

# Okadaic Acid Disrupts Clusters of Synaptic Vesicles in Frog Motor Nerve Terminals

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**Abstract.** The fluorophore FM1-43 appears to stain membranes of recycled synaptic vesicles. We used FM1-43 to study mechanisms of synaptic vesicle clustering and mobilization in living frog motor nerve terminals. FM1-43 staining of these terminals produces a linear series of fluorescent spots, each spot marking the cluster of several hundred synaptic vesicles at an active zone. Most agents we tested did not affect staining, but the phosphatase inhibitor okadaic acid (OA) disrupted the fluorescent spots, causing dye to spread throughout the terminal. Consistent with this, electron microscopy showed that vesicle clusters were disrupted by OA treatment. However, dye did not spread passively to a uniform spatial distribution. Instead, time

lapse movies showed clear evidence of active dye movements, as if synaptic vesicles were being swept along by an active translocation mechanism. Large dye accumulations sometimes occurred at sites of Schwann cell nuclei. These effects of OA were not significantly affected by pretreatment with colchicine or cytochalasin D. Electrophysiological recordings showed that OA treatment reduced the amount of acetylcholine released in response to nerve stimulation. The results suggest that an increased level of protein phosphorylation induced by OA treatment mobilizes synaptic vesicles and unmasks a powerful vesicle translocation mechanism, which may function normally to distribute synaptic vesicles between active zones.

**N**EUROTRANSMITTERS and other substances are secreted by nerve terminals in multimolecular packets, or quanta (Katz, 1962), and considerable ultrastructural evidence suggests that synaptic vesicles are the physical source of quanta (reviewed in Heuser, 1989). At the neuromuscular junction, vesicles are gathered together in clusters, and during repetitive nerve activity they are mobilized and moved to the presynaptic membrane, where they undergo exocytosis at specialized release sites (active zones). The mechanisms governing vesicle clustering, mobilization, and movement are only partly understood. The protein dephospho-synapsin I binds synaptic vesicles, arrests some microtubule-based movements of organelles in extruded squid axoplasm, reduces transmitter release when injected into squid presynaptic terminals, and is colocalized with synaptic vesicles at the neuromuscular junction. It has been proposed that vesicle mobilization during nerve activity is mediated by phosphorylation of synapsin I, which reduces its affinity for synaptic vesicles (reviewed in DeCamilli et al., 1990; Greengard et al., 1993; see also Landis et al., 1988; Hirokawa et al., 1989). Other components of the cytoskeleton have also been implicated in governing vesicle trafficking, including microtubules in axons (reviewed in Schroer and Sheetz, 1991) and actin in nerve terminals (Hirokawa et al., 1989; Valtorta et al., 1992; Benfenati et al., 1992).

Recently, we have developed fluorescent dyes which stain motor nerve terminals in an activity-dependent fashion, apparently by labeling the membranes of recycled synaptic vesicles. Thus, the dyes (in particular, FM1-43) may be useful tools for studying the trafficking of synaptic vesicles in living nerve terminals. The conclusion that the dyes stain recycled synaptic vesicle membranes rests primarily upon three observations (Betz et al., 1992; see also Nicholls et al., 1992). First, the staining of nerve terminals is activity dependent. That is, if a preparation is exposed to dye in the absence of stimulation, little staining occurs. Second, the pattern of staining—fluorescent spots which align with postsynaptic receptors—agrees well with the distribution of synaptic vesicles observed in the electron microscope. Each fluorescent spot corresponds to the cluster of several hundred synaptic vesicles at an active zone. Third, stained nerve terminals destain in an activity-dependent fashion. That is, the fluorescent spots persist for many hours in the absence of further nerve stimulation, but disappear in a few minutes during repetitive nerve stimulation. Presumably, destaining occurs when preloaded vesicles undergo exocytosis and release their dye.

In previous work, we have studied several aspects of frog motor nerve terminal staining and destaining with FM1-43. For example, we observed that the fluorescent spots do not move or change shape very much during destaining produced by nerve stimulation; they just grow dimmer (Betz et al., 1992a; Betz and Bewick, 1992, 1993). This suggested

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to us that there must be mechanisms for confining vesicles to a single active zone even while they move to the presynaptic membrane. Possibilities included active vesicle translocators (Hirokawa et al., 1989), and/or a cytoskeletal "cage" to restrain randomly moving vesicles. In a few experiments, we observed clear signs to active vesicle translocation between vesicle clusters. For example, dye was sometimes observed to flow from one spot into a neighboring spot. However, within individual clusters we saw no signs of directed vesicle motion; our results could be explained most simply by assuming random mixing of vesicles (Betz et al., 1992).

These experiments, which were performed entirely on normal preparations stained with FM1-43, naturally led us to consider ways to perturb the normal vesicle traffic, in hopes of learning more about the underlying mechanisms by which vesicle clusters are created and maintained. Most agents we tested—including cytochalasin D, colchicine, TPA, 8-Br-cAMP, and high temperature—had no significant effects on FM1-43 staining or destaining. However, the protein phosphatase inhibitor okadaic acid (OA)<sup>1</sup> disrupted the fluorescent spots, causing dye to spread throughout the nerve terminals. Staining and destaining with FM1-43 were also affected by OA, and combined optical, ultrastructural, and electrophysiological observations have revealed some new aspects of synaptic vesicle trafficking. (A brief account has been given; Betz, W. J. *Mol. Biol. Cell.* 1992. 3:121a.)

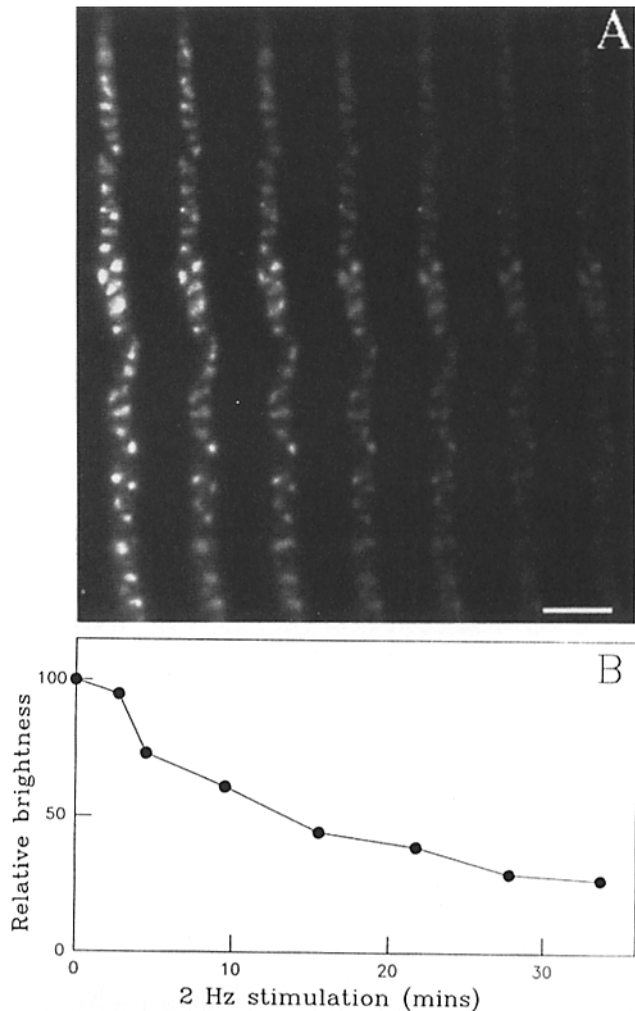
## Materials and Methods

Most of the techniques we used have been described in detail before (Betz et al., 1992a; Betz and Bewick, 1993). Briefly, frog (*Rana pipiens*) *cutaneous pectoris* nerve-muscle preparations were dissected and pinned in a Sylgard-lined dish. They were bathed in normal Ringer containing 2  $\mu$ M FM1-43 (Molecular Probes, Eugene, OR) and the nerve was stimulated at 10 Hz for 4.5–5 min. They were then washed for 30–90 min, and placed on the stage of a Leitz upright epi-fluorescence microscope. In experiments requiring muscle paralysis (e.g., those requiring repeated imaging or electrophysiological recordings during nerve stimulation), curare was added to the Ringer solution (4–5  $\mu$ g/ml for electrophysiology; 9–12  $\mu$ g/ml for imaging). In almost all experiments, the contralateral muscle served as control.

Illumination was from a mercury lamp (100 W; 5–20% neutral density transmittance filter; 530–540 excitation filter); other optical components included a Leitz H3 dichroic mirror and 550–650 nm emission filter. Images were acquired with a Photometrics Star 1 chilled CCD camera (3-s exposure, gain 4) and analyzed with a Silicon Graphics Personal Iris computer running software from G. W. Hannaway (Boulder, CO). Prints were made with a Kodak XL 7700 printer.

As in previous work, during any single experiment we acquired and processed all images identically. Image processing involved adjusting grey scales to 256 levels of brightness (8-bit resolution) and aligning each image with the first in the series. Then average brightness levels of selected regions were measured. In previous work, we measured the average brightness of individual fluorescent spots, whose positions were fixed over time. In the present work a somewhat different procedure was used because fluorescent spots were disrupted after exposure to okadaic acid. Thus, it was important to measure the average brightness of the entire terminal. To do this, we marked the area of interest (a rectangle which included the entire nerve terminal), and then determined the average brightness of all pixels in the area brighter than a certain threshold value. The threshold was chosen by trial and error to include nearly all pixels within fluorescent spots of the control (first) image, and none outside the spots. Care was taken to exclude or to mask artifactually bright regions (e.g., dead cells, myelin, endocytic vacu-

1. **Abbreviations used in this paper:** CD, cytochalasin D; 8-Br-cAMP, 8-bromoadenosine 3'5'-cyclicmonophosphate; EPP, end plate potential; MEPP, miniature end plate potential; OA, okadaic acid; TPA, phorbol 12-myristate-13 acetate.



**Figure 1.** Control preparation stained with FM1-43 revealed a characteristic array of fluorescent spots, and destained in response to electrical stimulation of the nerve. (A) Spots dimmed in an activity-dependent fashion. Immediately after the left hand image was taken, the nerve was stimulated continuously for 35 min at a frequency of 2 Hz, and repeated images were identically acquired and processed. The spots grew progressively dimmer during nerve stimulation. (B) The average brightness of the fluorescent spots during continuous nerve stimulation is plotted. Bar, 5  $\mu$ m.

oles in Schwann cells). We then determined how many pixels ( $N$ ) in the first image exceeded the threshold level of brightness; usually this was 10–20% of the total number of pixels in the area of interest. Then, in subsequent images, we determined the average brightness of the  $N$  brightest pixels (and confirmed that they all lay within the confines of the terminal). In other words, we assumed that a stained vesicle which moved out of a spot carried with it a fixed increment of fluorescence brightness.

## Drugs and Other Agents

FM1-43 was obtained from Molecular Probes. It was stored frozen; aliquots of 1–3 mg/ml were used for 1–2 mo.

OA and calyculin were purchased from LC Services (Woburn, MA), dissolved in stock DMSO, and stored frozen. The effects of OA were tested on more than 40 preparations; calyculin was tested on two preparations. Concentrations ranged from 1–5  $\mu$ M, dissolved in DMSO (1  $\mu$ l/ml). At this concentration, both OA and calyculin should act as nonspecific inhibitors of phosphatases.

Colchicine (No. C9754; Sigma Immunochemicals, St. Louis, MO) was

used at a concentration of 2  $\mu$ M. Preparations ( $N = 5$ ) were pretreated for 2 h, and then stained with FM1-43.

Cytochalasin D (No. C-8273; Sigma Immunochemicals) was dissolved in stock DMSO (1  $\mu$ l/ml) and applied to 17 preparations at 2–20  $\mu$ M (usually 20  $\mu$ M). Preparations were pretreated for 2–3 h before FM1-43 staining. Some preparations were destained by exposure to 30–45 mM potassium (3–4 min) rather than by electrical stimulation of the nerve.

TPA (phorbol 12-myristate-13 acetate; No. P-145; Research Biochemicals, Natick, MA) was tested in three experiments. One preparation was incubated for 1 hr in 200 nM TPA before staining. In two cases, muscles were first stained with FM1-43 and then incubated for 1 h in TPA.

8-Br-cAMP (8-bromoadenosine 3':5'-cyclicmonophosphate; No. B7880; Sigma Immunochemicals) was tested on two preparations. Terminals pre-stained with FM1-43 were not affected by 0.5 mM 8-Br-cAMP (2 h).

Bath temperature was elevated with a thermo-electric module, and monitored with a bead thermister. Two preparations were tested.

### Electron Microscopy

Some muscles were stained with FM1-43 before fixation. In these cases, they were visually checked before fixation to verify that the staining was successful. Muscles were fixed in 2.5% glutaraldehyde for 1 h, post-fixed in 1% OsO<sub>4</sub> for 1 h, dehydrated, embedded in Epon, and sectioned. Sections were stained with 5% uranyl acetate in 70% methanol for 10 min, placed in Reynold's lead citrate for 5 min, and viewed with a Philips CM10 electron microscope.

## Results

### Control Muscles

A typical control experiment is shown in Fig. 1. The prepara-

tion was first stained with FM1-43 and washed. The motor nerve terminal shown in Fig. 1 *A* (left) shows characteristic fluorescent spots, and previous work suggests that each spot is a cluster comprising several hundred stained synaptic vesicles (Betz et al., 1992*a,b*; Betz and Bewick, 1992, 1993). Repeated images were acquired while the nerve was stimulated at a frequency of 2 Hz (Fig. 1 *A*). The fluorescent spots progressively dimmed, without moving or changing shape, during more than 30 min of nerve stimulation. The average brightness of all spots during destaining is plotted in Fig. 1 *B*. The rate of destaining is highly dependent on the rate of nerve stimulation. For example, at 30 Hz, destaining occurs in about 3 min (Betz and Bewick, 1993). We interpret the dimming as reflecting loss of dye during exocytosis of stained synaptic vesicles.

### Treatments Giving Negative Results

We tested a number of drugs and other agents for effects on staining, on prestained terminals, and on destaining. With one exception (okadaic acid; see below), results were negative. In two experiments, we attempted to disrupt fluorescent spots with high temperature. The temperature was first raised to 33°C for 30 min, and then to 38–40°C for 15–20 min. Some dimming occurred at the elevated temperatures, which may have arisen due to increased spontaneous exocytosis. The fluorescent spots did not change shape or blur. Other treatments giving negative results included TPA

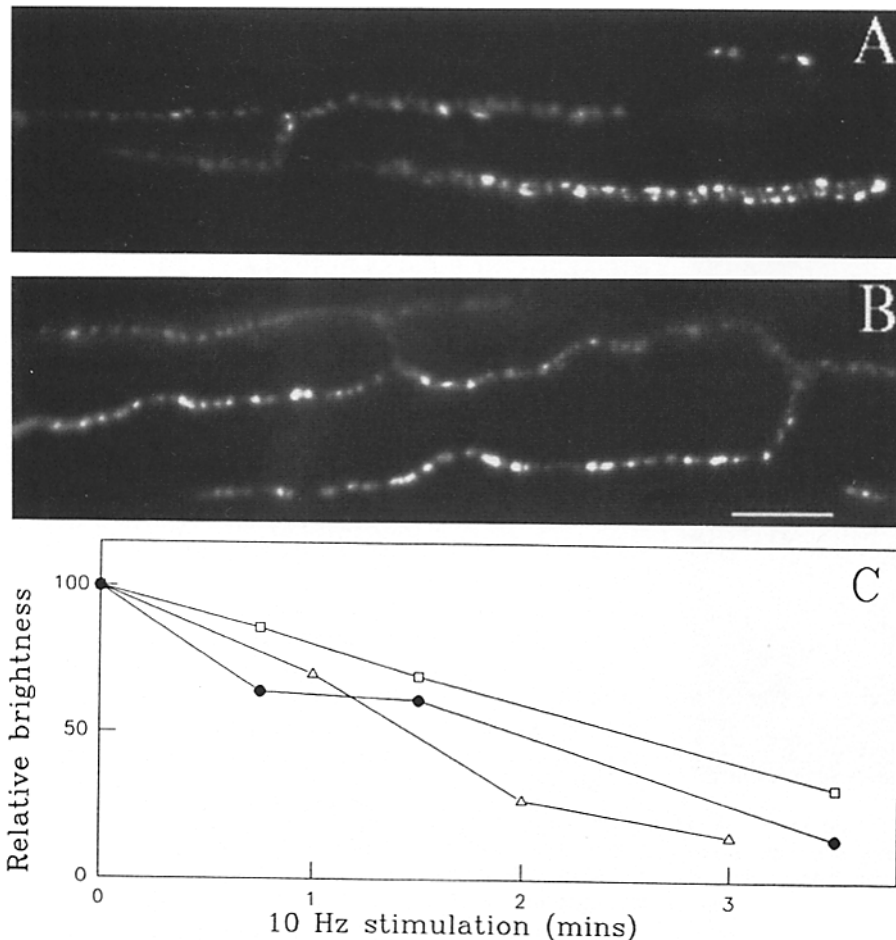


Figure 2. Cytochalasin D had no significant effect on staining or destaining. (*A*) Control terminal stained with FM1-43. (*B*) Terminal from the contralateral muscle, which was exposed to cytochalasin D (CD; 20  $\mu$ M) for 3 h before staining with FM1-43 (in the presence of CD). Both terminals show typical fluorescent spots, and, in general, nerve terminals from control and CD-treated muscles were indistinguishable. (*C*) Time course of destaining of contralateral control terminal ( $\square$ ), average of three terminals destained with 10 Hz stimulation in an earlier study ( $\Delta$ ; from Betz and Bewick, 1993, see Fig. 5), and CD-treated terminal ( $\bullet$ ). Bar, 10  $\mu$ m.

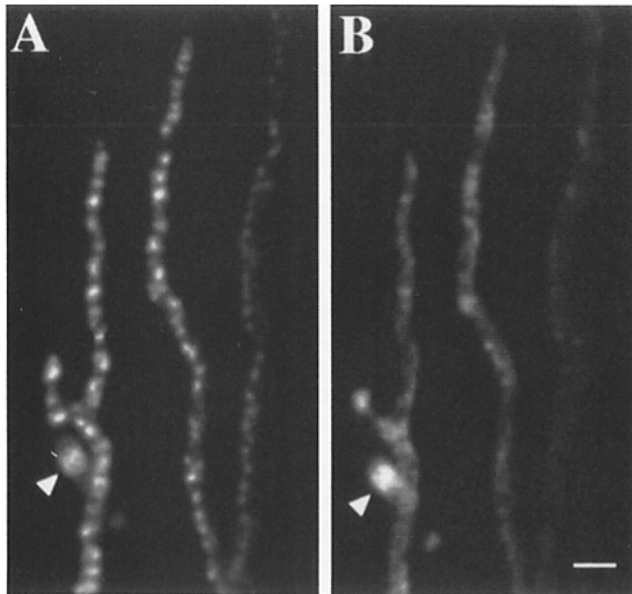
(PMA, activates protein kinase C), 8-Bromo-cyclic AMP (activates protein kinase A), colchicine (disrupts microtubules), the vehicle DMSO (1–10  $\mu$ l/ml), and cytochalasin D (disrupts actin polymerization and depolymerization). For example, Fig. 2 shows results from an experiment in which a preparation was exposed to 20  $\mu$ M cytochalasin D (CD) for 3 h, then stained, examined, and finally destained. The CD had no significant effect on any of these processes. Control terminals (Fig. 2 *A*) and CD-treated terminals (Fig. 2 *B*) were not distinguishable, nor were the rates of destaining of controls (Fig. 2 *C*, *open symbols*) and CD-treated terminals (Fig. 2 *C*, *filled symbols*) significantly different.

Finally, we wondered if intense nerve stimulation in the absence of transmitter release might disrupt vesicle clusters. We bathed stained preparations ( $N = 2$ ) in  $\text{Ca}^{2+}$ -free Ringer ( $\text{Ca}^{2+}$  replaced with  $\text{Mg}^{2+}$ ) and stimulated the nerve at 30 Hz for 5 min. Fluorescent spots destained slightly ( $\sim 10\%$ ), but did not move or change shape (data not shown).

### Blurring of Fluorescent Spots after Exposure to Okadaic Acid

Typical results with OA are shown in Fig. 3. Before OA was applied, the fluorescent spots were crisply punctate (Fig. 3 *A*), but after 30 min in OA, the fluorescent spots had become less distinct (Fig. 3 *B*). This blurring was not an artifact of suboptimal focussing of the microscope. For example, in a few experiments we took images in focal planes spanning the optimum focus, both before and after exposure to OA. Later, when we compared best focus images at each time point, it was clear that spots in OA-treated preparations had blurred (data not shown).

Terminals usually dimmed by 10–25% during exposure to OA for 30–60 min (22% in Fig. 3). Thus, another possible



**Figure 3.** OA disrupted fluorescent spots. (*A*) Control image, taken before OA was applied. (*B*) Same field, 40 min after exposure to 2  $\mu$ M OA. Nearly all spots have become blurred. The arrow head points to a fluorescent spot which grew brighter during exposure to OA. This spot was located adjacent to a Schwann cell nucleus.

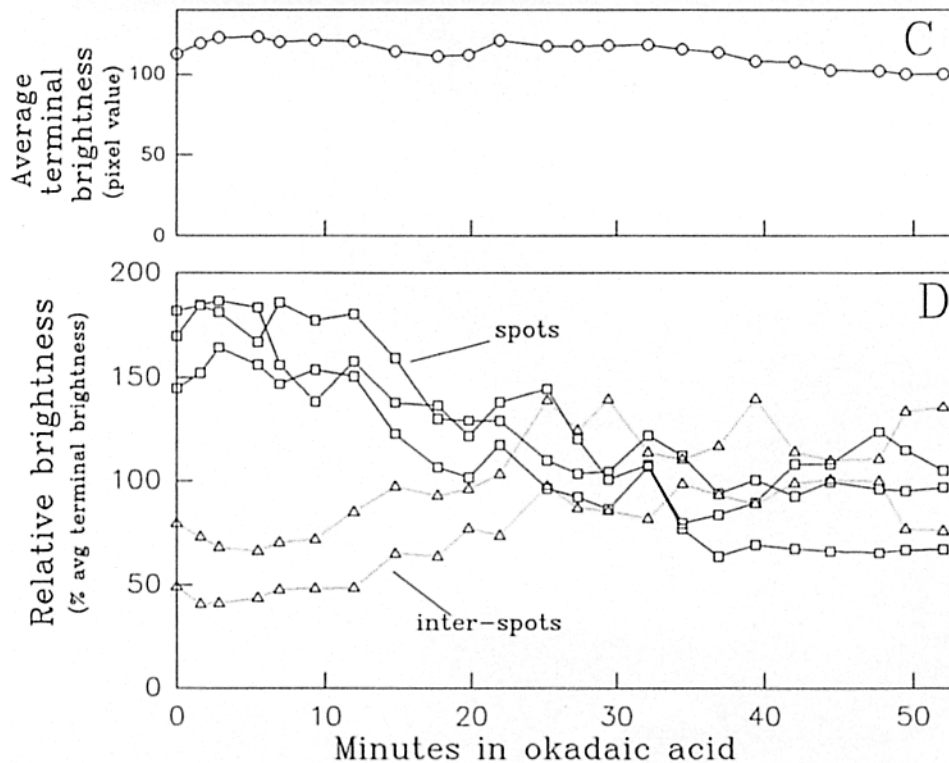
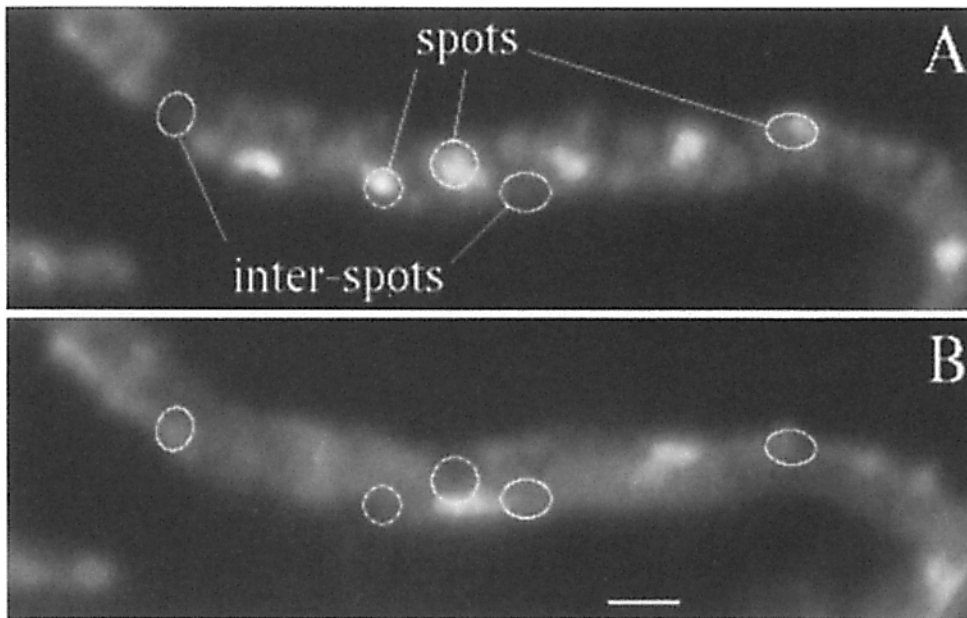
explanation for the blurring is loss of contrast as the fluorescent spots grew dimmer. To test this possibility, we followed the brightness of selected regions within the terminal over time. We found that the dimming of fluorescent spots was usually accompanied by brightening of regions between spots. In Fig. 4, several bright spots and dim “inter-spot” regions are marked before (Fig. 4 *A*) and after (Fig. 4 *B*) exposure to OA. In Fig. 4 *C* is plotted the average brightness of the entire terminal during OA exposure; it dimmed by 11%. In Fig. 4 *D* are plotted the brightnesses of the spots and inter-spots marked in Fig. 4 (*A* and *B*). Data in Fig. 4 *D* are plotted as a percentage of the average brightness of the entire terminal at each time point. Thus, 100% represents the average brightness of the entire terminal at any particular time. The bright spots (*squares*) grew dimmer and the dim regions between spots (*triangles*) grew brighter during exposure to OA. Fig. 4 *D* also shows the time course of OA action: there was a 5–10 min latency after OA was added, and most blurring occurred over the next 10–20 min.

### Electron Microscopy

We examined three muscles exposed for 1 h to OA; contralateral muscles served as controls. Nerve terminals were easier to find in cross sections, and with these we confirmed that the ultrastructure of individual organelles in OA-treated terminals was not readily distinguishable from controls. Longitudinal sections provided information about the distribution of vesicles within the terminals. Fig. 5 *A* shows typical examples of control and OA-treated terminals. The positions of synaptic vesicles in those terminals are shown in Fig. 5 *B*, and the densities of vesicles along the length of the terminals (ignoring distances from the presynaptic membranes) are shown in Fig. 5 *C*. It is clear that vesicles were clustered in the control, and distributed more diffusely in the OA-treated terminal. This difference was present even in terminals which, by virtue of relatively large Schwann cell processes interposed between muscle and nerve terminal, appeared to have been sectioned near their lateral margins.

### Active Dye Movements in Okadaic Acid

The impression gained by inspecting single fluorescence images was straightforward: in most regions of OA-treated terminals, dye appeared to have spread passively, producing a reasonably uniform spatial distribution throughout the terminal. This conclusion, however, belied a more complex, dynamic process which became evident when we viewed the same images as time lapse movies (i.e., images displayed in rapid succession). It then immediately became clear that most dye movements did not at all resemble simple passive diffusion down a gradient of dye concentration. On the contrary, evidence of active movements were plentiful. Most commonly, dye appeared to stream longitudinally inside the terminal. Some spots appeared to fragment and coalesce with their neighbors. Sometimes, an entire spot appeared to churn in place. Dye movements occurred in both proximal and distal directions: however, the tips of terminals usually became depleted of dye. These different types of movement could exist within a distance of only a few micrometers. For example, in wide terminals fluorescent spots are sometimes arranged in lateral pairs (Betz et al., 1992a). In these cases the members of a pair often behaved independently; some-

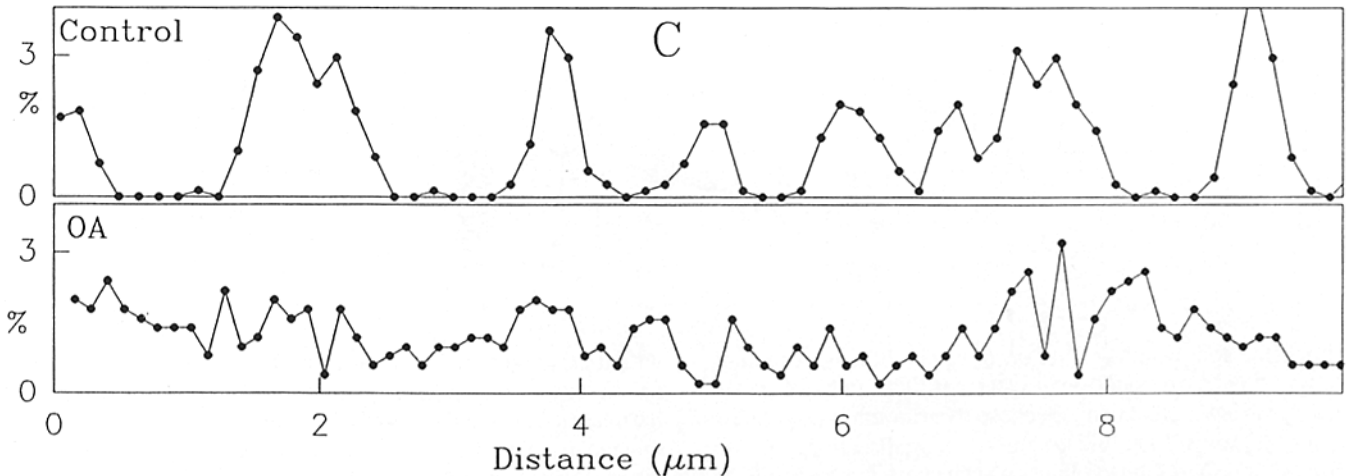
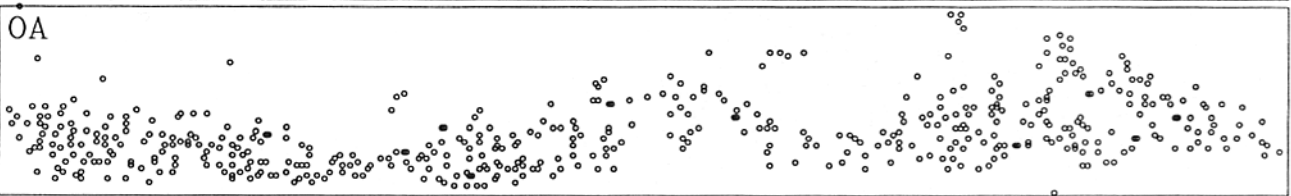
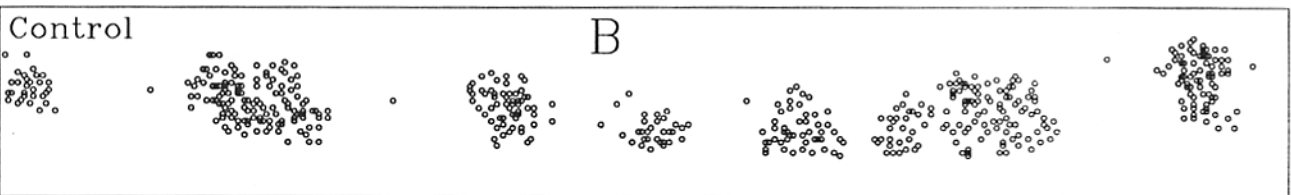
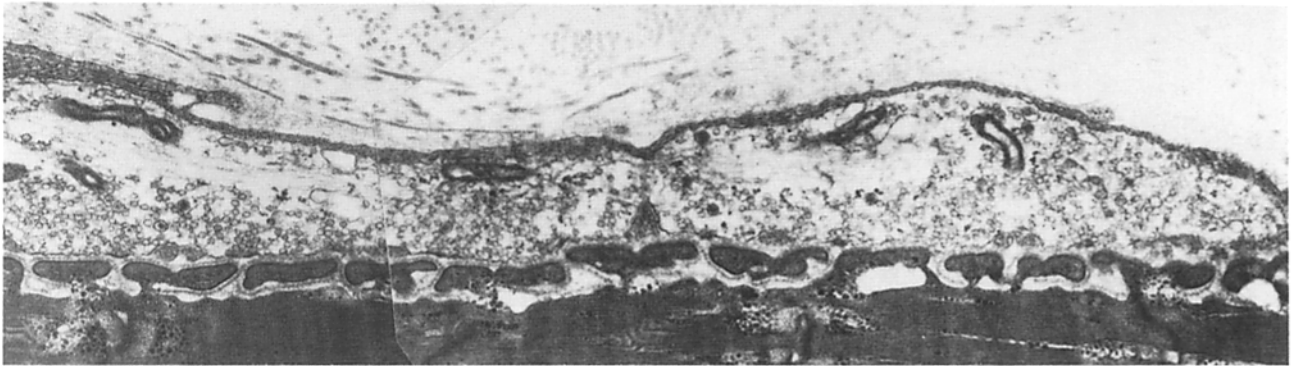
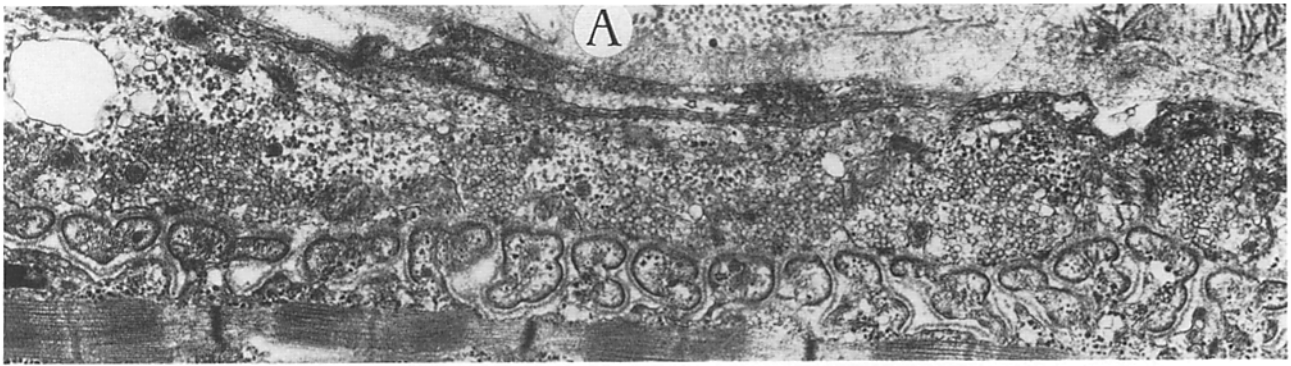


**Figure 4.** Spots began to break up about 10 min after exposure to OA. (A) Control image before OA exposure. Three fluorescent spots and two dim "inter-spots" are marked. (B) Same terminal after a 50-min exposure to OA. Fluorescent spots have nearly disappeared, and the brightnesses of the marked areas are approximately equal. (C) The average brightness of the entire terminal in A changed little during exposure to OA. (D) The average brightnesses of the regions marked in A are plotted against time in OA. Starting 5–10 min after OA was added, spots began to grow dimmer, and "inter-spots" began to grow brighter. After 20–30 min in OA, they were equally bright. Bar, 2  $\mu$ m.

times one spot would remain unchanged while dye streamed longitudinally to and from its partner. The rates of movements varied a great deal. Because dye moved in both directions, it was impossible to measure unidirectional movements, and therefore to quantify accurately the velocities of movements. In general, it appeared that the fastest rates were several micrometers per minute. We followed terminals for up to 1 h, and during this time the movements did not inactivate, but continued unabated. Some spots remained identifiable for the entire time, while others blurred and coalesced

with their neighbors into uniformly stained regions of terminal. In summary, while OA treatment disrupted the normal fluorescent spots and caused dye to spread out within the terminals, the spatial distribution was not entirely uniform, and the movements were clearly driven at least in part by active processes.

With one exception, we were unable to predict the type of dye movement at any particular region of the terminal. The exception concerns the location of Schwann cell nuclei, which were good predictors of sites of large dye accumula-

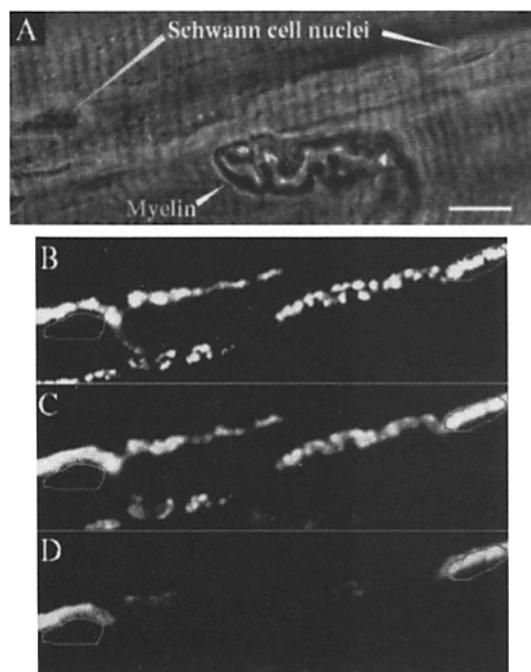


**Figure 5.** Electron microscopy showed that vesicle clustering was disrupted by OA. The preparation was stained with FM1-43, treated with OA ( $5 \mu\text{M}$ , 1 h), and then fixed and prepared for electron microscopy. (A) Longitudinal sections of control (top) and OA-treated muscles. The ultrastructure appears generally normal, with intact synaptic vesicles, mitochondria, and other organelles. (B) Each circle marks the position of a vesicle in the micrographs of A and B. Distinct clusters are evident in the control (top), but not in the OA-treated preparation (bottom). (C) Vesicle density profiles along the length of the terminal. The greater degree of clustering along the terminal length in the control (top) compared to the OA-treated preparation (bottom) is evident. Data are normalized for each terminal (total numbers of vesicles: 517 [control]; 500 [OA]). Distances from the presynaptic membrane were ignored in calculating densities. The scale on the bottom X axis applies to all panels.

tions during exposure to OA. That is, dye accumulated not inside the Schwann cells, but in the nerve terminals at sites adjacent to Schwann cell nuclei. Two examples are shown in Fig. 6. Schwann cell nuclei can be seen in the brightfield image (Fig. 6 *A*). Their outlines are drawn in the three fluorescence images. Fig. 6 *B* shows the control image. Fig. 6 *C* shows the same terminal after 20 min in OA. The fluorescent spots have become blurred, and the regions near Schwann cell nuclei have become relatively brighter than the other regions (this was particularly so for the terminal branch on the right). Fig. 6 *D* was obtained after the nerve had been stimulated (10 Hz for 2 min). The difference between the two regions is more apparent, not because dye loss was inhibited in regions near Schwann cell nuclei, but because they contained more dye when the stimulation began. Other examples of dye accumulation adjacent to Schwann cell nuclei can be seen in Figs. 3 and 7 (*arrowheads*). While such dye accumulations were good predictors of locations of Schwann cell nuclei, they did not occur at every one.

#### Attempts to Block the Action of OA

We tried to block the actions of OA in three different ways: exposure to colchicine, cytochalasin D, and  $\text{Ca}^{2+}$ -free Ringer ( $\text{Ca}^{2+}$  replaced with  $\text{Mg}^{2+}$ ). None of these treatments significantly altered the effects of OA. As illustrated in Fig. 7, colchicine ( $2 \mu\text{M}$ ) was applied for 2 h, and then the preparation was stained with FM1-43 (Fig. 7 *A*), then OA ( $2 \mu\text{M}$ ) was applied, and the fluorescent spots blurred in the usual fashion (Fig. 7 *B*). Finally, the nerve was stimulated, and the terminal destained (Fig. 7 *C*). Thus, colchicine had no sig-



**Figure 6.** OA caused dye to accumulate near Schwann cell nuclei. (*A*) Bright field image of muscle fiber. Myelin and two Schwann cell nuclei are visible. (*B*) Fluorescence view of same field as *A*, before OA was applied. Regions of Schwann cell somata are outlined in this and following panels. (*C*) Same field, 40 min after exposure to  $2 \mu\text{M}$  OA. (*D*) Same field after partial destaining produced by stimulation of the nerve (20 Hz for 2 min). Bar,  $10 \mu\text{m}$ .

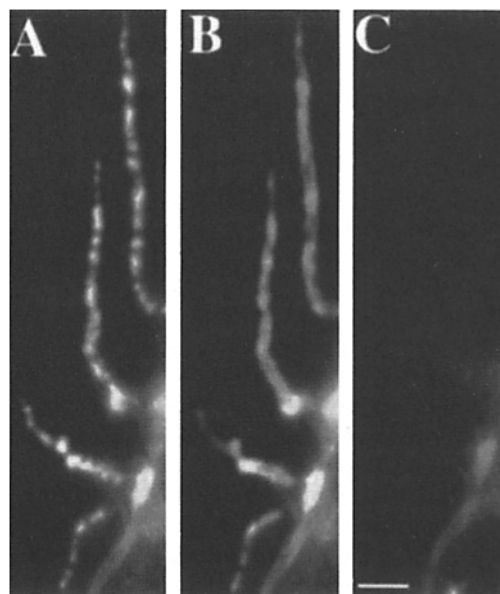
nificant effect on FM1-43 staining, or on the blurring of fluorescent spots caused by exposure to OA, or on subsequent destaining produced by nerve stimulation. Similar results were obtained with CD treatment and with removal of external  $\text{Ca}^{2+}$  (results not shown).

#### Irreversibility of Okadaic Acid Treatment

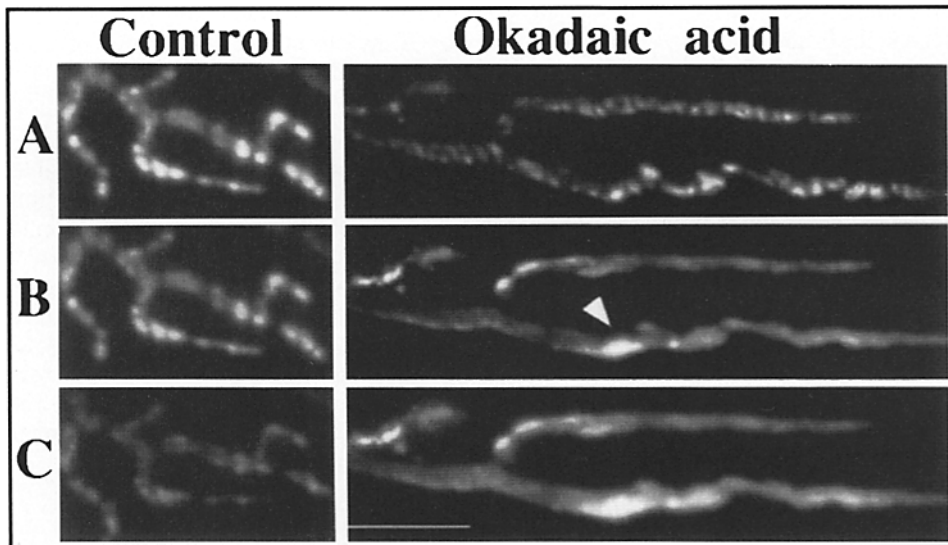
As shown in Fig. 8, the effects of OA were not reversible; fluorescent spots did not reform after OA was washed from the chamber. A control terminal from the contralateral muscle is shown on the left. Control spots dimmed by  $\sim 40\%$  over a 6-h period, but the spots did not move, change shape, or become blurred. The spots in experimental terminals (right side) were severely disrupted by treatment with OA for 1 h (middle panel), but after a 6-h wash in normal Ringer, no sign of recondensation of bright spots was observed. Little or no dimming occurred during the wash period, although the terminal appeared slightly swollen at the end.

#### Electrophysiology

OA had complex effects on evoked transmitter release (Fig. 9). In this experiment, the nerve was stimulated at 30 Hz for 2 s every 1–3 minutes. End plate potentials (EPPs) were recorded from a muscle fiber impaled with an intracellular microelectrode. The preparation was curarized to block muscle contractions. Control EPP peak amplitudes (Fig. 9 *A*) showed a characteristic envelope during high frequency stimulation: EPP amplitudes first increased, and then decreased to a low level during the 2 s stimulus train. When OA was added to the chamber, EPP amplitudes increased immediately (Fig. 9 *B*), and then declined, usually to 50–80% of the control amplitude (Fig. 9 *C*). Finally, when OA was washed from the chamber, EPP amplitudes declined further,



**Figure 7.** Colchicine did not block the effects of OA. The preparation was exposed to  $4 \mu\text{M}$  colchicine during the entire experiment (for 2 h before staining with FM1-43, 45 min during washing, and about 30 min during OA exposure and destaining). (*A*) Control image shows typical punctate staining pattern. (*B*) Spots have become blurred after 20 min in OA. (*C*) The terminal destained thoroughly (30 Hz nerve stimulation for 3 min).



**Figure 8.** Once disrupted by OA treatment, fluorescent spots did not reform. (A) Images taken after staining with FM1-43 but before OA treatment. (B) Images of the same terminals taken after the preparation in the right hand panel had been exposed to 2  $\mu$ M OA for 1 h. The fluorescent spots in the OA-treated preparation were disrupted. (C) Both preparations were then washed for 5 h in normal Ringer, and reimaged. The fluorescent spots in the control muscle dimmed by  $\sim$ 40%, but did not change position or shape. The appearance of the OA-treated terminal did not change very much. One spot (arrowhead) in the lower branch grew larger and brighter. This was the site of a Schwann cell nucleus. Bar, 10  $\mu$ m.

to about one-third of their initial values (Fig. 9 D). The amplitudes of the first EPP in each train are plotted in Fig. 9 E. We observed similar effects in four other experiments. In five separate experiments, without added curare, we studied spontaneous miniature EPPs (MEPPs). Although not quantified in detail, MEPP amplitudes and frequencies were clearly affected by OA little, if at all. Finally, in two experiments we observed that post-tetanic potentiation could be evoked (by 50 Hz nerve stimulation for 1 min after OA treatment; 4–5  $\mu$ g/ml curare present to block muscle contractions). Its amplitude and time course, like that of MEPPs, did not appear to be distinguishable from normal. Thus, while further electrophysiological studies will be necessary to quantify electrophysiological effects, it is clear that the major change is a gradual reduction in the amplitude of evoked EPP amplitudes, which probably reflects a reduction in the amount of transmitter released per stimulus.

#### **Destaining after Okadaic Acid**

As shown in Fig. 10, terminals treated with OA could undergo activity-dependent destaining. A low power view of a terminal is shown in Fig. 10 A. The region in the rectangle is shown enlarged in Fig. 10 B (control), after treatment with OA (Fig. 10 C), and after repetitive nerve stimulation (30 Hz for 10 min; Fig. 10 D). The rates of destaining of several separate regions are shown in Fig. 10 E (open circles). The rates are considerably slower than control muscles (filled circles; average of three experiments, control data from Betz and Bewick, 1993, see Fig. 5).

The effects of calyculin were tested in two preparations, and were indistinguishable from OA. Fluorescent spots blurred, and the terminals could still be destained by nerve stimulation. Further studies will be required to determine if more subtle differences exist between these two structurally different phosphatase inhibitors.

#### **Pretreatment with Okadaic Acid**

Having studied the effects of OA on prestained terminals, we

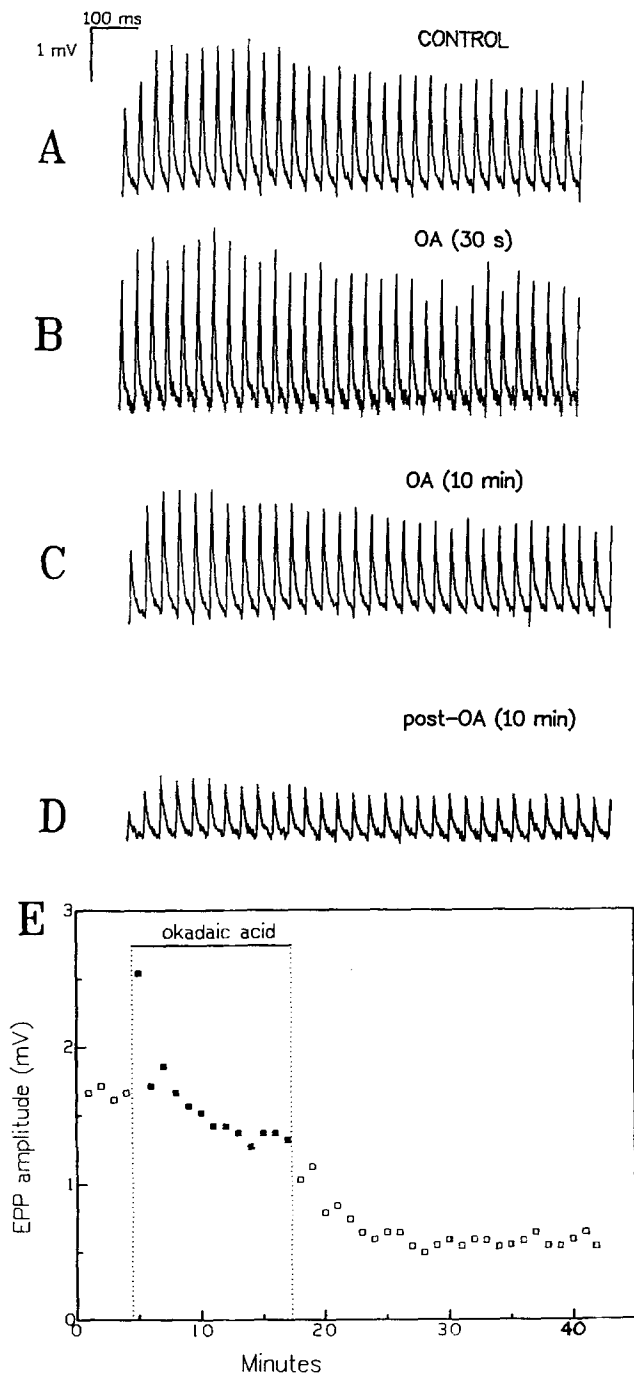
next reversed the order, first treating with OA, and then staining with FM1-43. As shown in Fig. 11, activity-dependent staining clearly occurred after OA pretreatment, but the staining was abnormal (Fig. 11 A). Crisp fluorescent spots typical of a normal preparation were infrequent, and the rather diffuse quality of the staining resembled terminals in which OA was applied after, rather than before staining with FM1-43. Terminals pretreated with OA could be destained with repetitive nerve stimulation (Fig. 11 B), but the rate was slower than normal (Fig. 11 C). Moreover, the terminals lost only about one-half of the dye. The incomplete destaining was not due to a loss of nerve terminal excitability, since subsequent exposure to 60 mM  $[K^+]$  for 5 min (a depolarizing stimulus sufficient to destain completely control preparations) caused additional destaining of only about 10% (data not shown). Perhaps OA treatment partially inhibits the vesicle recycling process, so that dye becomes trapped in cisternae or nascent recycling synaptic vesicles, and thus cannot be released by subsequent nerve stimulation.

#### **Discussion**

We have shown that the phosphatase inhibitor OA disrupts fluorescent dye spots in frog motor nerve terminals stained with FM1-43. The parallel disruption of synaptic vesicle clusters observed with the EM lends further support to the hypothesis that FM1-43 marks recycled synaptic vesicles. We conclude, therefore, that vesicle clustering depends on the state of protein phosphorylation in the terminal. This conclusion is consistent with the theory of synapsin I action (Greengard et al., 1993), for which increasing evidence suggests that synaptic vesicles are immobilized in resting terminals by the binding action of dephospho-synapsin I, and that, during nerve activity, appropriate phosphorylation of synapsin I frees the vesicles from their resting bonds.

How vesicles, once mobilized, then get to the presynaptic membrane is not well understood. Our previous work (Betz et al., 1992b) was consistent with movement by Brownian





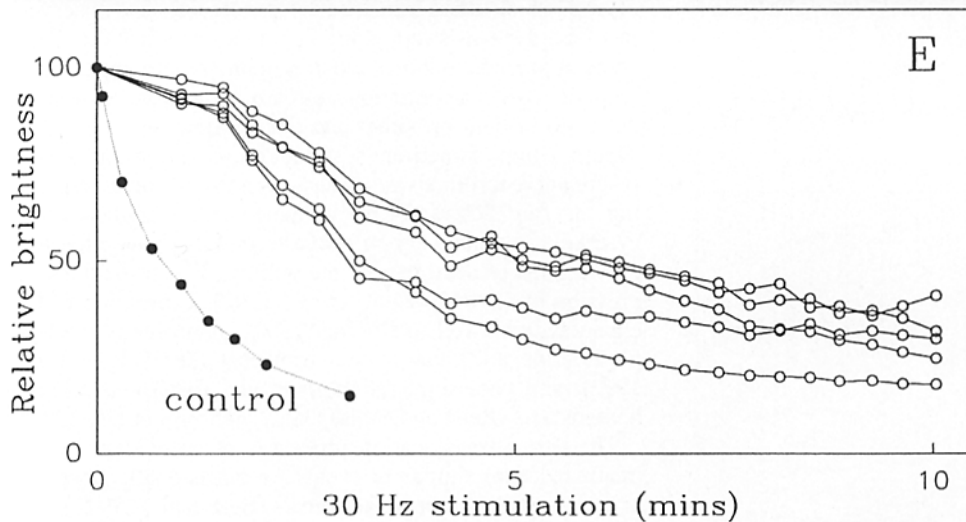
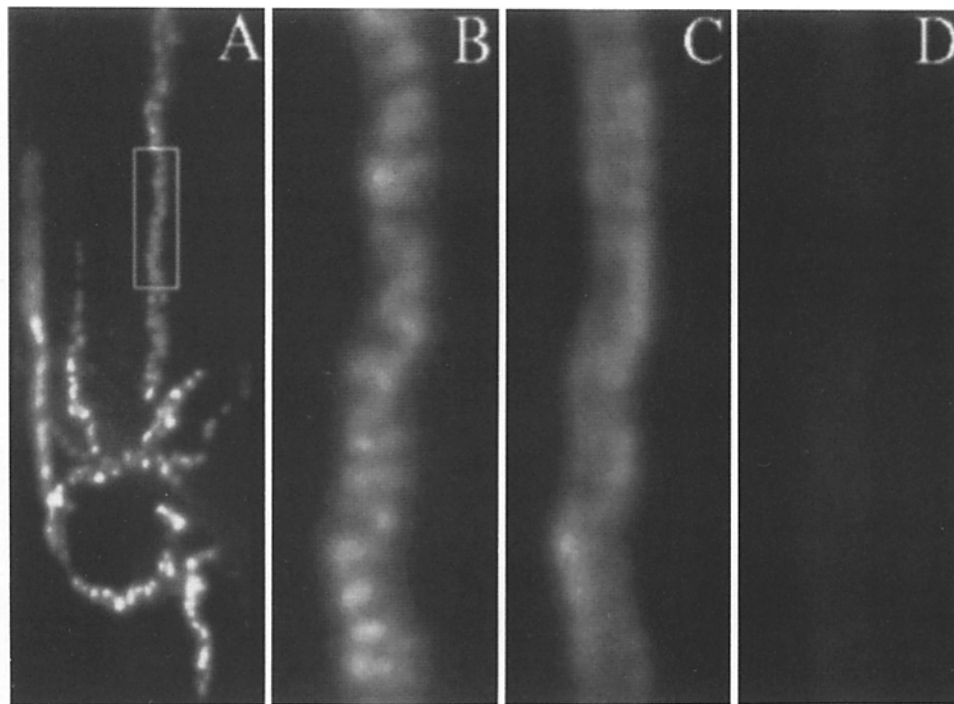
**Figure 9.** OA treatment reduced the amplitudes of evoked synaptic potentials. The effect of OA ( $2 \mu\text{M}$ ) on nerve-evoked EPPs was recorded with an intracellular microelectrode. The preparation was bathed in normal Ringer containing  $4.5 \mu\text{g/ml}$  curare to block muscle contractions. Stimulus trains (30 Hz for 2 s) were given once a minute. (A) Control EPPs revealed a typical pattern: amplitudes increased during the first few EPPs, and then decreased to a steady level. (B) Immediately after OA was added, EPP amplitudes increased transiently. (C) After 10 min in OA, EPP amplitudes reached a steady state which was  $\sim 80\%$  of control amplitudes. (D) When OA was washed from the chamber, EPP amplitudes decreased further, to  $\sim 30\%$  of controls.

motion, but did not rule out active translocation processes. Since fluorescent spots in FM1-43-stained terminals rarely change shape during nerve stimulation, some kind of cytoskeletal "cage" would be required to restrain freely moving vesicles and keep the cluster intact during nerve stimulation. Ultrastructural studies reveal no "bars" around vesicle clusters, but two cytoplasmic domains, distinguished by cytoskeletal differences, have been described in nerve terminals (Landis et al., 1988; Hirokawa et al., 1989). Thus, within clusters most cytoskeletal elements, including microtubules, neurofilaments, and actin (but see Hirokawa et al., 1990) are lacking, compared to the regions outside clusters. Perhaps synaptic vesicles are normally incapable of partitioning into the cytoplasm outside a cluster. This agrees with results of studies of diffusional movements of inert particles of different sizes, microinjected into tissue culture cells, which have shown that diffusion is increasingly hindered for larger particles. Particles larger than 50 nm were virtually immobile; this is close to the size of a synaptic vesicle (Luby-Phelps et al., 1987).

OA caused fluorescent spots to blur, but the dye, having escaped from a spot, did not spread uniformly throughout the terminal, as might be expected if synaptic vesicles were moving passively to a uniform spatial distribution. Instead, clear signs of active translocation processes were observed, in which dye was swept along in a concerted fashion. Some spots appeared to churn and fragment, others appeared to empty of dye in a continuous, streaming fashion. Movement occurred in both proximal and distal directions. Giant dye accumulations sometimes resulted; these were found at sites where nerve terminals passed a Schwann cell soma, suggesting that the Schwann cell influences the organization of cytoskeletal machinery in nerve terminals. This adds to a growing list of neuron-glia interactions, which includes expression of GAP43 (Woolf et al., 1992), expression of ion channels (reviewed in Ritchie, 1992), neurofilament phosphorylation and slow axonal transport (de Waegh et al., 1992), and potassium (Nicholls et al., 1992) and calcium homeostasis (Reist and Smith, 1992; Jahromi et al., 1992).

The active translocation processes made evident by OA treatment were similar to events we occasionally observed in earlier work on normal terminals (Betz et al., 1992b). For example, on relatively rare occasions we observed dye movements between fluorescent spots, as if one spot was "captured" by its neighbor. Such events were qualitatively indistinguishable from movements induced by OA. Perhaps OA simply activated this latent endogenous mechanism, whose normal function appears to be to move vesicles between clusters. However, it is also possible, although unlikely, that OA does just the opposite, namely to slow down rapid (but infrequent) constitutive vesicular traffic between clusters, causing a larger fraction of vesicles at any instant to reside in regions between clusters, and thereby blurring fluorescent spots. Measurement of unidirectional dye movements will be required to test rigorously this possibility.

The nature of the active vesicle translocators is unknown. Our observation that colchicine had no effect on the actions of OA suggests that microtubules are not involved in the process. Rat brain synaptic vesicles injected into squid preterminal axons are transported in an anterograde direction at a rate of about  $0.5 \mu\text{m/s}$ , and this movement was blocked by reducing external  $[\text{Ca}^{2+}]$  (Llinas et al., 1989). The move-



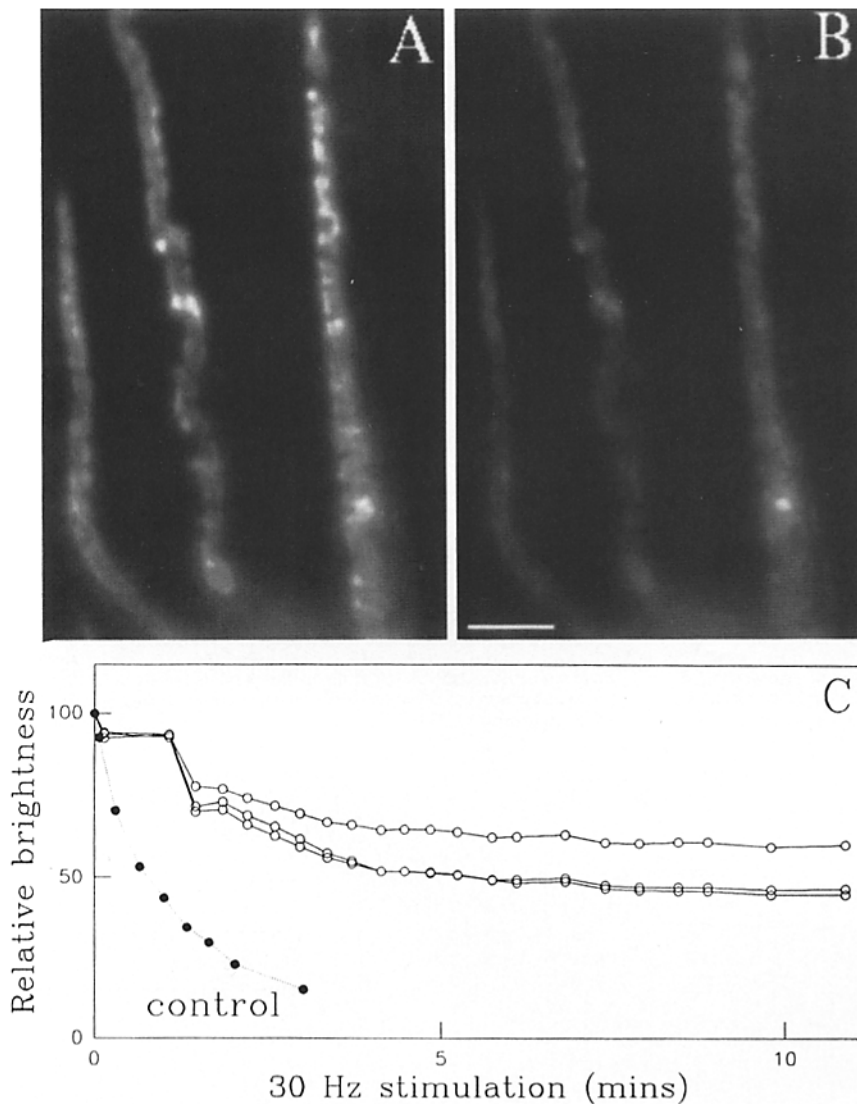
**Figure 10.** OA-treated terminals destained in an activity-dependent fashion, but the rate of destaining was slower than normal. (A) Low power view of terminal stained with FM1-43. The region within the rectangle is shown enlarged in B-D. The rectangle is 15  $\mu\text{m}$  in height. (B) Control image showing typical punctate staining. (C) Same field after exposure to 2  $\mu\text{M}$  OA for 40 min. (D) Same field after continuous nerve stimulation (30 Hz) for 10 min. (E) Time course of destaining. Open circles show brightness of several fluorescent regions during 30 Hz nerve stimulation. Filled circles show destaining rate of normal terminals (30 Hz stimulation; from Betz and Bewick, 1993, see Fig. 5).

ments we observed were about an order of magnitude slower than this, occurred in both directions, and were not blocked by removal of external  $\text{Ca}^{2+}$ .

The physiological effects of OA in general matched the optical effects. For example, the amplitudes of evoked EPPs and the rate of destaining in response to nerve stimulation were reduced by comparable amounts (40–60%) after OA treatment, as might be expected if vesicles were no longer clustered at presynaptic release sites. Inhibitory effects of OA on exocytosis have been reported for several cell types, including endocrine (Michimata et al., 1992; Ratcliff and Jones, 1993; Corvera et al., 1991) and exocrine (Wagner et al., 1992; Takuma and Ichida, 1991) cells. However, two other electrophysiological studies of frog neuromuscular transmission have shown that OA increases transmitter release, perhaps reflecting increased action of protein kinase

C (Abdul-Ghani et al., 1991; Swain et al., 1991). In these studies, transmitter release was reduced to low levels (by reducing  $[\text{Ca}^{2+}]$  and raising  $[\text{Mg}^{2+}]$ ); in the present work, release levels were normal. Perhaps under conditions of low release the delivery of replacement vesicles to the presynaptic membrane is not rate limiting, so that other effects of OA treatment become apparent.

None of the other agents we tested disrupted vesicle clusters, or significantly affected activity-dependent staining or destaining. We were particularly interested in cytochalasin D, since it has been proposed that actin filaments (coupled to a molecular motor) might mediate vesicle movements to the presynaptic membrane (Hirokawa et al., 1989). Moreover, changes in the state of actin polymerization have been postulated to govern exocytosis (Aunis and Bader, 1988; Sontag et al., 1988; Nuae and Lindau, 1988; Bernstein and



**Figure 11.** Pretreatment with OA disrupted the normal staining pattern. The preparation was exposed to 2  $\mu$ M OA for 1 h, and then washed. It was next stained with FMI-43 in the usual way. (A) The brightness of staining was approximately normal, but the pattern of staining pattern was abnormal. Relatively few distinct fluorescent spots, characteristic of controls, formed. For example, the upper region of the right hand terminal branch was fairly normal, but the other regions in this terminal were not. In general, such preparations were not readily distinguishable from terminals treated with OA after staining with FMI-43. (B) Same field after continuous nerve stimulation (30 Hz) for 10 min. (C) Time course of destaining. Open circles show brightness of several spots during 30 Hz nerve stimulation. Filled circles show destaining rate of normal terminals (30 Hz stimulation; from Betz and Bewick, 1993, see Fig. 5). The destaining after OA pretreatment was slower than normal, and incomplete.

Bamburg, 1989; but see Nakata and Hirokawa, 1992), and synapsin I in vitro interacts with actin in a phosphorylation-dependent manner (Valtorta et al., 1992). Cytochalasin D disrupts processes which depend on changes in actin polymerization or depolymerization (Cooper, 1987; Smith, 1988). The absence of effects of cytochalasin D in our studies suggests that changes in the state of actin polymerization do not play a large role in vesicle mobilization or movement to the presynaptic membrane at the frog neuromuscular junction.

Steve Fadul provided unfailing technical assistance throughout this work. Drs. Bruce Wallace, John Heuser, Antony Ridge, and Paul Fuchs provided valuable comments on the manuscript. Ms. Marjorie Alders provided expert assistance with electron microscopy.

Supported by National Institutes of Health research grants NS10207 and NS23466 to W. J. Betz, and a Human Frontiers Science Fellowship to A. W. Henkel.

Received for publication 24 November 1993.

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