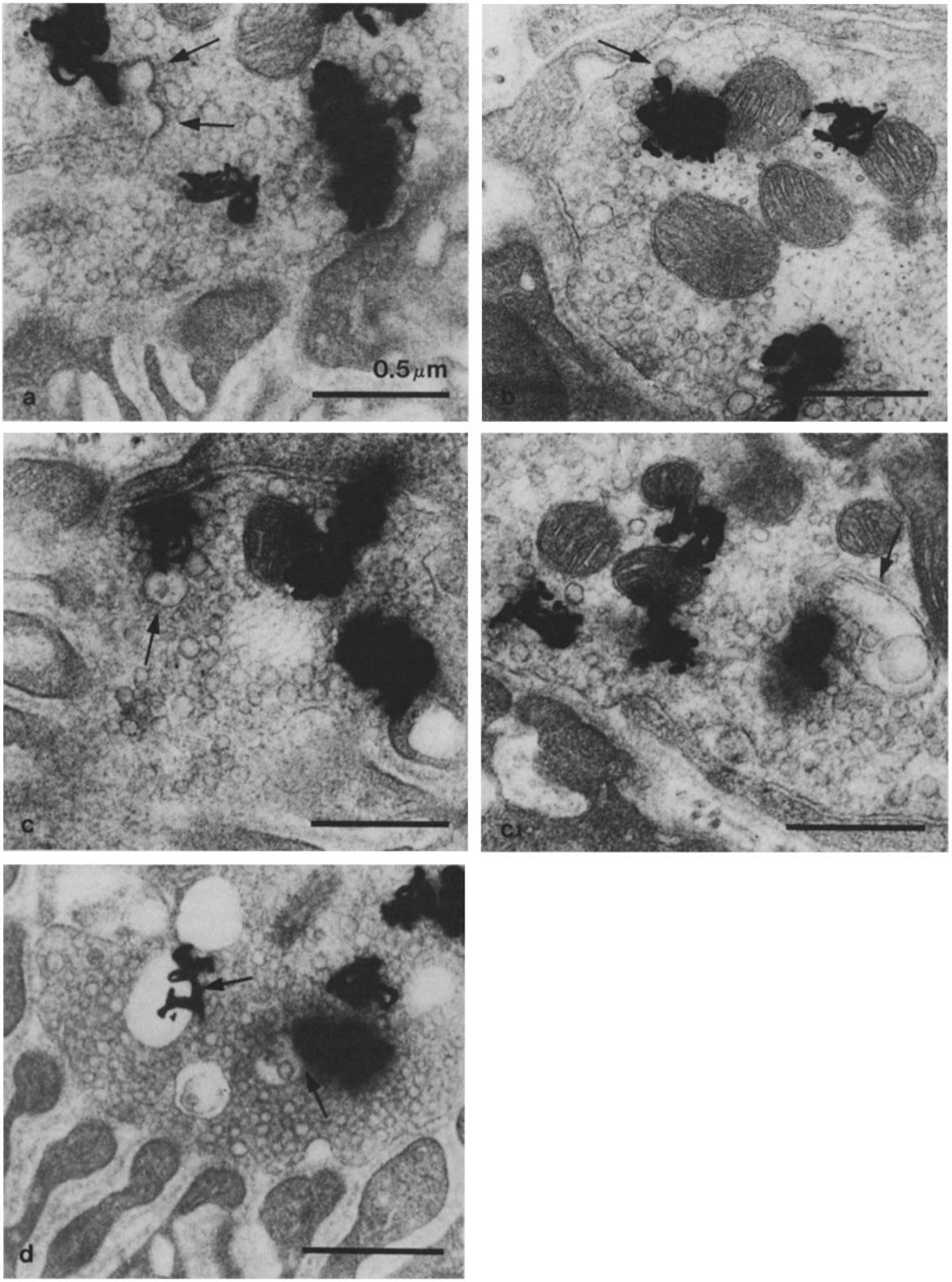

Figure 6. Structures of the motor nerve terminal involved in the process of acceptor-mediated endocytosis. Coated pits (*a*), coated vesicles (*b*), endosomes (*c*), and prelysosomes (c_1) are present (*arrows*) in these cholinergic nerve terminals showing that acceptor-mediated endocytosis can take place therein. (*d*) Nerve endings from a hemidiaphragm preparation treated with ^{125}I-BoNT in which silver grains can be seen associated with the membrane of intracellular vesicles (*arrows*).

processing by the biologically significant molecules is supported by reports that both BoNT (50) and diphtheria toxin (6) must be nicked, before application, if maximum toxicity is to be expressed. Additionally, in the case of Sendai virus, the fusion protein must be proteolytically cleaved to be active. The rich supply of proteolytic enzymes in lysosomes would lead one to expect that, if BoNT entry involved the lysosomal compartment, both nicked and unnicked forms of the molecule would be equally potent (31).

Based on this postulated endocytic route for BoNT, the antagonistic effects of toxin-induced neuromuscular paralysis by the lysosomotropic agents chloroquine, ammonium chloride, and methylamine can now be explained. In the presence of chloroquine, BoNT remains at an antitoxin sensitive site (46). Autoradiograms of motor nerve endings incubated with ^{125}I-labeled toxin in the presence of chloroquine showed that, under these conditions, the toxin still binds to the membrane and is subsequently internalized. Silver grains within the terminal were associated almost exclusively with vacuolar structures; therefore, to allow the antibodies to achieve their protective action they must have had access to the vesicular pool with which the toxin was associated. This could occur in two ways: antibodies could reach the same vesicles from the extracellular medium (17) and/or the toxin could be re-exposed to the surface of the cell (29). Given the known effects of chloroquine in other systems, the second scheme seems more likely (15). A possible explanation of events in the presence of the drug could, thus, be the following: the toxin still binds to extracellular acceptors and is taken up into endosomes. Because of the rise in the endosomal pH due to the presence of chloroquine, BoNT does not undergo the conformational change postulated to be required for entry into the cytoplasm. The acceptor–toxin complex is, therefore, returned to the cell surface intact where it is inactivated by antitoxin; this is based on the fact that the failure of a ligand to detach from its acceptor in the endosomal compartment may result in its return to the cell surface with recycling membrane patches (42). Recycling of the BoNT acceptor was suggested by the presence of more silver grains in nerve endings exposed to ^{125}I-BoNT (at least the higher concentrations) in the absence than in the presence of metabolic inhibitors (and thus inhibitors of the energy-requiring recycling process) (see Table I). The short chain amines, ammonium chloride and methylamine, also maintain BoNT molecules at an antitoxin-sensitive site (47). In addition to raising endosomal/lysosomal pH and thus preventing ligand–acceptor dissociation and lysosomal processing (10), these drugs are thought to prevent fusion of endosomes with lysosomes, possibly explaining the net increase in the proportion of grains seen on the plasma membrane. Collectively, the effects of lysosomotropic agents on the action of botulinum toxin (46, 47) as well as on the distribution of molecules at the nerve terminal, together with their known actions in other systems, provide strong evidence for the involvement of acceptor-mediated endocytosis in the uptake of BoNT.

Autoradiograms of nerve terminals treated with ^{125}I-BoNT in the absence of Ca^{++} showed that neither binding nor internalization is affected, despite pharmacological studies (44) having shown that under these conditions toxin remains accessible to antibodies. The lectins abrin and modeccin do not inhibit protein synthesis in the absence of Ca^{++} and ricin is less effective under these conditions (40). The protective effect of Ca^{++} deprivation in these cases is not due to reduced toxin binding to the cell membrane or to inhibition of internalization. These results are compatible with the suggestion (31) that Ca^{++} efflux from intracellular compartments is required for toxin transport to the cytoplasm. Similarly, the lack of BoNT toxicity in the absence of Ca^{++} (44) could be attributed to its inability to enter the cytosol from the endosome. Williams (54) has shown that the extent of ^{125}I-BoNT binding to synaptosomes is not appreciably different between pH 5 and 7; toxin could thus remain bound to its acceptors within the acidic endosomes and be returned to the cell surface to be inactivated by antitoxin.

The data presented here and elsewhere suggest strongly that acceptor-mediated endocytosis is involved in the uptake of BoNT into the nerve terminal. This proposal is viable in so far as the structures involved in this process are found at motor nerve terminals. Moreover, evidence that the toxin is taken up into an acidic compartment, from which it may gain access to the cytosol, is apparent from the demonstrated ability of lysosomotropic agents to perturb the internalization and to delay the onset of BoNT-induced blockade of neurotransmission (46). The need for nicking of BoNT in the expression of toxicity provides indirect evidence that it enters the cytoplasm from the endosome rather than a lysosome. In the case of other toxins and viruses, the low pH in the endosome is believed to induce a conformational change in the protein (exposing hydrophobic regions) that results in transfer of an active fragment into the cytosol. It is interesting that, under these conditions, the heavier subunit of BoNT forms 1.8-nm channels in asolectin membranes (20) when a positive potential is applied across the membrane and the pH is low (~4.5) in the toxin-containing chamber.

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