Fast Axonal Transport of Foreign Synaptic Vesicles in Squid Axoplasm

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ABSTRACT Translocation of intracellular organelles requires interaction with the cellular cytoskeleton, but the membrane and cytoskeletal proteins involved in movement are unknown. Here we show that highly purified synaptic vesicles from electric fish added to extruded squid axoplasm can show ATP-dependent movement. The movement is indistinguishable from that of endogenous vesicles and has a slight preference for the orthograde direction. In the presence of a nonhydrolyzable ATP analog, the synaptic vesicles bind to axoplasmic fibers but do not move. Elastase treatment of vesicles inhibits both binding and movement. We conclude that a protein component on the surface of cholinergic synaptic vesicles from electric fish is conserved during evolution and so can be recognized by the organelle-translocating machinery of the squid axon, resulting in ATP-dependent movement. Synaptic vesicles apparently retain the capacity for fast axonal transport, even after they reach their intracellular destination.

Membrane-bound organelles are actively translocated in most cell types (1). A particularly well-studied type of organelle translocation is the movement of membrane components in the axons of nerve cells at very rapid rates (2–5 μm/s [see references 2 and 3]). Morphological studies have shown that some organelles, such as mitochondria, can be transported in both the retrograde and anterograde directions, whereas small vesicles and tubules prefer the anterograde, and larger multivesicular membranes the retrograde direction (4–7). Included in the axonal components that migrate at fast speeds in both directions are the proteins of the synaptic vesicle (8, 9), although it is not known what type of membrane organelle transports them. Synaptic vesicles accumulate at nerve terminals, often filling much of the cytoplasmic space. Accumulation of synaptic vesicles in nerve terminals could be explained if, for example, their membrane lacked some component required for retrograde translocation.

Recently developed reconstituted systems that accurately reproduce the translocations seen in whole axons provide an opportunity to ask if purified synaptic vesicles retain their translocation capacity. Allen video-enhanced contrast–differential interference contrast (AVEC-DIC) microscopy (10, 11) allows us to see the movement of endogenous synaptic vesicle–sized organelles moving along axonal cytoskeleton even after it is extruded from the axon (12, 13) and hypotonically or mechanically dissociated (14–16). Membrane fractions isolated from squid axoplasm retain their capacity to move when added back to extruded axoplasm or purified microtubules from brain or flagella (17–19).

We have purified and characterized acetylcholine-containing synaptic vesicles from the terminals of neurons that innervate the electric organ of marine rays (20, 21). Despite the species difference, we find that such synaptic vesicles are translocated efficiently by the extruded squid axoplasm. A protein component of the vesicle membrane appears to be required for the ATP-dependent translocation and for the binding of vesicles to cytoskeleton seen in the presence of a nonhydrolyzable ATP analog that inhibits translocation.

MATERIALS AND METHODS

Fluorescent Labeling of Synaptic Vesicles: Synaptic vesicles were prepared from the electric organ of the marine ray Dicophyra ornata as described in references 20 and 21. 10 μg of synaptic vesicles were incubated for...
4 h at 4°C in 200 µl of a pre-cleared solution of Texas Red (Molecular Probes Inc., Junction City, OR) in homogenization buffer (0.4 M NaCl, 10 mM HEPES, pH 7.4, and 10 mM EGTA). The labeling reaction was stopped by pelleting (30 min at 100,000 g in a Beckman airfuge) (Beckman Instruments, Inc., Palo Alto, CA) and the synaptic vesicles were washed twice by cycles of pelleting and resuspension in homogenization buffer.

As a control, the movement of fluorescent beads was analyzed. In two of five experiments, carboxylated latex/polystyrene beads ("fluoresbrite microspheres") (Polysciences, Inc., Warrington, PA) were preincubated with 15% bovine serum albumin in 150 mM NaCl for 1 h at 4°C, then washed as above. In the remaining three experiments, the beads were simply washed twice in homogenization buffer.

**Manipulations of Axoplasm**

Giant postganglionic axons were dissected from the squid *Loligo pealei* and the axoplasm was extruded onto a coverslip (13). Approximately 50–100 ng of fluorescent synaptic vesicles in 0.5 µl of half-strength buffer X (final composition of 175 mM K aspartate, 65 mM tartrate, 30 mM betaine, 67.5 mM MgCl₂, 5 mM EGTA, 1.5 mM CaCl₂, 0.5 mM ATP, 0.5 mM glucose, 10 mM HEPES, pH 7.2) was pipetted onto the axoplasm using polyethylene tubing (Clay Adams, Div. of Becton, Dickinson & Co., Parsippany, NJ) and a Hamilton syringe (Hamilton Co., Reno, NV). The axoplasm was gently spread apart with a forceps to disrupt it slightly and facilitate entry of fluorescent synaptic vesicles. The preparations were covered with a second cover glass and incubated at 4°C for 30 min, then at 17°C for 30 min to 3 h before they were examined. We looked for motility of vesicles in the center of the bulk axoplasm at least 10 µm below the coverslip.

To examine the effects of apyrase and 5'-adenylylimidodiphosphate (AMP-PNP), the chelator that contained apyrase was perfused (13) with ~20 µl of apyrase (Sigma Chemical Co., St. Louis, MO; 0.5 mg/ml) or AMP-PNP (Boehringer Mannheim Biochemicals, Indianapolis, IN; 5 mM) in half-strength buffer X.

**Synaptic Viscle Treatments**

Trypsin and pronase digestions were performed on 10 µg of vesicles with 6 µg/ml enzyme in a final volume of 40 µl for 16 h at 4°C. These incubation conditions have been shown to alter synaptic vesicle proteins (22). Elastase digestions were performed on 10 µg of synaptic vesicles with 1 µg/ml elastase for 12 h at 4°C. All the above manipulations were carried out in homogenization buffer. The protease digestions were stopped by the addition of 1% diisopropylfluorophosphate in 95% ethanol to a final concentration of 0.02%. All treated particles were washed twice as described above before they were fluorescently labeled.

Trypsin, elastase, and diisopropylfluorophosphate were obtained from Sigma Chemical Co.; pronase was obtained from Calbiochem-Behring Corp., San Diego, CA.

**Video Microscopy**

Endogenous particle movement in axoplasm was viewed on a Zeiss Axiovert microscope equipped with a differential interference contrast 100x planachromatic objective (12, 13). Observations of fluorescent vesicles were made with a 100-W Hg light source and rhodamine excitation/barrier filter set (Carl Zeiss, Inc., Thornwood N.Y.); DIC images used either a Chalnicon camera. Still photographs were made from the video monitor on a Hamamatsu C-1000-12 silicon-intensified target camera and a SONY V05830 3/4-inch videocassette recorder. Some DIC images were recorded using a Chalnicon camera. Still photographs were made from the video monitor on Trn-X pan film developed in Microdol-X (both from Kodak) (Eastman Kodak Co., Rochester, NY).

**Velocity and Directionality Measurements**

Movement of vesicles were made by manually tracing uninterrupted vesicle movements on the video screen and measuring time and displacement. Velocities were calculated for individual movements of several particles. To determine the directionality of synaptic vesicle movements, the number of particles traversing an arbitrary line (perpendicular to the long axis of the axoplasm) in either direction in a video field during a 30-s to 2-min interval was determined. The percent of vesicles moving anterogradely was calculated for each field. The values given in Table I are the means (± SD) of the percent anterograde movement for all fields (i = the number of fields, n = the total number of particle movements tallied).

**RESULTS**

Synaptic vesicles (80 nm in diameter) from the electric organ of the elasmobranch *Discopyge onnata* were purified as previously described (20, 21) and fluorescently labeled with the sulforhodamine derivative Texas Red (see Materials and Methods). This fluorochrome covalently labels amino groups of macromolecules under mild conditions. The free dye is hydrophilic and is easily separated from the synaptic vesicles by pelleting and washing. Fluorescent vesicles were added to extruded axoplasm from the giant axon of the squid *Loligo pealei* (12) in a buffer with similar ionic composition but half the ionic strength of squid axoplasm (half-strength buffer X, described in Materials and Methods). Addition of this buffer to extruded axoplasm disperses the highly organized axoplasmic matrix somewhat and promotes "fraying" at the edges (13–16). Using AVEC-DIC microscopy (10, 11), we observed the movement of endogenous small (50–100 nm diameter) vesicles in the axoplasm at velocities of 1–2 µm/s at 21°C.

This is comparable to the velocities of similarly sized particles in the intact squid axon (23), extruded axoplasm (12, 13), and for the movement of squid vesicles in dissociated axoplasm (15) or on purified microtubules (18, 19). When the same preparations were examined by fluorescence video microscopy using a silicon-intensified target camera, fluorescent synaptic vesicles were observed to exhibit movement that was indistinguishable from the behavior of endogenous squid vesicles of similar size in the same field observed by DIC optics. The fluorescent vesicles moved at ~2 µm/s (Table I), often changing direction or disappearing from the plane of focus, like the endogenous organelles. The disorganized movement of both the fluorescent synaptic vesicles and endogenous particles compared to intact axoplasm was due to the introduction of vesicles in half-strength buffer X to the preparation (see Materials and Methods). Transported vesicles moved in smooth highly directional traverses with a path length consid-

**Table 1. Characteristics of Fluorescent Synaptic Vesicle Movement**

| Treatment | Axoplasms showing motility | Binding to fibrils | Mean velocity | Percent anterograde movement |
|-----------|---------------------------|-------------------|--------------|------------------------------|
| Untreated | 19                        | 0                 | 1.6 ± 0.4 (100) | 64.4 ± 10.7 (i = 27; n = 161) |
| Trypsin   | 2                         | 0                 | 1.6 ± 1.0 (25)  | 49.7 ± 5.5 (i = 4; n = 110)   |
| Pronase   | 2                         | 0                 | 1.6 ± 0.4 (25)  | 48.5 ± 9.1 (i = 6; n = 77)     |
| Elastase  | 2                         | 6                 | NA           | NA                           |
| Beads     | 0                         | 5                 | NA           | NA                           |

To score the axoplasm for motility and to measure velocity and directionality of transport, we examined particle movement in the center of bulk axoplasm, where movement was common and more organized than at the axoplasmic "fringe." Only axoplasms exhibiting good endogenous particle motility when viewed with AVEC-DIC microscopy are considered here.

*Individual axoplasms were scored positive for motility if any non-Brownian fluorescent vesicle movement was detected. Quantitative differences between preparations are discussed in the text.*

*To determine if particles bound to isolated fibrils, preparations were perfused with 5 mM AMP-PNP and scored for the presence of aligned fluorescent particles that co-localized with fibrils at the axoplasmic periphery seen through AVEC-DIC microscopy (examples are shown in Fig. 2). The velocities given are the mean ± SD of a number of individual fluorescent synaptic vesicle movements (the number of movements is given in parentheses).*

*The values given are the mean ± SD of the percent of vesicles moving in the anterograde direction. n, the number of individual vesicle movements tallied; i, the number of video fields examined. See Materials and Methods for further details on the velocity and directionality measurements. NA, not applicable.*
erably longer than the amplitude of Brownian motion. Fluorescent vesicles outside the axoplasm exhibited only Brownian movement.

To illustrate fluorescent synaptic vesicle movement, six sequential 1-s photographs were taken from a region of the video monitor. One vesicle was sufficiently bright and distant from others so that its movement was easy to follow (Fig. 1). Since the vesicles were moving at 1–2 μm/s, the translocating vesicle appears as a streak on the photographs (arrowheads, Fig. 1) in the direction of movement. Fluorescent vesicle movement was seen in every active axoplasm preparation that we examined (Table I), and many (10–100) fluorescent synaptic vesicles moved in each video field. At least 70% of all active fields containing fluorescent vesicles showed movement. The fluorescently labeled synaptic vesicles moved in both anterograde and retrograde directions but showed a modest preference for anterograde movement; ~64% of the vesicle translocations were in the anterograde direction (Table I). The movements of fluorescent synaptic vesicles and similarly sized endogenous particles were essentially indistinguishable, although the low contrast endogenous particles were much harder to follow; thus, it was difficult to make directionality measurements.

Like endogenous organelle movement, that of fluorescent vesicles was ATP dependent (13, 24). The enzyme apyrase, which hydrolyzes ATP and ADP and stops endogenous fast axonal transport (13), inhibited the long, directed translocations of fluorescent and endogenous vesicles. The vesicles made small jittery movements corresponding to Brownian motion, behaving as if they were free to diffuse in the viscous axoplasm and not bound to cytoskeleton. In contrast, when preparations were treated with AMP-PNP, a nonhydrolyzable ATP analog (13, 25), all endogenous particle and fluorescent synaptic vesicle movement in the bulk axoplasm, including Brownian motion, was inhibited. Brownian movement of free vesicles outside the axoplasm was unaffected. It appears therefore that the ATP analog permits binding but not translocation of fluorescent vesicles. Binding was reversible since the addition of a 10-fold excess of ATP caused the release of fluorescent synaptic vesicles from the fibrils (see below) and a restoration of movement in the axoplasm, as is seen for endogenous particles. (For details of the AMP-PNP-induced inhibition, see reference 25.)

The low strength buffer that was used promotes fraying of the cytoskeleton at the edge of the axoplasm, making it easier to see individual cytoskeletal fibrils (16). These fibrils support ATP-dependent movement of endogenous organelles and are occasionally decorated with nonmoving particles. Fluorescent vesicles were not bound to cytoskeletal fibrils, although they could be seen in Brownian motion in the surrounding buffer. When the peripheral “fringe” region is examined in the presence of AMP-PNP, moving endogenous particles are observed to freeze and accumulate on the fibrils (25). Similarly, when axoplasm that contained active fluorescent synaptic vesicles were perfused with AMP-PNP, stationary vesicles appeared in the fringe region. When fields that contained aligned stationary fluorescent vesicles were then examined using AVEC-DIC microscopy, the positions of particles seen in fluorescence often co-localized with fibrils seen by AVEC-DIC microscopy. (Fig. 2, a and b). In many cases, stationary fluorescent vesicles could be identified in AVEC-DIC microscopy as particles indistinguishable from endogenous particles bound to a fibril. If other particles serve to attach the fluorescent synaptic vesicles to the fibrils they were too small to be seen in the video images.

Polystyrene beads injected into fibroblasts (26) and axons (27) are translocated. When fluorescent, negatively charged 0.1-μm latex/polystyrene beads were introduced into the axoplasm, movement was not observed (Table I). Latex/polystyrene beads have a tendency to adsorb proteins (18, 26), and so could bind nonspecifically to axoplasmic elements. After preincubation with bovine serum albumin to reduce nonspecific binding, the pretreated beads still did not move. When AMP-PNP was used to inhibit endogenous particle transport yet allow binding, no beads were seen to accumulate on fibrils. These results suggest that the fluorescent beads can neither bind nor be translocated on cytoskeletal fibrils in squid axoplasm under conditions where exogenously added synaptic vesicles do.

To examine the nature of the interaction between axoplasm and fluorescent synaptic vesicles, we attempted to inhibit their movement by altering their surface proteins using different proteases (see Table I). Digestion with elastase inhibited fluorescent vesicle translcation and binding in the presence of AMP-PNP. Brownian motion continued, suggesting that the vesicles were not simply “stuck” in axoplasm. In these experiments, the motility of endogenous particles viewed using AVEC-DIC microscopy was unaffected, providing an internal control. In six of eight experiments, no movement of elastase-treated vesicles was seen in 50–100 video fields of active axoplasm that contained fluorescent vesicles; without elastase pretreatment >70% of the fields show movement. Even in

![Figure 1](https://example.com/image1.png)

**Figure 1** Sequential photographs (1-s exposures, 1/5, photographed at f-11) of the video monitor. The irregular streak (closed arrowhead) is the track of a moving fluorescent synaptic vesicle during the exposure. The open arrow marks a stationary reference point. Bar, 2 μm. × 4,000.
FIGURE 2 AMP-PNP induces binding of fluorescent synaptic vesicles to cytoskeletal filaments. Extruded axoplasm that was engaged in active transport of fluorescent vesicles was perfused with 5 mM AMP-PNP in half-strength buffer X. When all movement had stopped, the peripheral “fringe” region was examined for stationary, aligned fluorescent synaptic vesicles (3-s exposures, photographed at f-8). (a) Untreated fluorescent vesicles attached to filaments. (b) AVEC-DIC image of the same field. (c and e) Trypsin-digested vesicles also bind to filaments in the presence of 5 mM AMP-PNP. (d and f) AVEC-DIC images of the respective fields. Arrows (a and b) and arrowheads (c-f) indicate the fluorescent vesicles. Bar, 2 μm. x 4,375.

From these data we conclude that highly purified synaptic vesicles isolated from nerve terminals of a cartilaginous fish are competent to move in squid axoplasm with the same velocity and ATP-dependence as endogenous squid vesicles of the same size. These synaptic vesicles represent a population that was not actively engaged in axonal transport when isolated. Apparently the ability to be translocated is not lost on arrival at the nerve terminal. Preparations of squid axoplasmic membranes of unknown origin or purity are also transported when added back to axoplasm (17) or to purified microtubules from brain or flagella (18, 19). The transport of exogenous synaptic vesicles described here shows that a simple membrane from a different species is transported efficiently and specifically.

The attachment and movement of synaptic vesicles along the two cases where movement was observed, it occurred in only one or two out of the many video fields examined and was only observed in areas where endogenous particles were extremely active. Perhaps soluble factors (18) may be adsorbing onto the fluorescent vesicles or they may be interacting with other vesicles in these particularly active regions. In the presence of AMP-PNP, endogenous motility was inhibited. However, we saw no accumulation of elastase-treated fluorescent vesicles on fibrils at the axoplasmic periphery, although endogenous particles did accumulate.

We also treated vesicles with trypsin or pronase. At the concentrations of the enzymes used in this study, synaptic vesicle motility was not inhibited. In almost every video field that contained fluorescent vesicles and active endogenous vesicles, the fluorescent vesicles moved with the same velocity. These proteases did not affect the ability of the vesicles to accumulate on fibrils at the “axoplasmic fringe” in the presence of AMP-PNP (see Fig. 2, c-f). However the trypsin- and pronase-treated vesicles no longer had a preferred direction of movement, but moved equally well in both anterograde and retrograde directions. The mean values obtained for percent movement of treated vesicles in the anterograde direction (~50%) are significantly different (P < 0.01) from untreated vesicles (64%) (see above and in Table I).
cytoskeletal elements of squid axoplasm is destroyed by elastase treatment (Table I). After protease treatment, endogenous membrane vesicles from squid axoplasm also lost their ability to move on cytoskeletal elements, either in extruded axoplasm (17) or a reconstituted system (18). It should be relatively simple to identify the vesicle proteins involved in binding and motility, since only a few synaptic vesicle polypeptides are present at greater than one copy per vesicle (22), and not all of these are sensitive to external proteases. In addition, an alkaline wash did not perturb the translocation capacity of synaptic vesicles (data not shown). It is likely, therefore, that attachment of synaptic vesicles to cytoskeletal elements involves an integral membrane protein. The preliminary data with pronase and trypsin digestion imply that such membrane proteins may also play a role in the preferred directionality of fluorescent vesicle movement.

Recent evidence suggests that squid axoplasm contains soluble factors that can adsorb to glass or to latex/polystyrene beads with a negative surface charge. In the presence of ATP, purified microtubules move with respect to the bound factors (18). Nonspecific adsorption of such factors to protease-treated membranes apparently does not occur, perhaps because their charge density is different from that of glass or latex. When latex beads are injected into axons or the cytoplasm of cells, those that move do so at close to normal speeds (26, 27). In our case, however, addition of latex/polystyrene beads to extruded axoplasm yielded no detectable movement. There are several possible explanations of the apparent discrepancy between the movement of beads in the different systems. For example, in the extruded axoplasm, there might be an insufficient amount of free motor to coat beads, an inhibitor of movement might bind beads (but not membranes), or the beads might be bound to stationary structures such as microtubules. Too little is known at present about the nonspecific adsorption of cytoplasmic factors to beads to be able to distinguish between such possibilities.

An additional distinction between the behavior of vesicles and of beads coated with soluble factors is particle velocity. In all cases in the squid system (Table I and references 15, 17, 18, and 19), membrane vesicles move at 1.6 μm/s or more, whereas beads move at <0.5 μm/s (14). Comparison of the movement of soluble factors attached specifically to membranes and nonspecifically to beads should help elucidate what is required for specific and directional transport in cells.

We would like to thank R. J. Lasek and the members of the Kelly lab for many helpful discussions, and Leslie Spector for her assistance with this manuscript.

This work was supported by the following grants from the National Institutes of Health: NS09878, NS15927, and AM33937 (to R. B. Kelly), and NS18361 and AG00795 (to S. T. Brady). Additional funding was provided to T. A. Schroer from University of California Patent Funds and National Cancer Institute Institutional Training Grant ST3209270.

Received for publication 4 February 1985, and in revised form 22 April 1985.

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