INTRODUCTION

Daily treatment of rats with the disulfide reducing agent 2,4-dithiobiuret (H₂N-CS-N-CS-NH₂, DTB) causes a delayed-onset and ascending neuromuscular weakness (Atchison and Peterson 1981; Atchison et al. 1981a,b, 1982). The precise mechanism underlying this effect is still unknown, although it is clear that disruptions of presynaptic processes resulting in a decrease of acetylcholine (ACh) release are involved (Atchison 1989; Weiler et al. 1986). Both Ca²⁺-dependent and -independent release of ACh (Atchison 1989; Weiler et al. 1986), as well as frequency-dependent facilitation, augmentation, and potentiation, are impaired in DTB-treated motor nerve terminals (Xu and Atchison 1996b). At the time of hindlimb muscle weakness, diaphragmatic neuromuscular transmission remains intact but is more susceptible to failures of transmission in the presence of diminished extracellular Ca²⁺ (Atchison 1990), suggesting that even in asymptomatic tissues subtle and as yet undetectable changes are occurring in the release apparatus. Acute application of DTB to isolated neuromuscular preparations causes some but not all of the electrophysiological signs associated with the muscle paresis, indicating that DTB itself, rather than some toxic metabolite, is responsible for the ultimate effects on ACh release and that some degree of “functional reserve” remains in the nerve terminal target(s) associated with DTB-induced muscle weakness and requires chronic treatment with the toxicant to deplete (Spitsbergen and Atchison 1990). Moreover, acute treatment of PC12 cells with DTB reduces ACh release subsequent to entry of Ca²⁺ during depolarization and does not involve an effect on Ca²⁺ buffering (Ireland et al. 1995). Ultrastructural examinations suggest that DTB alters the number of synaptic vesicles and causes the presence of abnormal tubulovesicular structures in rat motor nerve terminals (Jones 1989; Rheuben et al. 1998; Sahenk 1990). Therefore it has been proposed that the effects of DTB include a disruption of vesicle trafficking and/or exocytosis in motor nerve terminals. The processes underlying vesicular release of neurotransmitter are still not well defined, and numerous cellular reactions no doubt contribute to mobilization of vesicles from “reserve” to “active” status, docking, priming and exocytosis, and subsequent membrane recycling. Several chemicals such as the Clostridial toxins, α-latrotoxin, and vesamicol (Humeau et al. 2002) alter vesicle recycling and exocytosis, indicating that there may be defects in either or both exocytosis and internal vesicular processing.
been useful in elucidating events underlying this process. Thus agents such as DTB which disrupt the cholinergic exocytotic process may be valuable tools in understanding the steps involved in ACh release. Furthermore, several poorly understood human neurological disorders present with clinical signs similar to those seen during DTB-induced muscle paresis. These include several congenital myasthenic syndromes (Engel and Ohno 2002a, b), botulism (Sellin 1981, 1984), and neuroaxonal dystrophy (de Leon and Mitchell 1985; Kimura et al. 1987). As such, understanding the mechanisms responsible for the muscle weakness induced by DTB may also provide clues into mechanisms associated with these neurological disorders.

Exocytosis and synaptic vesicle recycling has been studied in a number of preparations by use of the styryl fluorescent dye, FM1-43 (Betz and Bewick 1993; Betz et al. 1992b; Kuromi and Kidokoro 1998; Ribchester et al. 1994; Ryan et al. 1993). Current hypotheses suggest that FM1-43 can be taken up into the nerve terminal by one or more endocytotic mechanisms during activity (Pyle et al. 2000; Richards et al. 2000; Südhof 2000; Teng and Wilkinson 2000) and released by subsequent vesicular exocytosis. This methodology has unique advantages over more conventional electrophysiological assays, as it allows the characterization of several subcellular processes involved in vesicle recycling such as the size of recycling vesicle pools, the kinetics of endocytosis, and the organelles involved, the course of vesicle repriming, and vesicle movement (Betz and Henkel 1994; Ryan et al. 1993, 1996a,b; Wu and Betz 1996). In this report, we used uptake of FM1-43 and horseradish peroxidase (HRP) in combination with conventional microelectrode recording to test directly whether the mobilization of synaptic vesicles with subsequent exocytosis and endocytosis is impaired in motor axon terminals of DTB-treated mice and, if so, to attempt to identify at what point or points the effect occurs. Additionally, we sought to determine if HRP accumulates in the aberrant tubulovesicular structures seen in DTB-treated terminals (Jones 1989; Kempley 1984; Rheuben et al. 1998) in hopes of determining if these unusual structures are directly involved in an abortive vesicular recycling process.

Preliminary reports of parts of this study were presented at the 28th Annual Meeting of the Society for Neuroscience, Los Angeles, CA (Xu and Atchison 1998).

**Methods**

**Chemicals**

Purified, recrystallized DTB was obtained from Ash Stevens (Detroit, MI). FM1-43 was obtained from Molecular Probes (Eugene, OR) and stored frozen in distilled H2O (1 mg/ml). Purified α-latrotoxin (α-LTx) was obtained from Alomone Labs (Jerusalem, Israel). α-tubocurarine and all other reagents were obtained from Sigma Chemical (St. Louis, MO).

**Preparations and solutions**

Experiments were performed using the isolated triangularis sterni (TS) muscle from untreated (normal) and DTB-treated ICR male mice (20–25 g, Harlan Sprague-Dawley Laboratories, Madison, WI), which were killed by cervical dislocation followed by exsanguination. This preparation was chosen because its thin size allows ready access of reagents to, and visualization of, the motor axon terminals (McArde et al. 1981). DTB, dissolved in 0.9% NaCl (wt/vol) to a concentration of 1 mg/ml, was injected (intraperitoneally) into mice at a dose of 15–20 mg · kg⁻¹ · day⁻¹ for 5–7 days. This results in development of muscle weakness that is qualitatively similar to that occurring in rats treated with a dose of 1 mg · kg⁻¹ · day⁻¹ for 6 days (Atchison and Peterson 1981). This weakness is most noticeable in the hindlimbs but also affects other muscles. No attempt was made to characterize further the constellation of effects that occur at this point of DTB intoxication in mice as the syndrome has already been well described in rats (Atchison and Peterson 1981; Atchison et al. 1981a,b). The TS muscles with their intercostal nerve were isolated and superfused in a recording chamber with physiological saline solution (1–3 ml/min) that had the following composition (mM): 135 NaCl, 5 KCl, 2 CaCl2, 1 MgCl2, 12 NaHCO3, 1 NaH2PO4, and 11 d-glucose (Xu and Atchison 1996a). Solutions were aerated with 95% O2, 5% CO2, giving a pH of 7.3–7.4 at room temperature (25–27°C). Muscle contractions evoked by intercostal nerve stimulation were prevented by perfusing the preparation with 1–3 μM α-tubocurarine.

**Fluorescence determination**

Preparations were incubated for 1–2 min in 8 μM FM1-43 diluted in physiological saline from a stock solution of 1 mg/ml (1.6 mM) in distilled water. The nerve was then stimulated with trains of pulses for 7 min. Typically, stimulus frequency within the train was 30 Hz, train duration was 10 s, and interval between trains was 20 s. A similar nerve stimulation protocol was used to examine the kinetics of destaining. For staining with KCl-induced depolarization, the preparations were incubated with solution containing 30 mM KCl for 1–2 min, and then bathed in this high [KCl] solution with 8 μM FM1-43 for 5 min.

Two types of experiments were performed using α-LTxs to either load or unload FM1-43. The first is similar to that described initially by Henkel and Betz (1995). FM1-43 loading involved preincubation of the preparation in dye-free saline in the presence of 2 μg/ml of α-LTx for 15–30 min, by which time some of the muscle fibers began to twitch as determined by visual inspection. The solution was then changed within seconds to one containing FM1-43 and α-LTx (2 μg/ml) for 5 min. After removal of the FM1-43, preparations were subsequently washed with α-LTx-free physiological saline for 30 min. In the second type of experiment, the terminals were loaded with FM1-43 using the nerve stimulation protocol described in the preceding text. Destaining was then induced by treatment of the muscle for 60 min with 2 μg/ml of α-LTx in the absence of FM1-43, and residual fluorescence was measured. α-LTx was made in a stock solution of 40 μg/ml in 50% distilled water and 50% glycerol (vol/vol). Working solutions were diluted with physiological saline from this stock solution.

In all experiments, preparations were washed for 30 min with normal physiological saline after loading with FM1-43 and viewed using a Nikon upright epifluorescence microscope equipped with a ×40 water-immersion objective (Nikon Optics, Tokyo), a 100 W Hg lamp, and 0.7–100% neutral density transmission filter. For FM1-43, the excitation filter was set at 420–490 nm, dichroic mirror set at 505 nm, and emission was at 520 nm. Images were captured with a Sony color digital camera (Cooke, Tonawanda, NY) and processed on a microcomputer using Image Pro-Plus software (Media Cybernetics, Silver Spring MD). To reduce the possibility of photobleaching and phototoxicity, illumination was kept at a minimum. Images were acquired with a 300- to 1,200-ms exposure, usually with 2–5% excitation light transmittance.

For each preparation, five to seven surface nerve terminals were selected by eye for quantitation of fluorescence intensity of staining. Time-lapse sequences for destaining were usually recorded at rates of 2/s to 5/s. When capturing images for destaining, the best focus position was adjusted manually at every 40–500 s to ensure that it was maintained throughout the experiment. Due to the difference in exposure time for capturing images or small changes of light intensity.
produced by the solution level, the intensity of the respective backgrounds differs. Consequently we used the brightness control in the “contrast enhancement” dialog box of Image Pro-Plus to decrease all pixel values of the area of interest of the image until the background was adjusted to a constant level of 8–10 pixels. After subtraction of the intensity of each background, fluorescence images were aligned, and the outline of each selected terminal, area, or spot was marked, and average fluorescence intensity of all pixels inside the outline calculated. In some cases, areas of interest were enlarged (×2 or ×3) and realigned.

Electrophysiological measurements

End-plate potentials (EPPs) were recorded intracellularly using conventional microelectrode recording techniques and borosilicate glass tubing (1.0 mm ID; W. P. Instruments, Sarasota, FL). The electrodes had impedances of 5–15 MΩ when filled with 3 mM KCl. EPPs were amplified (MT70, WP Instruments), displayed on a storage oscilloscope (Model 4090, Nicolet Instruments, Verona, WI), and recorded to computer using the software program SCAN kindly provided by Dr. John Dempster (University of Strathclyde, Scotland). Suprathreshold electrical stimuli (duration of 30 s) were applied to an intercostal nerve trunk using a stimulator (S88, Grass Medical Instruments, Quincy, MA) with stimulus isolation unit (SIU, Grass Medical Instruments) connected to a glass suction electrode filled with physiological saline. The muscle fiber innervated by the selected terminal was impaled with a microelectrode, and, after a series of recorded control EPPs at a frequency of 0.5 Hz, the nerve was stimulated continuously at a constant frequency of 15 Hz for 1–2 min.

Vesicle labeling with HRP

For HRP labeling, DTB treatment and TS dissection were identical to those described in the preceding text. The TS muscle is divisible into three regions, each having a separate innervating intercostal nerve (see McCarril et al. 1981). One of the three nerves was selected for stimulation with the muscle fields belonging to the other two nerves serving as intrinsic unstained controls. The intercostal nerve was stimulated at 50 Hz for 7 min in the preceding-described mouse physiological saline (including δ-tubocurarine, as for the physiological experiments) containing dialyzed 1.5% Type VI HRP (wt/vol, Sigma) while monitoring the EPPs. The tissue was incubated in HRP solution for 15 min, then rinsed in physiological saline for 10 min before beginning fixation.

The muscle was fixed while pinned by perfusion of 2.5% glutaraldehyde (vol/vol) and 0.25% paraformaldehyde in 0.1 M sodium cacodylate buffer for 30 min and then removed to a beaker containing a large volume of fresh fixative (King et al. 1996). After 2 h, the tissue was rinsed in 0.1 M cacodylate buffer for 40 min with four changes. At this time, small pieces of muscle were dissected from each of the stimulated and unstained regions of the TS muscle. The tissue was soaked in 0.5% cobalt chloride (wt/vol) for 15 min and rinsed briefly with warmed cacodylate buffer. Muscle fibers were then incubated for 60 min at 37°C in a solution of 10 mg 3,3′-diaminobenzidine·4 HCl (DAB), 40 mg D-glucose, 8 mg ammonium chloride, and 0.14 mg glucose oxidase in 20 ml of 0.1 M cacodylate buffer (Itoh et al. 1979).

After incubation, the tissue was rinsed in 0.1 M cacodylate buffer for 40 min and post fixed in 2% osmium tetroxide for 2 h. Selected muscles were block-stained with 1% uranyl acetate (wt/vol). All tissue was dehydrated in a graded series of ethanol and embedded in Araldite resin according to routine methods.

Thin sections (75–90 nm) were cut and collected on copper Formvar-coated slot or uncoated mesh grids and examined with a JEOL 100CXII (Tokyo) transmission electron microscope at 80 kV. Sections were stained with methanolic uranyl acetate or Van Wile uranyl acetate, followed by lead citrate, or left unstained.

Individual terminals and boutons were identified in the blocks and followed for short distances with serial sections before removing several micrometers of the block face and going on to another part of the same junction or to different junctions in the muscle. Terminal profiles were photographed in their entirety at ×29,000 and enlarged to ×64,000. All synaptic vesicles, coated vesicles, and other vesicular structures in a given profile were counted. Structures with diameters up to 135 nm were included in the “vesicle” population because DTB produces an increase in the variability of vesicle dimensions such that the larger vesicles cannot be distinguished from small endosomes (Jones 1989; Rheuben et al. 1998) Vesicular structures were identified as “labeled” if any of the following features were present: entire lumen filled with dark precipitate, dark precipitate around the edges of the vesicle, or precipitate that only adhered to part of the vesicle. In most cases, unstained sections were photographed to facilitate identification of the HRP-DAB reaction product. Stimulated and unstained regions of the muscle were sampled from three untreated and three treated animals.

Statistical analysis

Data from FM1-43 staining experiments and the ultrastructural counts of synaptic vesicles were analyzed using Student’s unpaired t-test (Steel and Torrie 1960). Data from destaining experiments were analyzed by mixed design ANOVA followed by Student’s t-test for paired samples. Differences were considered significant at P < 0.01 for all experiments. Measurements are expressed as means ± SE of separate nerve terminals from 6 to 12 preparations.

RESULTS

Nerve terminal morphology as characterized by FM 1-43 staining in untreated and DTB-treated mice

When mouse motor nerve terminals were stimulated intermittently at 30 Hz for 7 min in the presence of 8 μM FM1-43, significant uptake of dye occurred. After washing the preparation for 30 min with dye-free physiological saline, stained nerve terminals became visible and bright (Fig. 1A). Labeled terminals in untreated mice lacked the discrete, punctate spots as described in frog (Betz et al. 1992b) and Drosophila motor nerve terminals (Kuromi and Kidokoro 1998, 1999). Rather, the fluorescence generally appeared uniform, although the terminals occasionally contained regions that were somewhat more brightly fluorescent than others (Fig. 1A, left). Unstimulated terminals incubated in FM1-43 for comparable periods of time exhibited little staining (results not shown).

In mice treated with DTB for 5 days, labeled terminals appeared swollen and the intensity of FM1-43 fluorescence was relatively dim and nonuniform in most terminals (referred to hereafter as dim terminals). However, occasional terminals in the same preparations exhibited an overall fluorescence intensity equivalent to that of untreated mice (bright terminals), but fluorescence intensity was more unevenly distributed than that of untreated terminals (Fig. 1A, right). In terminals from DTB-treated animals, brightly stained regions sometimes appeared to move or to destain spontaneously (Fig. 1B). There was little uniformity in staining intensity from terminal to terminal within the same preparation; that is, a mixture of “bright” and “dim” terminals were often adjacent. When mice were treated with DTB for 7–9 days, few nerve terminals would take up dye. Consequently, the study was restricted to mice that were treated for 5–6 days, which corresponds to the onset of muscle weakness.

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Size of the recycling vesicle pool is reduced in DTB-treated mice

To test the hypothesis that the size of the total synaptic vesicle pool in DTB-treated motor nerve terminals was reduced, steady-state labeling was attained by intermittent stimulation of the nerve at 30 Hz for 7 min in the presence of FM 1-43. Typically, pulse trains of 10-s duration were presented at 30 Hz every 20 s. The preparation was then exposed to dye for an additional 5 min to allow for completion of slow endocytosis. This protocol would be expected to load both the “recycling vesicle pool” and the “reserve pool.” After washing the preparation with dye-free, normal saline for 20–30 min, the brightness of stained nerve terminals was maintained for several hours, and the average brightness of several terminals could be measured. Figure 2A shows that the average fluorescence brightness for equivalent trains of action potentials was reduced in DTB-treated mice to 60% of that seen in nontreated mice. The overall histogram distribution of fluorescence intensities in DTB-treated mice was shifted to lower values and was broader than that of control (Fig. 2B). A similar difference in FM1-43 labeling between untreated and DTB-treated motor nerve terminals was seen when depolarization was induced by 60 mM KCl. The average fluorescence of DTB-treated terminals was likewise 50–60% of that in untreated mice as well (results not shown). FM1-43 labeling and destaining by KCl-induced depolarization and tetanic nerve stimulation were indistinguishable.

HRP is taken up into vesicles and cisternae but not taken up directly into tubulovesicular structures in DTB-treated terminals

Previously we found in rat neuromuscular junctions (Rheuben et al. 1998), that terminals from any single DTB-treated muscle exhibited abnormalities that included, but were not limited to, a reduction in the numbers of vesicles near active

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FIG. 1. 2,4-dithiobisurett (DTB)-induced morphological changes of mouse motor nerve terminals. A: representative images of motor nerve terminals of triangularis sterni muscles in normal and DTB-treated mice showing fluorescence staining with FM1-43 triggered by intermittent nerve stimulation. Typically, pulse trains lasting 10 s were presented at 30 Hz and repeated every 20 s for 7 min. Preparations were then washed for 20–40 min by perfusing them continuously with dye-free physiological saline. Note that nerve terminals of DTB-treated mice appear swollen, and the overall intensity of fluorescence is less and is not uniform throughout the terminals. B: in DTB-treated terminals, the nonuniform distribution of FM1-43 fluorescence could change rapidly with time, either due to movement of labeled organelles within the terminal or within the overlying Schwann cell processes, or due to the release of dye associated with spontaneous activity. Control 0 s: the preparation was incubated with 8 μM FM1–43 for 5 min and then washed for 10 min before imaging for the first time. Time-lapse sequence images were taken every 2 s thereafter to observe dye movement or spontaneous destaining in the unstimulated terminal. Changes in fluorescence distribution over an 8-s time period are evident in the circled regions.

FIG. 2. The size of the recycling vesicle pool is reduced in DTB-treated mice. A: average fluorescence levels after 30-Hz intermittent nerve stimulation for 7 min in the presence of FM1-43 in untreated (control) and DTB-treated nerve terminals. For each preparation, 5–7 surface nerve terminals were selected by eye for quantitation of fluorescence intensity. The average intensity for each terminal was normalized to the average fluorescence intensity of terminals in normal mice. Values are the means ± SE of 48 normal and 70 DTB-treated motor nerve terminals from 8 and 10 mice, respectively. The average intensity of fluorescence is reduced in DTB-treated motor nerve terminals to about 60% of normal. B: histogram of the distribution of fluorescence intensities in untreated and DTB-treated motor nerve terminals. The outline of each nerve terminal was marked automatically, and the average value of fluorescence intensity within the area was calculated. Note that the distribution of average fluorescence intensities is shifted to lower values in DTB-treated mice. However, terminals with fluorescence intensity equivalent to that of untreated controls are also seen. The values are from 9 (21632 μm²) untreated and 11 (29751 μm²) DTB-treated preparations, respectively.
zones, differences in vesicle size distributions, and the presence of tubular or densely packed vesicular structures in the central core region of the bouton. Tubulovesicular structures were also noted by Kemplay (1984) and Jones (1989) and seem to be particularly characteristic of the later stages of DTB treatment, but their origin and functional implications are not known.

The present experiments had several goals. First, we sought to determine if similar morphological changes were occurring in the mouse TS muscle at the specific time period being examined with FM1-43. Second we sought to see if the abnormal tubular or vesicular structures were actively involved in the recycling process. Third, we wished to see if we could determine an ultrastructural basis for the difference between bright and dim terminals. We used a fluid phase marker for endocytosis that could be followed ultrastructurally, HRP, and compared stimulated and unstimulated muscle regions to accomplish these goals.

Uptake of HRP was examined under a comparable stimulation regime to that used above for FM1-43 loading. The intercostal nerve was stimulated for 7 min at 50 Hz in an HRP-containing solution and then allowed to rest for 15 min before rinsing for 10 min in physiological saline, and fixing. Because HRP was not completely removed in a 10-min rinse, and only terminals clearly having HRP remaining in the extracellular space were examined, this gave a consistent 25-min time period for completion of uptake. By this regime, both vesicles involved in a rapid recycling method as well as any organelles involved in subsequent slower methods of endocytosis could have been labeled.

In untreated mouse terminals, HRP-DAB reaction product was found in synaptic vesicles, coated vesicles, endosome-like structures, and cisternae as has been seen in many other studies on synaptic vesicle recycling in vertebrate nerve terminals (cf. Heuser and Reese 1973, 1981). In terminals from untreated mice, the synaptic vesicles, coated vesicles, and endosomes are largely concentrated in a band in the peripheral part of the bouton, a “vesicle domain,” while neural filaments, mitochondria, small amounts of smooth endoplasmic reticulum, and microtubules are primarily concentrated in a central region, the “core region.”

Endocytosis of label at the time of fixation appeared to occur both at or near the active zones where “hot spots” or clusters of labeled vesicles were seen and along the surface of the terminal in apposition to the Schwann cell (Fig. 3B). Figure 3B also illustrates the uptake of HRP via a coated vesicle at the time of fixation, so it is likely that the slow method of endocytosis is responsible for at least part of the HRP-labeled structures.

After 5–6 days of DTB treatment, the terminals of mice were similar ultrastructurally to those of rats examined at the onset of muscle weakness (Jones 1989; Rheuben et al. 1998). In moderately to slightly abnormal terminals, HRP was taken up into cisternae and multivesicular bodies, some of which were found in the centers of the terminals, as well as into synaptic and coated vesicles (Fig. 3, A and B). The distinction between the vesicle domain and the core region was less clear in DTB-treated terminals, and the appearance of the core region varied greatly. In more abnormal terminals (Figs. 4 and 5), the core regions contained a high density of tubular or spherical structures of varying dimensions. In some, there was a large cluster of closely packed vesicles, whose dimensions were similar to those in the synaptic vesicle region (Fig. 5A), whereas in others, there were masses of small-diameter, tubular structures with dimensions similar to microtubules (Fig. 5B). In comparison, Jones (1989) described the presence of a quite different branched tubular complex, similar to smooth endoplasmic reticulum, in DTB-treated terminals; it was striking that this abnormality could take so many forms. Nevertheless in these severely affected terminals, HRP reaction product was not found in any of the variously shaped tubulovesicular complexes in the core region.

In a previous ultrastructural study of effects of DTB on rat neuromuscular junction (Rheuben et al. 1998), one of the earliest and most conspicuous changes observed after DTB treatment was an alteration in the morphology of the terminal Schwann cells. These cells became more sheetlike in their covering of the nerve terminals, and their processes became thicker. We found in the present study that HRP reaction product was taken up into conspicuous cisternae of the Schwann cells covering the terminals in both untreated (results not shown) and DTB-treated mice (Fig. 3B). However, the significance of this is unclear.

**Uptake of HRP into vesicles is reduced in DTB-treated terminals**

We compared the fractions of synaptic vesicles that were labeled in DTB-treated and untreated terminals in preparations in which part of the muscle received evoked stimulation (50 Hz for 7 min), and part, because of the segmental innervation pattern, did not, thus acting as an unstimulated control (Table 1). In unstimulated terminals from both untreated and DTB-treated animals, there was some uptake of HRP into vesicles during the incubation period and during fixation; approximately 14–15% of the total vesicles present were labeled. [Because of the increased variability in vesicle diameters in DTB-treated terminal, as previously noted by Jones (1989) and Rheuben et al. (1998), and the difficulty of determining whether a coat was present or absent in some HRP-labeled vesicles, we included in these counts all vesicular structures ≤135 nm in diameter. This population may thus have included small endosomes as well.] In both untreated and DTB-treated terminals, stimulation increased the fraction of vesicles that contained HRP reaction product, but the increase was much less, on average, in DTB-treated terminals—36.9% for untreated versus 23.6% for DTB-treated. $P \leq 0.01$. The average uptake of HRP into treated terminals was thus 63% of that in untreated terminals, which was similar to relative uptake as measured using FM1-43.

**Total number of vesicles is reduced only in DTB-treated terminals having tubulovesicular profiles**

We examined the terminals for a net difference in the total number of vesicles, both labeled and unlabeled, available to participate in the recycling process, including any that might be considered “reserve pool” vesicles. Because DTB-treated terminals swell (Jones 1989; Kemplay 1984; Rheuben et al. 1998), we did not calculate vesicles per square micrometer of terminal (which would automatically give a lower density of vesicles). Instead we averaged the total number of vesicles per bouton profile in random planes of section, sampling boutons
at several levels and junctions from different parts of the muscles. The average total number of vesicles per profile from all the terminal profiles observed in unstimulated muscles was similar in untreated animals, 202.9 ± 22.0/profile, n = 48, and in DTB-treated animals, 231.0 ± 26.2, n = 49 (means ± SE); and the total number per profile from stimulated muscles was 209.4 ± 16.2/profile, n = 78 from untreated animals, and 223.6 ± 22.4, n = 69 from treated animals. These data suggest both that stim-

FIG. 3. In motor nerve terminals slightly affected by DTB, stimulation for 7 min in the presence of horseradish peroxidase (HRP) induces uptake into a variety of structures. A: HRP is found in synaptic vesicles, coated vesicles (not readily distinguishable from uncoated vesicles at this magnification), endosomes, cisternae (short arrows), and multivesicular bodies (long arrow). By endosomes we mean spherical vesicular structures 2–4 times the typical diameter of a synaptic vesicle. For experiments in which the fraction of vesicles labeled was determined, we examined only terminals lying in regions of the muscle that were well exposed to HRP. In these regions, HRP diamino benzidine (DAB) reaction product was found in the extracellular space, here seen coating the collagen fibrils, surrounding the terminal and filling the subsynaptic clefts. Note that in this slightly affected terminal, the density of the organelles in the core region is fairly normal, but there are a few microtubules running at differing angles. In untreated terminals microtubules tend to be arrayed in parallel with the long axis of the terminal branch; ×35,900. B: in this lightly affected bouton, a coated vesicle can be seen forming at the time of fixation (short arrow). This portion of the membrane is facing the terminal Schwann cell. A mixture of labeled vesicles and cisternae are seen. Several unlabeled endosomes are present, one directly above the short arrow. The Schwann cell has also taken up HRP into very large vacuoles or cisternae (long arrows); ×74,000.
ulation has no effect on the average total number of vesicles observed in either treated or untreated populations, and that DTB-treatment per se does not reduce the average total number of vesicles present in the terminals at the time period examined. However, the FM1-43 studies suggested that there might be two functionally different types of terminals present in muscles at the stage of DTB intoxication that we examined: bright terminals and dim terminals, reflecting differing degrees of release and/or endocytosis. Present and previous ultrastructural (Jones 1989; Rheuben et al. 1998) as well as functional studies (Atchison 1989, 1990) indicated that there was quite a range of abnormality seen in the terminals of any given muscle with DTB treatment. Therefore we reanalyzed the data after first dividing the sample into severely affected and moderately or lightly affected on the basis of specific morphological criteria (disregarding synaptic vesicles), with the presence of tubulovesicular profiles, or disorganized or abnormal structures in the “core region” signifying a severely affected terminal.

In the subpopulation of DTB-treated terminals with tubulovesicular profiles in the core region as shown in Fig. 5, the average total number of vesicles per profile was smaller, 102 ± 16.2/profile versus 280 ± 28.4/profile for the less-affected terminals. Furthermore, the fraction of vesicles that was labeled with HRP in stimulated terminals was much lower in terminals with tubulovesicular masses in their core regions, 11.9 ± 3.1%, \( n = 22 \), compared with 29.1 ± 2.52%, \( n = 47 \) for the less-affected terminals. In this case, \( n \) represents separate boutons from the same or different terminals. The two subpopulations were significantly different from each other, both with respect to the fraction labeled and the total number of vesicles present, \( P < 0.01 \). Thus the more severely affected type of terminal as defined on ultrastructural grounds has substantially fewer synaptic vesicles present (as observed when fixed after stimulation) and undergoes less endocytosis. This type of terminal presumably corresponds to the dim terminals seen in the studies of FM1-43 uptake.
Time course of FM1-43 endocytosis is unaltered in DTB-treated motor nerve terminals

The reduction of uptake of FM1-43 and HRP after stimulation and the swelling seen in DTB-treated nerve terminals suggest that a defect of endocytosis in those terminals might ultimately give rise to a loss of vesicles, with vesicle membrane gradually being incorporated into the plasma membrane. Thus a reduced level of FM1-43 fluorescence in terminals of DTB-treated animal could result from reduced exocytosis, impaired endocytosis, or a combination of the two. Endocytosis can be studied in isolation by examining its time course after tetanic stimulation. This time period should include primarily slow endocytotic mechanisms (Ryan et al. 1993; Sun and Wu 2001; Wu and Betz 1996).

Therefore we measured and compared the time course of endocytosis during and after a 6-min tetanus in untreated and DTB-treated nerve terminals to determine whether we could detect a defect of membrane retrieval. The time course of endocytosis was determined by measuring the amount of dye taken up into nerve terminals as a function of the delay time between the onset of nerve stimulation (30 Hz for 6 min) and the delivery of the dye to the terminal (Fig. 6). The longer the delay was, the dimmer the fluorescence was. The total dye incubation time was long enough to permit endocytosis to reach completion (15 min).

### TABLE 1. Fraction of vesicles labeled with HRP in motor nerve terminals of triangularis sterni muscles of control and DTB-treated mice

<table>
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<tr>
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<th>Unstimulated Muscles, %</th>
<th>Stimulated Muscles, %</th>
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<tr>
<td>Untreated</td>
<td>14.99 ± 2.76</td>
<td>36.9 ± 1.99*</td>
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<tr>
<td>n</td>
<td>48</td>
<td>78</td>
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<tr>
<td>DTB-treated, all</td>
<td>13.98 ± 2.18</td>
<td>23.57 ± 2.19†</td>
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<tr>
<td>n</td>
<td>49</td>
<td>69</td>
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Values are means ± SE; n is the number of terminal boutons sampled. HRP, horseradish peroxidase; DTB, 2,4-dithiobiuret. * value significantly different from the unstimulated preparations of the same treatment regimen (P < 0.01). † Value significantly different from the non-DTB treated (untreated) terminals at the same extent of nerve stimulation (P < 0.01).
The time course of endocytosis is unaltered in DTB-treated motor nerve terminals. The time course of endocytosis was determined by measuring the amount of FM1-43 uptake into nerve terminals as a function of the delay time between the onset of nerve stimulation (30 Hz for 6 min) and delivery of the dye. The longer the delay time was, the dimmer the fluorescence was. The dye incubation time was long enough to permit endocytosis to reach completion. Solutions were changed in a few seconds by removing one solution and adding another. Additional solution changes were made every 5 min. The data were normalized with respect to the average amount of dye taken up when the dye was present during the full tetanus. The time constant for endocytosis in untreated and DTB-treated terminals is 370 and 400 s, respectively. This difference is not statistically significant (P > 0.05). The values are the means ± SE of 5 untreated and 8 DTB-treated mice.

The graph in Fig. 6 shows two things: first, during the time periods when dye is present during the tetanus and endocytosis could presumably occur by both fast and slow routes, the rate of uptake is not detectably greater than during the time periods when dye was applied at or after the termination of stimulation. There is at best a very slight change in slope at 6 min, when stimulation was stopped. This suggests that contributions via forms of rapid endocytosis are undetectable in this preparation by this method or that the relative amount of dye taken up and re-released in the rapidly recycling process is an undetectably small proportion of the entire pool.

Second, the durations and total kinetics of synaptic vesicle endocytosis are similar in untreated and DTB-treated preparations under these conditions. These data, coupled with the qualitative observations that HRP is taken up into a typical set of organelles, do not point to defects in the initial steps of slow endocytosis that are occurring in DTB-treated terminals.

**Release of dye from labeled structures is reduced in DTB-treated motor nerve terminals**

In terminals with FM1-43 labeled vesicle pools, there is a gradual decrease in fluorescence with subsequent stimulation. This is attributed to loss of dye from the membranes of individual vesicles. On fusion of vesicles with the plasma membrane, the dye diffuses into the surrounding membrane and is then washed out. Therefore the amount of destaining and the destaining rate reflect the number of vesicles involved in exocytosis, although the exact rate of loss reflects the properties of the dye itself, as well as the complexity of the tissue. In the TS, for untreated motor nerve terminals, 50% of dye destaining occurred in 20 min with 30-Hz nerve stimulation, and complete destaining required 50–60 min (Figs. 7A and 8).

In DTB-treated terminals, there were qualitative differences in destaining properties. Characteristic discrete fluorescent spots or clusters with different sizes were seen on loading with FM1-43 as shown in Fig. 1B. These fluorescent spots then appeared to move freely within the nerve terminals, to change in shape and size, and to destain spontaneously (without stimulation) in time lapse movies, as noted in the preceding text (Fig. 1B).

In dim fluorescent nerve terminals of DTB-treated mice, nerve stimulation caused enhanced movement of the brightly fluorescent spots but did not cause significant average dye destaining (Fig. 7). Some bright regions grew dimmer and some dim regions grew brighter during nerve activity. However, the average intensity of fluorescence in the entire nerve terminal was essentially unchanged during 60 min of nerve stimulation (Figs. 7B and 8), and fluorescence declined by only 50% after 120–150 min of nerve stimulation. The time course over which dye movement was observed during nerve stimulation varied among nerve terminals but typically occurred within 2–15 min after beginning nerve stimulation. The phenomenon of dye coalescence during destaining appeared more obvious in DTB-treated than untreated mice perhaps due to the lack of destaining during nerve stimulation and the swollen nerve terminals characteristic of DTB-treated mice (Fig. 7B) (Rheuben et al. 1998). Dye coalescence has been suggested to result from endocytosis that is not restricted to sites of exocytosis (Richards and Betz 1998).

Among bright nerve terminals from mice treated with DTB for only 5 days, partial destaining (Figs. 7C and 8), measured as the average fluorescence brightness over the entire terminal, as well as complete destaining (results not shown) occurred with nerve stimulation, but the average rate of destaining was reduced compared with untreated terminals (Fig. 8). In this comparison, the fluorescence brightness was normalized to that of the starting value, before initiating destaining, so that differences in exocytosis and subsequent endocytosis were accounted for. Therefore this suggests that DTB may affect a subsequent step of vesicle recycling such that once the dye is endocytosed it is less likely to reach the state of being readily released.

**Increased depression of synaptic transmission in DTB-treated mice**

FM1-43 only labels vesicles that have been directly involved with exocytosis/endocytosis cycling; vesicles that are prepared and arrive at the terminal from the cell body escape labeling during nerve activity (Betz and Bewick 1992), as do vesicles in the reserve pool that never entered the active cycle. Actual transmitter release, therefore could come via labeled or unlabeled vesicles during any given test period depending on
the processes underlying mobilization. Conceivably DTB-treatment could reduce the transport of newly synthesized neurotransmitter into existing vesicles, yet enhance utilization of preformed vesicles. This would be evident as a decrease in FM1-43 labeling but not as an overall decrease in synaptic function. Thus we were interested in comparing the time course of depression of synaptic transmission in treated and untreated nerve terminals using an electrophysiological assay to determine if DTB reduced release to multiple stimuli.

In muscles treated with d-tubocurarine to reduce postsynaptic responses below threshold for an action potential and contraction, EPPs could be recorded at end plates of DTB-treated preparations innervated by brighter staining nerve terminals that were preloaded with FM1-43 and identified with fluorescence microscopy but not from nearby dim terminals. Depression of EPP amplitudes during repetitive stimulation was more pronounced in DTB-treated end plates innervated by bright terminals as compared with those of untreated mice (n = 6–8). When high-frequency stimulation (15 Hz) was applied for 1 min in untreated mice, an initial sharp decrease in EPP amplitude was seen followed by a much slower decline (Fig. 9). Such stimulation in DTB-treated terminals resulted in a greater initial activity-dependent depression of EPP amplitudes, followed by a slow decline that paralleled that seen in untreated mice. As shown in Fig. 9, the EPP amplitude after 5 s of nerve stimulation is reduced to 36 ± 4.61% of starting amplitude in

FIG. 7. The number of vesicles released is reduced in DTB-treated motor nerve terminals. A: representative images showing destaining produced by intermittent nerve stimulation at 30 Hz in an untreated motor nerve terminal. Destaining appeared to be uniform throughout the terminal. Terminals were loaded with intermittent 30-Hz stimulation in the presence of FM1-43, then washed for 30 min prior to observation for destaining. B: representative images showing destaining by intermittent nerve stimulation at 30 Hz in a DTB-treated mouse. In a dim terminal, chosen by eye, stimulation of the nerve caused dye movement but didn’t cause dye destaining. Some bright regions grew dimmer and some dim regions grew brighter during nerve activity, but the average intensity of fluorescence in the entire nerve terminal was unchanged during 20 min of nerve stimulation. C: representative images showing destaining by intermittent nerve stimulation at 30 Hz in a bright terminal from a DTB-treated mouse. At 60 min after nerve stimulation, partial destaining had occurred, but fluorescence appeared to be uniform in specific branches of the terminal. Note, however, that some of the less bright branches of this terminal (top half) grew brighter after nerve stimulation.

FIG. 8. Quantification of destaining in untreated and DTB-treated mice. A: time course of destaining for the representative terminals of Fig. 7. ● is from Fig. 7A; ○ is from B; and • is from C. B: the average intensities of fluorescence of untreated, bright and dim terminals measured before and at 20 and 60 min after nerve stimulation. The outline of each selected terminal was marked automatically and average fluorescence intensity of all pixels inside the outline was calculated. The data are presented as the amount of the fluorescence normalized to the average fluorescence values before unloading nerve stimulation (control). The data are the means ± SE of 10 untreated and 11 DTB (dim terminals) or 9 DTB (bright terminals)-treated mice, respectively. * indicates values significantly different from untreated (Normal).
DTB-treated terminals and to 56 ± 5.03% of the initial value in untreated neuromuscular junctions.

**α-LTx fails to increase the recycling vesicle pool size and number of vesicles released in DTB-treated mice**

Both results of uptake of FM1-43 and labeling of HRP indicated that in DTB-treated terminals, once vesicle membrane (and FM1-43) has been endocytosed (implying both that some exocytosis and some endocytosis has occurred), it is not released as well as it is in untreated animals. However, it is unclear whether this is due to a decline in the number of vesicles in the readily releasable pool due to a block in an early step of recycling, an effect of DTB on the mobilization of readily releasable vesicles to the immediately releasable state, or a direct effect on exocytosis.

To begin to differentiate among these possible mechanisms, we tested whether the potent secretagogue α-LTx would increase the recycling vesicle pool size, as measured by uptake, or if it could enhance the release of vesicles previously labeled by nerve stimulation in DTB-treated motor nerve terminals. Both untreated and DTB-treated nerve terminals exposed to 2 μg/ml α-LTx for 30 min took up FM1-43; the respective staining patterns were indistinguishable from those obtained by electrical stimulation of the motor nerve. In DTB-treated terminals, the fluorescence patterns were again lower and uneven (results not shown). The amount of uptake of FM1-43 into DTB-treated motor nerve terminals produced by α-LTx is reduced to 60% of that in untreated terminals (Fig. 10D) comparable to levels produced by nerve stimulation.

In experiments in which FM1-43 was loaded by nerve stimulation, the untreated terminals mostly destained within 30 min after incubation with 2 μg/ml α-LTx and complete destaining occurred with further exposure to α-LTx for 60 min. (Fig. 10, A and B). In bright terminals, partial or complete destaining occurred with α-LTx treatment that was comparable to the effects of nerve stimulation. In dim DTB-treated terminals, α-LTx caused dye movement within the nerve terminals but did not cause dye destaining during 60 min of exposure to the toxin (Fig. 10, B and C). Some bright regions grew dimmer and some dim regions grew brighter, but the average fluorescence intensity over the whole nerve terminal was again unaltered. The areas that became brighter were usually close to the nerve bundle (Fig. 10B). These features are consistent with the observations on dim terminals obtained using nerve stimulation and provided no evidence that α-LTx was capable of inducing release of a set of vesicles that were otherwise unreleasable by normal nerve activity in DTB-treated terminals.

**FIG. 9. Increased depression of synaptic transmission in DTB-treated mice.**

A: representative traces showing end-plate potential (EPP) depression elicited by 15-Hz nerve stimulation at untreated and DTB-treated end-plates. Surface end plates with bright FM1-43 fluorescence were selected for study from untreated controls and DTB-treated mice. Although there appeared to be little difference in the rate of destaining between these two preparations, EPP depression was evident in DTB-treated end plates during 15-Hz repetitive nerve stimulation. B: the time course of EPP depression for the representative traces of A elicited by 15-Hz nerve stimulation at untreated and DTB-treated end plates, respectively.

DISCUSSION

We used the optical methodology developed by Betz and co-workers (Betz and Bewick 1992, 1993; Betz et al. 1992a,b) in conjunction with ultrastructural and electrophysiological techniques to examine the mechanism(s) underlying impaired synaptic transmission at mouse motor nerve terminals during neuromuscular weakness induced by the paretic agent DTB. Previous studies, while showing that many processes evoked release of ACh are reduced in DTB-treated terminals (Atchison 1989; Ireland et al. 1995; Weiler et al. 1986), have only provided indirect evidence that vesicular release/recycling was disrupted during DTB-induced muscle weakness. In the present study, we examined vesicle handling directly.

Our results are consistent with the following conclusions. First, the amount of both FM1-43 and HRP taken up by DTB-treated terminals is reduced substantially in DTB-treated mice. Second, the rate of slow endocytosis and the qualitative distribution of label into organelles are not, however, distinguishable from normal. Third, the total number of synaptic vesicles declines in dim terminals from DTB-treated mice, but additional membrane appears in the form of tubulovesicular structures in the core of the terminal, and as plasma membrane, as the terminal swells. Fourth, relative depression to repeated stimuli is greater in DTB-treated terminals than in untreated terminals. Fifth, neither electrical stimulation nor α-LTx could induce the normal proportion of dye loss from DTB-treated terminals. The results suggest that the depressant actions of DTB on ACh release are complex and may involve multiple sites. In considering where and how DTB may act, we divided the motor neuron secretory process into three simplified concepts, as illustrated in Fig. 11 — exocytosis, endocytosis, and internal vesicular processing. We use the term exocytosis to mean simply the Ca2+ -triggered fusion of a vesicle with the membrane and the release of its contents. We include in the term endocytosis all of the possible mechanisms by which vesicle membrane can be invaginated into the terminal and separated from the plasma membrane. We include in the term internal processing all of the steps from the initial endocytotic vesicle to the preparation of a filled, docked, and primed, releasable vesicle. Clearly each of these processes consist of multiple steps, many of which are yet unclear. Additionally, they may contain alternate processes such as partial exocytosis (so-called “kiss and run”) or rapid
versus slow endocytosis. Furthermore because of the cyclic nature of the secretory process, a direct effect on one step will likely ultimately have an indirect effect on the others. Table 2 lists the observations of the present and previous studies that support an effect of DTB on each of these three processes. Based on results of the present study in concert with previous electrophysiological, neurochemical, and ultrastructural findings, we propose that DTB affects internal vesicle processing and at least indirectly, and possibly directly, exocytosis.

Recycling vesicle pool is reduced during DTB-induced muscle weakness

The principal finding of the present study is that the size of the releasable vesicle pool of ACh is reduced in nerve terminals of DTB-treated mice. Both functional and anatomical results support the conclusion that the pathogenesis of neuromuscular weakness induced by DTB includes a progressive reduction in size of the functional vesicle pools. First, there is reduced uptake and subsequent release of FM1-43 in affected
terminals from DTB-treated mice, and fewer vesicles are labeled by HRP after stimulation. Second, nerve terminals from DTB-treated mice exhibit little destaining during 60 min of nerve stimulation, whereas terminals from untreated mice destain almost completely. Third, during repetitive activity depression of release is more notable in DTB-treated than control mice. Taken together, these results suggest that the functionality of the immediately releasable pool of ACh is reduced progressively and notably so during repetitive activity. Fourth, while the total number of synaptic vesicles in the less-affected terminals is nearly normal, the number of vesicles estimated from terminals with tubulovesicular profiles is reduced by about half. Additionally, in a previous study of DTB-treated rat neuromuscular junction, the number of vesicles in a specific 250-nm band overlying the active zones was reduced to about 50% of normal in severely affected terminals (Rheuben et al. 1998). These effects are progressive; more severely affected terminals from DTB-treated mice have little uptake of either type of label and reduced or no release of that which is taken up. Moreover, consistent with results from all other published reports, the effects of DTB are variable. That is, one terminal which is dramatically affected by DTB may be juxtaposed to another that is apparently unaffected.

A reduced size of the recycling vesicle pool in DTB-treated mice is further supported by the original electrophysiological studies of quantal release statistics in rats treated with DTB.

**TABLE 2. Experimental results supporting effects of DTB on components of the motor neuron secretory process**

<table>
<thead>
<tr>
<th>Key Experimental Results in DTB-Treated Terminals</th>
<th>References</th>
<th>Exocytosis</th>
<th>Endocytosis</th>
<th>Internal Vesicular Recycling</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPP amplitude and quantal content to a single shock are reduced in amplitude after chronic DTB treatment</td>
<td>Atchison (1989); Weiler et al. (1986)</td>
<td></td>
<td>X</td>
<td></td>
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<tr>
<td>Acute exposure to DTB causes a transient decrease in EPP amplitude</td>
<td>Spitsbergen and Atchison (1990)</td>
<td></td>
<td>X</td>
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<tr>
<td>MEPP frequency is reduced in both chronically treated and single dose DTB-treated animals</td>
<td>Weiler et al. (1986); Atchison (1989); Spitsbergen and Atchison (1990)</td>
<td></td>
<td>X</td>
<td></td>
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<tr>
<td>MEPP amplitudes are transiently reduced following single high doses of DTB; and in the presence of high Mg²⁺; they are not reduced following chronic administration</td>
<td>Spitsbergen and Atchison (1990, 1991); Atchison (1989)</td>
<td>(postsynaptic effects not fully excluded)</td>
<td>X</td>
<td>Spitsbergen and Atchison (1991)</td>
</tr>
<tr>
<td>Depression of EPP amplitudes to repeated stimulation is greater</td>
<td>Present study</td>
<td></td>
<td>X</td>
<td></td>
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<tr>
<td>There is an increase in frequency of very large MEPPs with slow rise and decay times</td>
<td>Atchison (1989)</td>
<td></td>
<td>X</td>
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<tr>
<td>In DTB-treated PC12 cells KCl− evoked release of ACh (measured chemically) was decreased, but intracellular levels of ACh and choline were not; release of dopamine was also reduced at higher DTB concentrations</td>
<td>Ireland et al. (1995)</td>
<td></td>
<td>X</td>
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<td>Decrease in the number of normal synaptic vesicles in the vicinity of the synaptic contact, or throughout the terminal</td>
<td>Rheuben et al. (1998); present study</td>
<td></td>
<td>X</td>
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<td>Uptake of FM1-43 and HRP are reduced in amount, but qualitatively, endocytosis appears normal and no difference in rate was detected</td>
<td>Present study</td>
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<td>X</td>
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<td>Unloading of FM1-43 is slower in bright terminals but did not occur in partially loaded dim terminals</td>
<td>Present study</td>
<td></td>
<td>X</td>
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<td>Nerve terminals are enlarged, with increased membrane surface area</td>
<td>Rheuben et al. (1998); present study</td>
<td></td>
<td>X</td>
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<tr>
<td>α-latrotoxin does not cause the unloading of FM1-43 from “un releasable sites” (to exclude indirect effects need to determine if vesicles are depleted or not)</td>
<td>Present study</td>
<td></td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Abnormal tubular and vesicular structures increase in density in the central part of the terminal</td>
<td>Jones (1989); Kemplay (1984); Rheuben et al. (1998); present study</td>
<td></td>
<td>X</td>
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EPP, end-plate potential; MEPP, miniature EPP.
The release statistic—\( n \), a variable that has been correlated with the number of activatable, or vesicle-primed release sites—was prominently reduced (Atchison 1989). Furthermore, in neurochemical studies, the concentration of newly synthesized ACh—which is stored and subsequently released preferentially during ongoing nerve activity (Collier and MacIntosh 1969)—was lower in extensor digitorum longus muscles of DTB-treated rats than in comparable controls (Weiler et al. 1986). Thus this observation too is consistent with a reduction in the active recycling pool of vesicular ACh. Although not tested specifically, the reduced vesicle pool size is probably not due directly to a block of ACh transport into vesicles by DTB, because empty cholinergic vesicles will label with FM1-43 and undergo exocytosis and recycling (Parsons et al. 1999), and empty glutamatergic synaptic vesicles are similarly competent to undergo complete cycles of exocytosis, endocytosis and docking in cultures of cerebellar granule cells (Cousin and Nicholls 1997).

**Effects of DTB on exocytosis**

A reduction in size of the recycling vesicle pool could arise directly or indirectly as a result of a defect in exocytosis. An effect of DTB on exocytosis was first suggested by the original electrophysiological studies of DTB-induced muscle weakness that included a reduction in quantal content of single EPPs, increased tendency to fatigue during repetitive stimulation, decreased frequency of occurrence of miniature EPPs (MEPPs), and an increase in the occurrence of giant MEPPs (Atchison 1989; Weiler et al. 1986). The effects on MEPPs and quantal content in response to single shock stimulation are strikingly similar to those observed during botulinum toxin poisoning (Cull-Candy et al. 1976), which is thought to disrupt exocytosis by actions on SNARE proteins involved in vesicular release. Taken in conjunction with the ability of agents such as 4-aminopyridine to increase SNARE proteins involved in vesicular release. Taken in conjunction with the increased entry into ACh-acetylating enzyme in DTB-treated rats (Atchison 1989) and muscle contractility (Atchison et al. 1982) in response to motor nerve stimulation in DTB-treated rats, this suggested that at least superficially a similarity to presynaptically acting toxins (Lundh et al. 1977). Acute administration of DTB either by single injection or bath application to naive neuromuscular junctions also transiently affects EPP amplitude (Spitsbergen and Atchison 1990), indicating that DTB can have an immediate, albeit short-lived, effect on ACh release. Moreover, comparison of transmission in respiratory muscles with hindlimb muscles during chronic DTB treatment revealed that even in diaphragm, which was not overtly paralyzed during chronic administration of DTB, junctional transmission was more susceptible to impairment by use of low [Ca\(^{2+}\)]/high [Mg\(^{2+}\)]. (Atchison 1990). This further supports a progressive defect in exocytosis but does not indicate whether this effect is direct or indirect. Results of both electrophysiological studies in which the release parameter \( n \) but not \( p \) was reduced during DTB-induced muscle weakness (Atchison 1989) as well as cell fluorescence studies in PC12 cells treated acutely with DTB, showed that the defect was not a simple result of impaired entry or handling of “trigger Ca\(^{2+}\)” (Ireland et al. 1995). Thus a more complex and potentially interesting effect on secretion was suggested.

In the present study, the reduced staining of nerve terminals with FM1-43 or HRP, the increased depression in response to repetitive stimulation, and the inability of \( \alpha\)-LTx to stimulate release in DTB-poisoned preparations are also all consistent with the conclusion that DTB affects exocytosis. Because of the activity-dependent nature of staining with these agents, if secretion is impaired, there is less opportunity for FM1-43 or HRP to be internalized. But it is equally true that exocytosis is reduced indirectly when fewer vesicles are present, and thus an impairment in processes responsible for internal vesicular processing among distinct vesicle “pools” could conceivably explain many of the original electrophysiological observations. Hence, while exocytosis clearly is reduced during DTB-induced muscle weakness, it is still not clear whether this effect is direct or indirect.

An especially interesting result from the present study was the inability of the potent secretagogue \( \alpha\)-LTx to stimulate release in DTB-poisoned terminals. While \( \alpha\)-LTx is known to bind to at least two membrane receptors—neurexin 1A and the so-called “Ca-independent receptor for latrotoxin” or CIRL—the exact mechanism by which \( \alpha\)-LTx induces secretion remains elusive (cf. Södhof 2001). However, black widow spider venom, from which \( \alpha\)-LTx is obtained, can induce an increase in MEPP frequency at rat neuromuscular junctions after poisoning with botulinum toxin A (Cull-Candy et al. 1976; Dreyer et al. 1987; Kao et al. 1976). Moreover, at frog neuromuscular junctions at which activity-dependent destaining of preloaded FM1-43 is blocked by botulinum toxins A, C, and D, black widow spider venom induced destaining (Henkel et al. 1996). Thus it is presumed that the “target of \( \alpha\)-LTx is “downstream” from or can act independently from the interactions of the SNARE proteins such as SNAP-25, the putative target of botulinum toxin A. As such, it was surprising that in the present study, \( \alpha\)-LTx was unable to induce destaining of FM1-43 from DTB-poisoned nerve terminals. Perhaps the inability of \( \alpha\)-LTx to overcome an effect of DTB reflects the fact that there are no further cholinergic vesicles present to be released or that the steps involved in internal vesicular processing are impaired. However, Broadie et al. (1995) were unable to induce increases in quantal release with black widow spider venom at neuromuscular junctions of *Drosophila* mutants lacking the SNARE proteins n-Synaptobrevin or Syntaxin. Thus depending on the location of a DTB-induced defect in the exocytotic apparatus, its action could also be refractory to reversal by \( \alpha\)-LTx if the defect is “downstream” of the \( \alpha\)-LTx receptor.

In summary, data from the present study clearly reinforce the notion based on earlier electrophysiological data that DTB reduces exocytosis at motor axon terminals. Whether this is due to a direct or indirect action remains unclear. Because of the obvious deficit of vesicles and the presence of the abnormal tubulovesicular structures, it is unlikely that a single step in exocytosis per se could be the sole target of DTB intoxication. This could only be the case if tubulovesicular structures form when vesicles are chronically impaired from being released.

**Effects of DTB on endocytosis**

Could endocytosis itself be directly impaired during DTB-induced muscle weakness giving rise to fewer releasable vesicles? Terminals from treated animals are swollen (Rheuben et al. 1998; present study); this could occur if vesicular membrane were added to the terminal membrane and not retrieved. The total amount of FM1-43 or HRP visualized within a terminal after stimulation depends directly on endocytosis and was less in DTB-treated terminals. Because decreased endocytosis could arise indirectly as a result of decreased exocytosis, separating a direct effect on endocytosis from an indirect effect is difficult experimentally. To
examine endocytosis in the present study, we used two complementary but separate techniques. First, we measured uptake of HRP into vesicles using electron microscopy. Even though the amount of uptake was reduced, we found no qualitative abnormalities in the type of structure that included tracer. HRP was found in coated vesicles, endosomes, cisternae, and synaptic vesicles as if the terminal could complete the cycle in the classical way, suggesting that no step in the formation of initial endocytotic structures or intermediate organelles was blocked completely. Second, we examined the rates at which FM1-43 was taken up during and after a 30-Hz stimulation for 6 min. No difference in rate of uptake could be detected between terminals of untreated and DTB-treated mice. Previous studies of rates of endocytosis in rat hippocampal and frog motor nerve terminals have shown that endocytosis persists after cessation of exocytosis and that the time course of endocytosis depends on the number or duration of stimuli (Ryan et al. 1993; Sun and Wu 2001; Wu and Betz 1996). Tetanic stimulation as they used, and as we use here, would likely invoke slow endocytosis as well as any ongoing fast recycling methods, such as the “kiss-and-run” method, during the tetanus; endocytosis following the tetanus would occur by definition entirely via a slow method or methods.

So while a defect in endocytosis cannot be definitively ruled out, we could find no evidence for a primary effect of DTB on this process other than the swollen terminals—which could arise by another mechanism.

Effects of DTB on internal vesicular processing

A number of our findings are consistent with the idea that DTB causes defects in the internal handling of synaptic vesicle membrane. First, there is a decrease in the number of synaptic vesicles and a progressive increase in tubulovesicular structures. The center of the terminal becomes packed with abnormal membrane-bound structures of various sizes and shapes. The mechanisms underlying the formation of these structures are unknown. However, the fact that abnormal tubular structures are also seen in the motor axons (Jones 1989; present study) suggests that DTB has a broad effect on some types of internal membranes and furthermore might affect the supply of new vesicle membrane arriving from the cell body.

Second, FM1-43 can be taken up in some DTB-treated terminals, apparently in a nearly normal quantity in the bright new vesicle membrane arriving from the cell body. It is also possible that FM1-43 and HRP do not label the same endocytic routes, and further experiments should include examination of photoconverted FM1-43 to identify the ultrastructural locations of unreleasable FM1-43.

Although abnormal MEPPs and altered release properties can be consistent with defects in exocytosis, some of the physiological findings could be equally consistent with defects in internal recycling processes. The presence of MEPPs of abnormal sizes and shapes suggests defects in either filling mechanisms or in the number of normally sized vesicles. There is structural evidence for a change in the size distribution of vesicles in DTB-treated rat terminals (Rheuben et al. 1998). Similarly, the greater relative depression to high-frequency stimulation suggests a deficit in vesicles belonging to a reserve pool or a defect somewhere just upstream of the actual releasable vesicles. Finally, as noted in the preceding text, the inability of α-LTx to induce secretion could reflect an inability to move vesicles from a reserve to a releasable status.

Summary and conclusion

In summary, the size of the vesicle recycling pool, as well as the number of vesicles present, is reduced in DTB-treated mice. Vesicular release is reduced during nerve activity, but there was no evidence that endocytosis is altered qualitatively. However, there is an increase in membrane outside the vesicle recycling pool in the form of plasma membrane and tubulovesicular profiles. These profiles do not accumulate HRP after stimulation, suggesting that any involvement in recycling is not by an immediate route. The potent secretagogue α-LTx did not enhance ACh release in DTB-treated terminals, suggesting that there were no further accessible vesicles to be released or that an effect of DTB was distal to the site of action of α-LTx. Taken together these observations suggest that the chronic effects of DTB have a major impact on the processes of cholinergic vesicular recycling.

However, it remains possible that in addition, DTB affects one or more of the molecular steps involved in exocytosis. The processes underlying normal mobilization, docking, and release of synaptic vesicles are still poorly understood, but through use of pharmacological probes such as α-LTx, botulinum, and tetanus toxin, our understanding of this crucial process is being unraveled. DTB may similarly prove to be a valuable tool in deciphering the processes of synaptic vesicle exocytosis and of internal vesicular recycling processes.

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