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FM1-43 dye ultrastructural localization in and release from frog motor nerve terminals

(photoconversion/exocytosis/neuromuscular junction/synaptic vesicles/fluorescence)

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ABSTRACT **Previous work has shown that the fluorescent styryl dye FM1-43 stains nerve terminals in an activity-dependent fashion. This dye appears to label the membranes of recycled synaptic vesicles by being trapped during endocytosis. Stained terminals can subsequently be destained by repeating nerve stimulation in the absence of dye; the destaining evidently reflects escape of dye into the bathing medium from membranes of exocytosing synaptic vesicles. In the present study we tested two key aspects of this interpretation of FM1-43 behavior, namely: (*i*) that the dye is localized in synaptic vesicles, and (*ii*) that it is actually released into the bathing medium during destaining. To accomplish this, we first photolyzed the internalized dye in the presence of diaminobenzidine. This created an electron-dense reaction product that could be visualized in the electron microscope. Reaction product was confined to synaptic vesicles, as predicted. Second, using spectrofluorometry, we quantified the release of dye liberated into the medium from tubocurarine-treated nerve–muscle preparations. Nerve stimulation increased the amount of FM1-43 released, and we estimate that normally a stained synaptic vesicle contains a few hundred molecules of the dye. The key to the successful detection of released FM1-43 was to add the micelle-forming detergent 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), which increased FM1-43 quantum yield by more than two orders of magnitude.**

FM1-43 and other styryl dyes have been used to monitor exocytosis, endocytosis, and endosomal traffic in a variety of cell types, including motor nerve terminals in amphibia (1–3), mammals (4), and *Drosophila* larvae (5); cultured hippocampal neurons (6–9); pituitary cells (10); *Dictyostelium* (11); and yeast (12). The amphipathic structure of the dyes, with ionically charged heads and hydrophobic tails, has led to the following proposed mechanism of action: From the extracellular fluid dye molecules reversibly partition into the outer leaflet of all surface membranes, but they cannot penetrate to the cytoplasm, owing to their charged headgroups. The relatively low dielectric constant of the membrane also increases the quantum yield of the dye, which is only weakly fluorescent in water. If dye-labeled membrane is internalized, for example by endocytosis, the dye becomes trapped in the resulting endosome. Subsequent washing of the preparation in dye-free medium removes dye only from surface membranes, leaving behind stained endosomes. Then, if a stained endosome undergoes exocytosis in dye-free medium, dye molecules dissociate from the membrane, causing the cell to destain.

Considerable indirect evidence supports this proposed mechanism of FM1-43 action. For example, in nerve terminals both staining and destaining are activity dependent (i.e., they require endo- or exocytosis, respectively), and the pattern of staining is generally consistent with the ultrastructural distribution of synaptic vesicle clusters (1, 13). However, several key aspects of the hypothesis have not been directly tested. In the present work, we addressed two of these, asking, In stained frog motor nerve terminals, is FM1-43 ultrastructurally localized in synaptic vesicles? Is FM1-43 released from the nerve terminals during nerve stimulation? We answered the first question by applying the photoconversion technique (14–18) to produce electron-dense precipitates at sites of FM1-43. We answered the second question by measuring FM1-43 concentration in extracellular fluid with a fluorometer. In both cases, the hypotheses were confirmed: photoconversion products were localized in synaptic vesicles, and extracellular dye concentration increased significantly during activity-dependent nerve terminal destaining.

EXPERIMENTAL PROCEDURES

General Staining and Destaining Procedures. Frog (*Rana pipiens*) cutaneous pectoris muscles were dissected and used immediately in all experiments. Nerve terminals were stained by exposing preparations to 4 μM FM1-43 {*N*-[3-(triethylammonio)propyl]-4-(4-dibutylaminostyryl)pyridinium dibromide; Molecular Probes} dissolved in buffered frog Ringer solution (115 mM NaCl/2 mM KCl/1.8 mM $CaCl_2$/10 mM Hepes, pH 7.0) or normal frog Ringer solution (NFR; 115 mM NaCl/2 mM KCl/1.8 mM $CaCl_2$/2.4 mM $NaHCO_3$), and stimulating the nerve electrically at 10 Hz for 4.5–5 min. Destaining of dye-loaded and washed (1–2 hr) nerve terminals was carried out in dye-free frog Ringer solution by stimulating the nerve at 30 Hz for 5 min (2).

Photoconversion. Stock solutions of diaminobenzidine [DAB (as the tetrahydrochloride); Sigma] at 30 mg/ml in double-distilled water were divided into aliquots, frozen, and stored for up to 3 months. For daily use, DAB aliquots were diluted 1:10 with double-distilled water, then the same volume of 200 mM sodium phosphate buffer (PB; pH 7.4) was added to obtain a final DAB concentration of 1.5 mg/ml in 100 mM PB. The solution was then filtered through a 0.2-μm-pore nitrocellulose filter before being applied to a preparation.

Cutaneus pectoris muscles were dissected in NFR, pinned out in Sylgard-lined dishes, and stained with 4 μM FM1-43 (2). The preparations were rinsed in NFR for 20–60 min at 22°C and checked for the quality of nerve terminal fluorescent staining. Only brightly stained preparations were used in experiments. In early experiments, we attempted to photoconvert living preparations, but we found that the resulting tissue

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Abbreviations: DAB, diaminobenzidine; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.

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damage was too extensive. Thus, stained preparations were fixed before photoconversion. Unfortunately, FM1-43 fluorescence decreased during fixation, and so we sought to develop a compromise fixation protocol that produced sufficient reaction product while simultaneously preserving sufficient ultrastructure. We found that fixation in 1% paraformaldehyde for 10 min at 22°C generally satisfied both criteria, although further attention to this problem might improve tissue preservation and DAB reaction product deposition. After fixation, the central region of the muscles, which contained most of the nerve terminals, was cut out and pinned in a Sylgard-lined dish. The preparations were then washed several times in 100 mM PB (5 min each step) and incubated in the DAB solution in the dark at 22°C for 20 min. The DAB solution was then refreshed, and the preparations were illuminated through a Leitz Fluotar L 25 × 0.35 n.a. objective with blue light (430 nm) from a 100-W mercury bulb at 100% transmittance for different lengths of time. During the experiments the objective was focused on surface nerve terminals. After 30–45 min of continuous illumination the terminal fluorescence completely faded, and after an additional 15 min brown deposits became visible in the illuminated terminals. After illumination was stopped the preparation was washed for 10–20 min with 100 mM PB and the photoconverted region was cut out under the dissection microscope. For electron microscopy the preparations were postfixed in 2% paraformaldehyde and 1% glutaraldehyde for 30 min at 22°C. After the fixation, preparations were thoroughly rinsed for 30 min with 100 mM PB and subjected to further electron microscope processing, following a standard protocol.

Electron Microscopy. The muscle pieces were osmium-postfixed (2% OsO_4/20 mM $CaCl_2$/50 mM NaCl/30 mM sodium barbital buffer, pH 7.4) at 4°C, dehydrated through an ascending series of ethanol solutions, embedded in Epon, and sectioned. Samples were not subjected to Reynolds lead or uranyl acetate block staining treatment. Sections were viewed and photographed with a Philips CM10 electron microscope.

Release of FM1-43 from Frog Nerve Terminals. Muscles were stained with 4 μM FM1-43 and then washed for 30 min in Ca-free Ringer solution. The ends of the muscle were then cut off and discarded. This was done to eliminate damaged cells near the tendons (mostly fibers from muscles adjacent to the cutaneous pectoris) that otherwise spontaneously released unacceptably high levels of dye. The remaining central region of the muscle, which contained all of the motor nerve terminals, was washed for 1.5 hr at 4°C in Ca-free Ringer solution. ($CaCl_2$ was replaced with 4 mM $MgCl_2$ to reduce spontaneous exocytosis, which might have been elevated due to leakage of potassium ions from the cut muscle fibers, although we did not examine this possibility experimentally.) The preparation was then placed in a 250-μl tube, its nerve was inserted into a stimulation electrode, and the muscle was washed three times within 1 min with 200-μl aliquots of Hepes-buffered Ringer solution (containing 1.8 mM calcium). In control experiments (not illustrated), we found that muscle contractions evoked by direct electrical stimulation of the muscle could increase the amount of dye collected; presumably this mechanical activity enhanced the clearance of dye from the interstices of the muscle. To block this mechanical activity, we paralyzed muscles by adding 6 μM tubocurarine to all solutions. The basal leak (background) of FM1-43 from the cut muscle was measured by incubating the preparation three times with 100 μl of Hepes-buffered Ringer solution. Samples were collected every 5 min and replaced with fresh Ringer solution. Then the preparation was stimulated (30 Hz for 5 min) via the nerve, and the fluid was collected immediately after the end of nerve stimulation. The samples were stored for up to 30 min before fluorescence was measured in a fluorometer.

Fluorometric Quantification of FM1-43 Released by Nerve Stimulation. All fluorometric measurements were performed on a Farrand (Valhalla, NY) manual spectrofluorometer S/N 213, equipped with a xenon lamp (200–1400 nm) and a magnetic xenon arc stabilizer. For determination of excitation–emission spectra, 2 μM FM1-43 was used in the assay. All samples, including calibration samples, were mixed with 2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and mixed in a Vortex mixer for 5 sec before measurement. Sample volume of the quartz cuvette was 100 μl, and FM1-43 concentration was measured at 465-nm excitation and 560-nm emission. These optimal wavelengths were empirically determined by scanning the excitation and emission spectra in 10- to 20-nm steps. Calibration (FM1-43 concentration versus fluorescence emission units) was between 0.16 and 160 nM FM1-43.

RESULTS

CHAPS Enhances FM1-43 Fluorescence. FM1-43, dissolved in water or buffer, showed very little fluorescence and was almost undetectable at concentrations below 2 μM. Its fluorescence emission peak fell in the orange-red at 600 nm when excited with blue light at 460 nm. As shown in Fig. 1*A*, addition of increasing amounts of CHAPS, a zwitterionic detergent, shifted the emission peak to 560 nm and produced a large increase in absolute fluorescence (about 350-fold; from 0.0043 to 1.51 fluorescence units). Fluorescence reached a maximum at a concentration of about 2% CHAPS (Fig. 1*A*).

Excitation–Emission Spectra of FM1-43. The fluorescence spectrum was determined by using a constant concentration (2 μM) of FM1-43 dissolved in Hepes-buffered Ringer solution that contained 2% CHAPS. Dye fluorescence was measured over a broad range of wavelengths of excitation (400–540 nm) and emission (480–620 nm). The maximal fluorescence occurred at 465-nm excitation and 560-nm emission (Fig. 1*B*; cf. ref. 19), and this wavelength pair was chosen for the fluorometric quantification of dye released from motor nerve terminals.

Quantification of Released FM1-43. All preparations (n = 7) were treated with tubocurarine during experiments. Unstimulated neuromuscular preparations released an average of 143 ± 98 fmol of dye (mean ± 1 SD) during each 5-min collection period; this was collected in 0.1-ml volumes, giving an average concentration of 1.43 ± 0.98 nM (Fig. 2). This spontaneous leakage varied widely in different preparations (range 0.2–2.1 nM), although it was quite constant over time in any single preparation ($P > 0.05$ over the three collection periods). Nerve stimulation caused a highly significant increase in liberated dye (average 3.2-fold increase, $P < 0.001$). For each experiment, subtraction of average basal release gave a measure of the stimulation-dependent portion of dye release; the average for all experiments was 318 ± 103 fmol. Samples collected during the period 5–10 min after the end of stimulation also contained a slightly elevated concentration of dye, but this was not significantly different from background ($P > 0.05$; Student's t test).

Ultrastructural Localization of FM1-43. Fig. 3 *Upper* shows an example of a nerve terminal that was stained with FM1-43, washed for 90 min, lightly fixed, exposed to intense blue light for 40 min in the presence of DAB, and prepared for electron microscopy. About half of the synaptic vesicles contained an electron-dense precipitate, which we interpret as reflecting the presence of FM1-43 in those vesicles. Curiously, the ultrastructural labeling of vesicles with DAB reaction product appeared to be all-or-none. Importantly, other intracellular organelles, such as mitochondria, were not labeled. Naturally, we wondered if the vesicles lacking DAB reaction product did not contain FM1-43 from the outset, or did contain FM1-43 but failed to produce reaction product. To test this, we increased the time of illumination in the presence of DAB to 65 min. An example from such an experiment is shown in Fig. 3 *Lower*. In

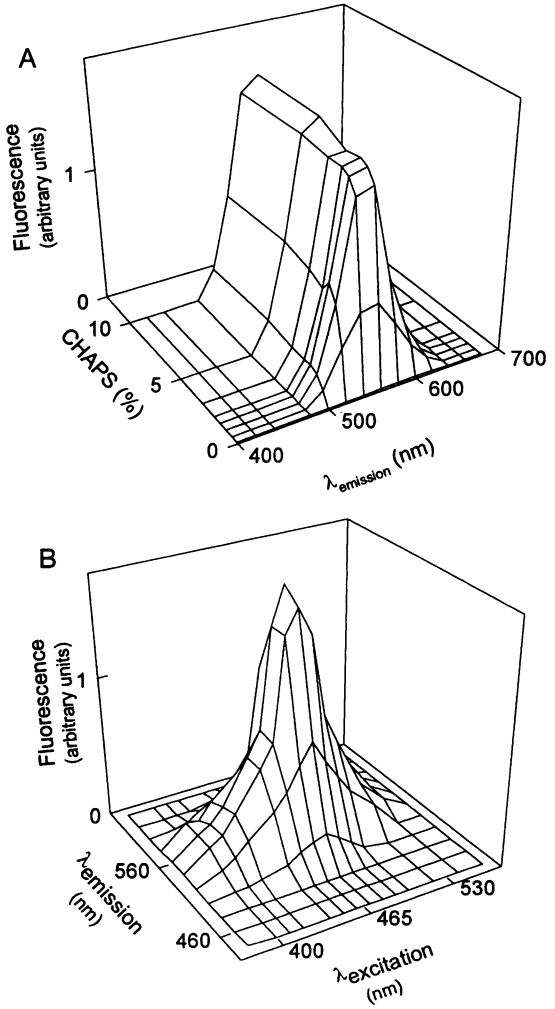

FIG. 1. (*A*) CHAPS enhances FM1-43 fluorescence. FM1-43 (2 μM) was dissolved in NFR and mixed with CHAPS at different concentrations, and the resulting fluorescence was measured in a fluorometer. The excitation wavelength was set to 460 nm and emission was measured between 400 and 680 nm. Addition of CHAPS caused a large increase in fluorescence, the increase saturating at a CHAPS concentration of about 1.5%. The peak emission wavelength shifted from 600 nm (no CHAPS) to 560 nm (≥0.5% CHAPS). (*B*) Excitation (470 nm) and emission (550 nm) maxima were determined for FM1-43 in 2% CHAPS.

this terminal, virtually all synaptic vesicles contained reaction product. In addition, other organelles in both the nerve terminal and muscle fiber were lightly labeled, although not nearly as heavily as the vesicles. Other than synaptic vesicles, the most heavily labeled structures in the most intensely illuminated preparations were mitochondria in small muscle fibers (presumably true slow fibers); an example is shown in Fig. 4 *Upper*.

We performed several control experiments in which preparations were treated identically to those described above, except they were not exposed to FM1-43 (although they were subjected to a sham staining procedure). Fig. 4 *Lower* shows an example of a nerve terminal from such a preparation, which had been exposed to intense illumination. Some intracellular organelles are labeled with DAB reaction product, although the synaptic vesicles are notably free of label.

DISCUSSION

Using the DAB photoconversion technique, we found clear signs that FM1-43 is located in synaptic vesicles. Heavy reaction product was observed in vesicles of preparations that had been stained with FM1-43 but not in control preparations (no FM1-43 exposure). Curiously, vesicles appeared to display label in an all-or-none fashion, rather than progressively increasing their amount of label with increasing illumination. For example, illumination for 30–40 min caused DAB label to appear in about 50% of the vesicles, while illumination for 60 min or longer caused virtually all vesicles to be labeled. Evidently DAB photoconversion is a polymerization process that requires a certain threshold for initiation, and thereafter continues to completion (20). It thus seems clear that FM1-43 is contained in synaptic vesicles.

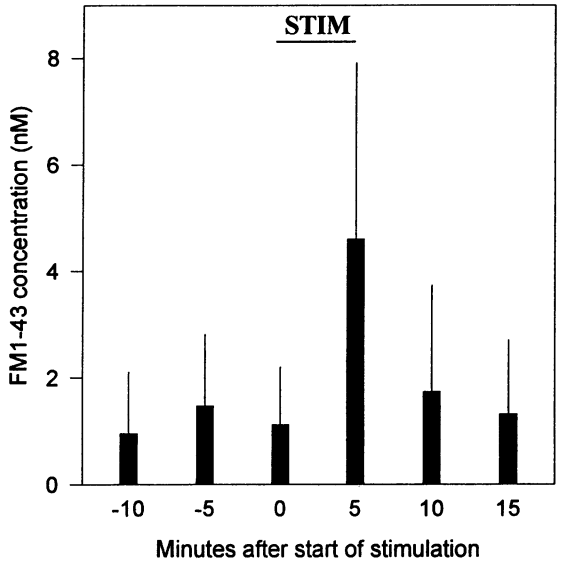

FIG. 2. FM1-43 is released from nerve terminals in an activity-dependent fashion. Stained preparations were incubated in a small volume (100 μl) of Hepes-buffered Ringer solution. Samples of extracellular fluid were collected after every 5 min of incubation and replaced with fresh Ringer solution. The graph shows the mean FM1-43 concentration (±1 SD; seven experiments) in 100-μl extracellular fluid samples. Control samples before nerve stimulation showed a background leakage of dye into the bath. Fluorescence was significantly higher in the sample collected immediately after nerve stimulation (STIM; 10 Hz for 5 min), and then it returned to control levels.

Is FM1-43 distribution restricted to synaptic vesicles? This question was more difficult to answer definitively, owing to the presence of endogenous peroxidases capable of catalyzing the polymerization of DAB into electron-dense material (17, 21). These enzymes are found especially in mitochondria and endoplasmatic reticulum (22, 23). We found that intense illumination caused reaction product to appear in such organelles; the most heavily labeled were mitochondria of small muscle fibers. Such labeling appeared whether or not preparations had been stained with FM1-43, and the amount of labeling appeared to be no different, although we did not quantify the labeling in detail. Importantly, even after the most intense illumination of control (no FM1-43) preparations, virtually no label appeared in synaptic vesicles.

In summary, it seems reasonable to conclude that the routine staining procedure we follow (10-Hz nerve stimulation in the presence of 2–4 μM FM1-43 for 5 min) leads to staining of most, and perhaps all, synaptic vesicles, which themselves have no endogenous peroxidase activity. If FM1-43 gains access to other intraterminal organelles, it is at a much lower concentration (lower DAB reactivity) than in synaptic vesicles. DAB photoconversion of FM1-43 may therefore be useful for exploring other aspects of vesicle recycling, in much the same way that horseradish peroxidase uptake by recycling vesicles has proven valuable (24).

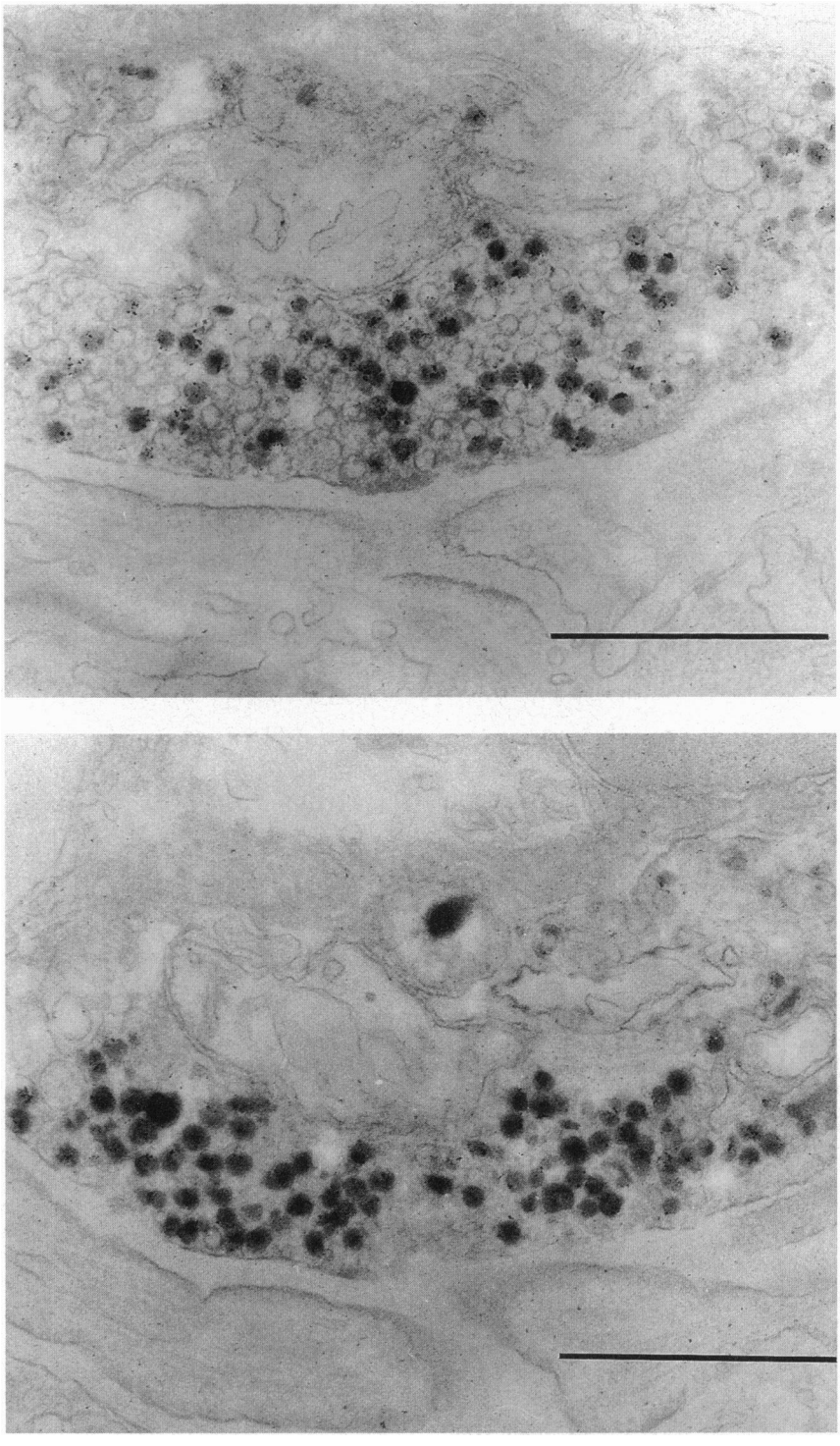

FIG. 3. Photoconversion of FM1-43 with DAB reveals ultrastructural localization of reaction product in synaptic vesicles. Lightly fixed neuromuscular preparations were incubated with DAB and illuminated with intense blue light for prolonged periods of time. (*Upper*) Example of a terminal stained with FM1-43, fixed, and illuminated for 40 min. About half of the synaptic vesicles contain reaction product. Labeling appeared to occur in an all-or-none fashion. (Bar = 500 nm.) (*Lower*) Same procedure as for *Upper* but with a longer illumination time (65 min), which caused virtually all synaptic vesicles to be labeled. (Bar = 500 nm.)

We turn next to the mechanism of destaining. Why does the fluorescence of a nerve terminal stained with FM1-43 decrease during repetitive nerve stimulation? Indirect evidence suggests that the dye is released from the terminal during exocytosis of stained vesicles, and then it diffuses away into the large volume of surrounding extracellular fluid (2, 25), although other mechanisms, such as activity-dependent metabolism of the dye to a nonfluorescent form, cannot be ruled out. To examine this question, we tried to detect FM1-43 released during nerve stimulation by collecting extracellular fluid and examining it in a fluorometer for signs of dye. Early control experiments revealed the futility of detecting nanomolar concentrations of FM1-43 dissolved in water; the fluorescence was too low. However, when we added the micelle-forming detergent CHAPS to an aqueous solution of FM1-43, fluorescence increased by more than two orders of magnitude. We interpret

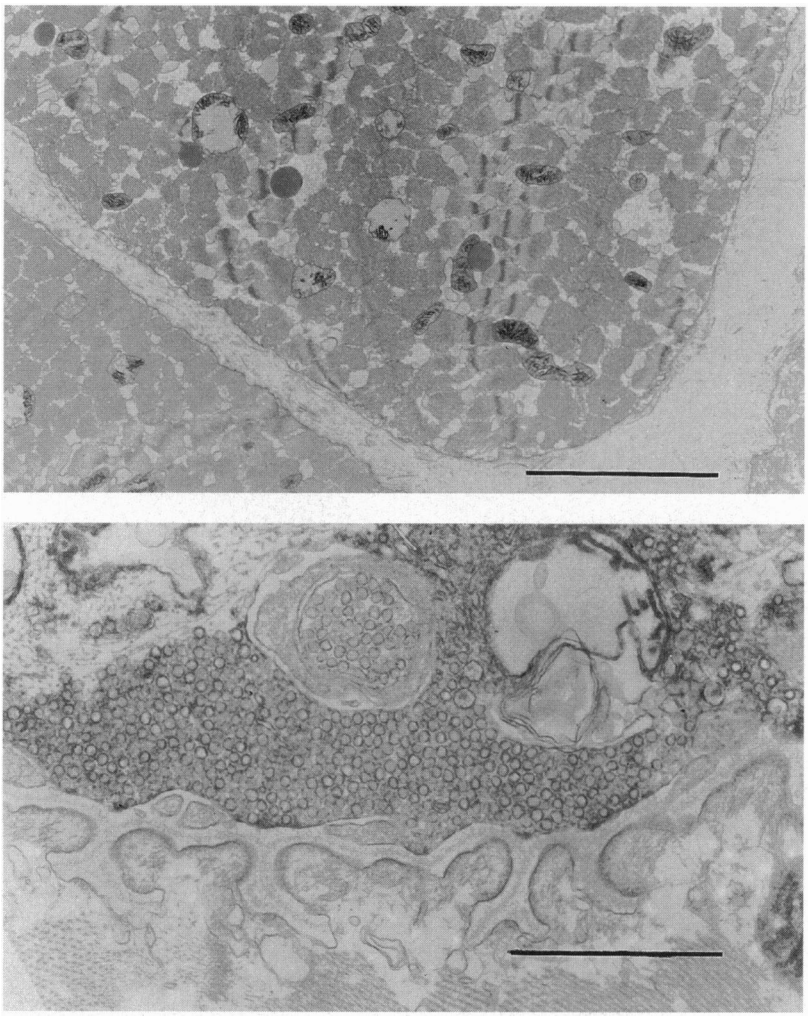

FIG. 4. (*Upper*) Mitochondria in this small muscle fiber in a preparation stained with FM1-43 are heavily labeled with reaction product after 65 min of illumination. Similar labeling was observed in mitochondria of small fibers in preparations that were not stained with FM1-43 (not illustrated). (Bar = 5 μm.) (*Lower*) Endogenous peroxidase activity of mitochondria and other organelles in a nerve terminal and muscle fiber. This preparation had not been exposed to FM1-43. The preparation was incubated with DAB for 30 min and then intensely illuminated for 65 min. Intraterminal mitochondria and other membranes are labeled. Synaptic vesicles, however, contain virtually no DAB reaction product, suggesting a complete lack of endogenous peroxidase activity in synaptic vesicles. (Bar = 1 μm.)

this as reflecting the partitioning of the dye into the CHAPS micelles in a fashion analogous to its partitioning into biological membranes. The lower dielectric constant of the lipid solvent (compared with water) is evidently responsible for the large increase in quantum yield as the dye enters the hydrophobic domain of the micelle (26). Consistent with this, the Stokes shift was reduced (the fluorescence emission peak shifted from 600 nm to 560 nm). Thus, collected samples of extracellular fluid containing FM1-43 were diluted with CHAPS and examined in a fluorometer. Further adjustments to reduce the amount of background release of dye from the preparation were also necessary (see *Experimental Procedures*).

In all experiments (seven preparations) the fluorescence of extracellular fluid samples was highest immediately after nerve stimulation (about 3-fold higher than during control periods). The average amount released by nerve stimulation was 318 fmol per muscle. If this dye came from 500 million synaptic vesicles (500 muscle fibers per muscle, each innervated by a nerve terminal containing one million stained vesicles; refs. 27–29) then each vesicle contained, on average, 383 molecules of FM1-43. In a vesicle with an interior diameter of 45 nm, the average spacing between these molecules would be 4.1 nm, and there would be about 70 phospholipid molecules per molecule of FM1-43 (assuming 0.5 nm per phospholipid molecule).

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