Cross-bridges Mediate Anterograde and Retrograde Vesicle Transport along Microtubules in Squid Axoplasm

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ABSTRACT To assay the detailed structural relationship between axonally transported vesicles and their substrate microtubules, vesicle transport was focally cold blocked in axoplasm that was extruded from the squid giant axon. A brief localized cold block concentrated anterogradely and retrogradely transported vesicles selectively on either the proximal or distal side of the block. Normal movement of the concentrated vesicles was reactivated by rewarming the cold-blocked axoplasm. At the periphery of the axoplasm, moving vesicles were located on individual microtubules that had become separated from the other cytomatrix components. The presence of moving vesicles on isolated microtubules permitted the identification of the structural components required for vesicle transport along microtubules. The results show that 16–18-nm cross-bridges connect both anterogradely and retrogradely moving vesicles to their substrate microtubules. These observations demonstrate that cross-bridges are fundamental components of vesicle transport along axonal microtubules. Thus, vesicle transport can now be included among those cell motile systems such as muscle and axonemes that are based on a cross-bridge–mediated mechanism.

Vesicle transport is an essential process in cellular membrane dynamics (Schliwa, 1984). This ATP-dependent process actively shuttles membranous organelles through the meshwork of the cytoplasmic matrix that otherwise limits the mobility of these organelles (Porter et al., 1983). The vesicle transport system is particularly refined in cells, such as neurons, that have long cytoplasmic extensions. Neurons can have axons that are centimeters or even meters in length and the end of the axon depends upon membranous elements that are conveyed from the nerve cell body by vesicle transport (Grafstein and Forman, 1980). To meet the needs of the axon, the vesicle transport system in neurons has become extremely efficient during the 700 million years since neurons first evolved (Lasek and Katz, 1977; Lasek et al., 1985).

The vesicle transport system is so efficient in axons that individual vesicles can move at their maximal rate of transport almost continuously for many hours and over distances of many centimeters (Brady, 1984). Moreover, the system is directionally selective. Newly synthesized vesicles that are produced in the nerve cell body are transported primarily anterogradely toward the axon terminal, and spent membranous structures are transported primarily retrogradely from the axon terminal back to the cell body where they are degraded by lysosomes (Tsukita and Ishikawa, 1980; Smith, 1980; Fahim et al., 1985).

Studies of vesicle transport of axoplasm isolated from squid giant axons demonstrate that microtubules are the lineal substrate for vesicle transport (Allen et al., 1985; Schnapp et al., 1985; Vale et al., 1985a). The following models for the mechanism of vesicle transport along microtubules have been proposed: (*a*) vesicle movement driven by microstreams created along the surfaces of microtubules (Weiss and Gross, 1982; Allen et al., 1985); (*b*) the direct interaction of the vesicle with the microtubule and propulsion by a proton pump in the vesicle membrane (Sheetz et al., 1984); and (*c*) vesicle movement produced by cross-bridges that connect the vesicles to the microtubule (Schmitt, 1968; Smith, 1971).

To understand the mechanism of vesicle transport along microtubules requires information about the structural organization of the motility complex formed by a transported vesicle and its substrate microtubule. Similarly, information about the structural organization of the motility complexes in muscle and axonemes was crucial in understanding the mechanism in these motile systems (Huxley, 1976; Gibbons, 1981; Satir et al. 1981). To examine the detailed structural organization of the vesicle–microtubule motility complex

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with the electron microscope, vesicles must be fixed while they are actively moving along microtubules. Ideally, the microtubules should be separated from the surrounding cytoplasmic matrix that normally complicates these studies (Porter et al., 1983).

Normally, the density of transported vesicles in the axon is low and those vesicles that can be found with the electron microscope may or may not be moving at the time of fixation. One way to overcome these problems is to concentrate moving vesicles in one region of the axoplasm by focally blocking the movement of transported vesicles (Tsukita and Ishikawa, 1980; Fahim et al., 1985). Cooling a localized region of the axon to 4°C provides a reversible block of vesicle transport (Brimijoin and Helland, 1976). When the moving vesicles encounter the cooled region they stop moving. Moreover, anterogradely transported vesicles collect specifically on one side of the cold block and retrogradely transported vesicles collect on the opposite side of the cold block (Tsukita and Ishikawa, 1980; Fahim et al., 1985).

We have used a modified form of the cold block method to obtain detailed information about the structural relationship between transported vesicles and microtubules. Previous cold block studies blocked axonal transport for 2 h or more (Tsukita and Ishikawa, 1980; Fahim et al., 1985). Under these conditions, the vesicles become so conjested that the structural relationships between the vesicles and the cytoskeletal elements were often disrupted (Fahim et al., 1985). To avoid this problem, we examined the effects of brief (15 min) cold blocks. Furthermore, to obtain moving vesicles at the time of fixation, the cold block was released by re-warming the cold-blocked axoplasm. We demonstrate that, by using this method, it is possible to analyze the detailed structural relationship between actively moving vesicles and their substrate microtubules in axoplasm.

MATERIALS AND METHODS

Nerve Preparation and Light Microscopy: Giant axons were dissected from the squid (*Loligo pealei*) within 48 h of the animal's capture and supply by the Marine Biological Laboratory at Woods Hole, Massachusetts. The axoplasm was extruded from the cortical sheath of the axon as previously described (Lasek, 1984; Brady et al., 1985). Isolated axoplasm was extruded directly on to a No. 1 coverslip and mounted in a dry "chamber" that consisted of 2-side fragments of coverslips (spacers) and a top coverslip to prevent excessive dehydration during the cold-blocking process. Dynamic analysis of vesicle transport was assessed using a video-enhanced contrast/differential-interference contrast (AVEC-DIC) microscope (Allen et al., 1981; Brady et al., 1982, 1985).

Cold Block: The cold block apparatus was identical to that described by Fahim et al. (1985). Briefly, it consisted of an aluminum block with a cooling pipe inserted in a groove in its surface. The temperature of the cooling pipe was maintained at −5 to 0°C by the circulation of a cooled ethanol/water mixture. The aluminum block was placed in a water bath and maintained at a constant temperature of 15 to 17°C. Temperatures were monitored using a Bailey microthermistor. A temperature gradient of 15 to 3°C was maintained from the edge of the aluminum block to the center of the cooling pipe (2 mm). The isolated axoplasm in the chamber was then placed in such a way that it spanned the region of the temperature gradient.

Preparations were cold blocked for periods of 15 min and were either fixed by perfusion in situ or allowed to re-warm for an interval of 3 to 7 min at 21°C before perfusion of the fixative. Reference points were scribed onto the bottom coverslip to mark the position of the center of the cooling pipe and the proximal and distal regions of vesicle accumulation.

Electron Microscopy: Cold-blocked preparations of squid axoplasm were perfused with a fixative containing 4% glutaraldehyde in EGTA–phosphate buffer (Fahim et al., 1985) with an osmolarity of 1,200 mosmol. Primary fixation was continued for a minimum of 2 h at room temperature and the "chamber" was then disassembled leaving the extruded axoplasm attached to the lower coverslip. Samples were postfixed in 1% OsO_4 solution in the same buffer, for 1 h on ice, washed extensively in deionized water, and stained en bloc in a 0.5% aqueous solution of uranyl acetate overnight at 4°C. Dehydration was accomplished by passage through graded alcohols and samples were embedded in PolyBed 812 resin. After polymerization, the reference points were marked in the epon block, and the coverslip removed by repeated immersion of the block in boiling water and liquid nitrogen.

Ultrathin sections were cut either longitudinally or transversely to the long axis of the axoplasm stained with uranyl acetate and lead citrate and examined on a Jeol 100 CX at 80 kV. In some experiments 0.2 M PIPES buffer was substituted for the phosphate. Sections taken 2–4 mm from the center of the cold block served as experimental material, while regions of the same axoplasm in excess of 10 mm from the cold block served as controls.

Quantitative Analysis: For quantitative analysis only the peripheral region of the axoplasm was used. One hundred vesicles each from the proximal zone and the distal zone of vesicle accumulation were examined. The distance of the vesicle from both its nearest microtubule and its nearest neurofilament was measured. The number and length of cross-bridges between the vesicle and either of these cytoskeletal elements were also analyzed. Vesicle size was estimated by calculating the average of the long and short axis of each vesicle. All measurements were made directly from the negatives of a previously calibrated microscope at a magnification of 50,000 using a light box and graticule eyepiece. On each negative a microtubule was measured in the same manner and served as an internal indicator of magnification. This method provided a relatively accurate means of estimating the size of structures in the range of the diameter of the microtubule. For example, 25 measurements of the size of a microtubule in a section resulted in a mean of 24.7 nm and an SEM of 1.5 nm.

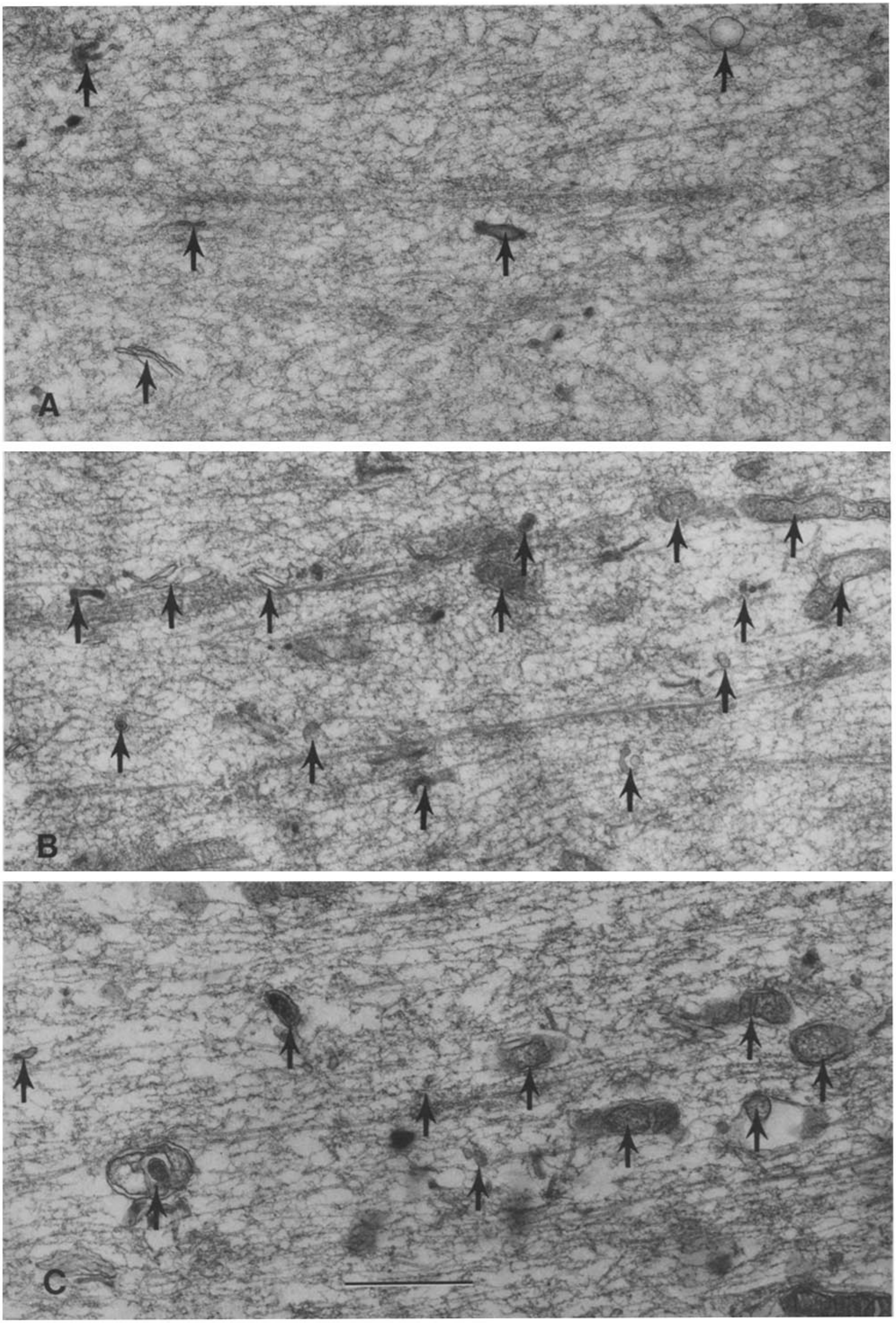
To examine the effects of the plane of sectioning on the distances estimated above, 10 vesicle–microtubule interactions were selected which showed some form of cross-bridge structure. By changing the stage goniometer, the specimen was tilted around the axis of the microtubule and the vesicle–microtubule complexes were photographed at different angles of tilt. Measurements of the maximal distances between the vesicle and its nearest microtubule and neurofilament were made from these negatives in a similar manner to that above.

RESULTS

Transported Vesicles Accumulate during a Short-term Cold Block

Using AVEC-DIC light microscopy, we found that large numbers of particles accumulated on either side of a cold block in axoplasm after a period of only 15 min. Furthermore,

FIGURE 1 Electron micrographs from longitudinal sections of extruded squid axoplasm either from a control preparation or from axoplasm that had been cold blocked for 15 min and then re-warmed for 3 min before fixation. (*A*) Control axoplasm, taken more than 10 mm from the center of the cold-blocked region. Note the presence of both microtubule and neurofilament domains and the scarcity of membranous organelles (arrows). (*B*) A section through the axoplasm 3 mm proximal to the center of the cold-blocked region. Note the accumulation of small-diameter vesicles and elongate mitochondria that are closely associated with the microtubule domains of the axoplasm (arrows). These organelles must have been moving anterogradely to accumulate on the proximal side of the cold block (see text). (*C*) A section through the axoplasm 3 mm distal to the center of the cold-blocked region. Note the accumulation of large diameter organelles, including complex vesicles and degenerating mitochondria (arrows). These organelles must have been moving retrogradely to accumulate on the proximal side of the cold block (see text). Bar, 1 μm.


A
B
C

when the axoplasm was re-warmed to 18–20°C, >85% of the particles that had accumulated proximal to the cold-blocked region moved anterogradely and >85% of those that had accumulated distally moved retrogradely.

Electron microscopy confirmed that the regions of the axoplasm on either side of the cold block contained many more membranous organelles than the control axoplasm (Fig. 1). Although many organelles accumulated at the cold block, they were usually spatially separated from one another. This result differs from that observed after long-term cold blocks of ≥2 h (Tsukita and Ishikawa, 1980; Fahim et al., 1985). In the long-term cold block experiments, the membranous organelles packed tightly into clusters. This tight packing can result in the displacement of organelles from the microtubules along which they were transported (Tsukita and Ishikawa, 1980; Fahim et al., 1985). In contrast, in the short-term cold block, the transported organelles rarely contacted each other and they retained their normal relationships to surrounding structures. Thus, the short-term cold block both concentrated a sufficient number of transported organelles for quantitative analyses and maintained the normal relationships between these organelles and their substrate microtubules.

Anterogradely Transported Organelles Differ from Retrogradely Transported Organelles

The transported organelles that accumulated proximal to the cold block differed structurally from those organelles that accumulated distal to the cold block (see Figs. 1, 2, 4, and 5), confirming the results of Tsukita and Ishikawa (1980) and Fahim et al. (1985). The anterogradely accumulated vesicles were mainly of two types, small vesicular or vesiculo-tubular structures and long tubular mitochondria (Fig. 1). In contrast, the most abundant retrograde organelles were complex membranous structures such as multivesicular and dense core bodies with mean diameters of between 80 and 200 nm.

The anterogradely transported vesiculo-tubular structures were composed of an 80-nm vesicle and a cylindrical tail region. This tail region had numerous varicosities along its length and a dense core (Fig. 2). Measurement of the anterogradely accumulated vesicles showed that 63% of these structures were <80 nm in diameter (Fig. 3). By contrast, 85% of the vesicles that accumulated distal to the cold block were >80 nm in diameter, and 38% were >200 nm in diameter (Fig. 3).

Long elements of the smooth endoplasmic reticulum did not accumulate on either the proximal or distal side of the cold block. The smooth endoplasmic reticulum does not appear to be transported rapidly in axons (Ellisman and Lindsey, 1983).

Mitochondria were numerous among the retrogradely transported organelles (Fahim et al., 1985). However, they differed morphologically from the anterogradely transported mitochondria. Anterogradely transported mitochondria had the morphological characteristics of healthy active mitochondria. In contrast, the retrogradely transported mitochondria were swollen and deformed. These results support the hypothesis of Fahim et al. (1985). They propose that mitochondria generated in the neuron cell body are transported primarily anterogradely. The mitochondria apparently undergo degenerative changes during some stage of their transit through the axon, and are then transported primarily retrogradely back toward the lysomal system in the neuron cell body.

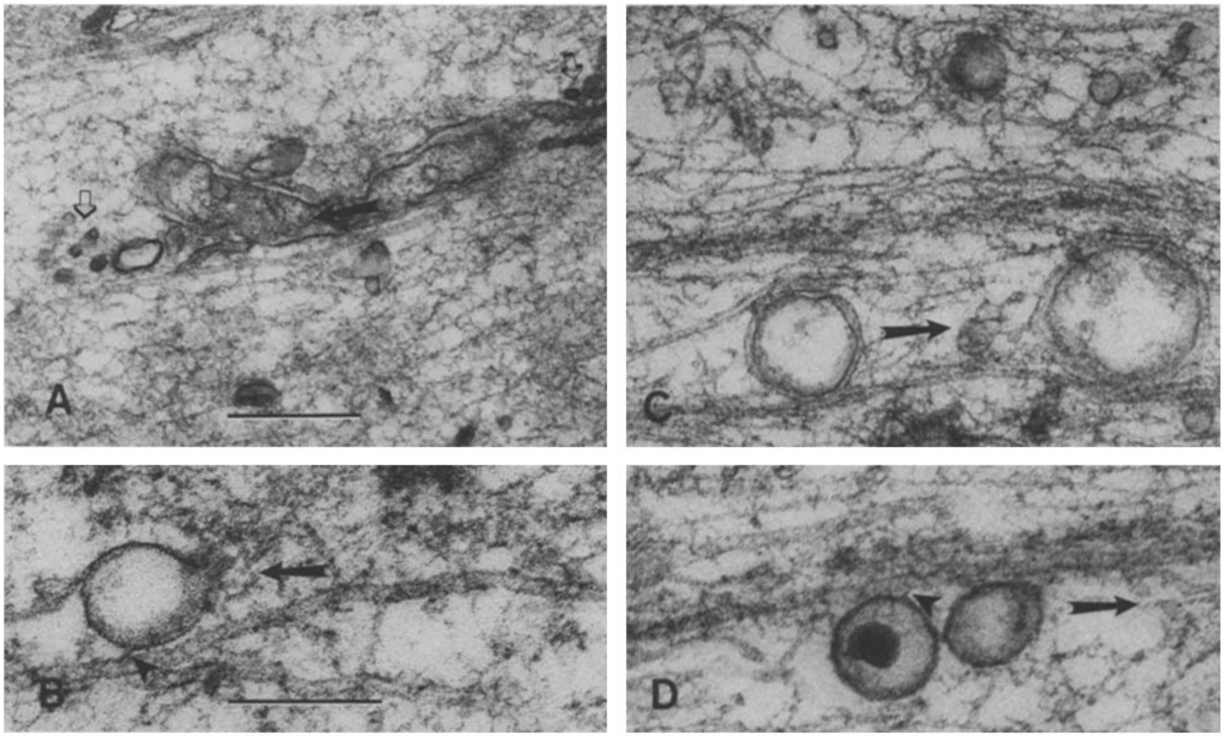


FIGURE 2 Electron micrographs showing microtubule domains in the region of vesicle accumulation that had been cold blocked for 15 min and re-warmed for 3 min. (*A* and *B*) Anterograde moving vesicles. (*C* and *D*) Retrograde vesicles. Note that the vesicles are closely associated with the microtubules and appear to be attached to the microtubule (arrowheads in *B* and *D*). Open arrows indicate vesicular-tubular bodies that contain a dense core. Large arrows indicate the direction of vesicle movement at the time fixation. Bars, (*A* and *C*) 0.5 μm; (*B* and *D*) 0.25 μm.

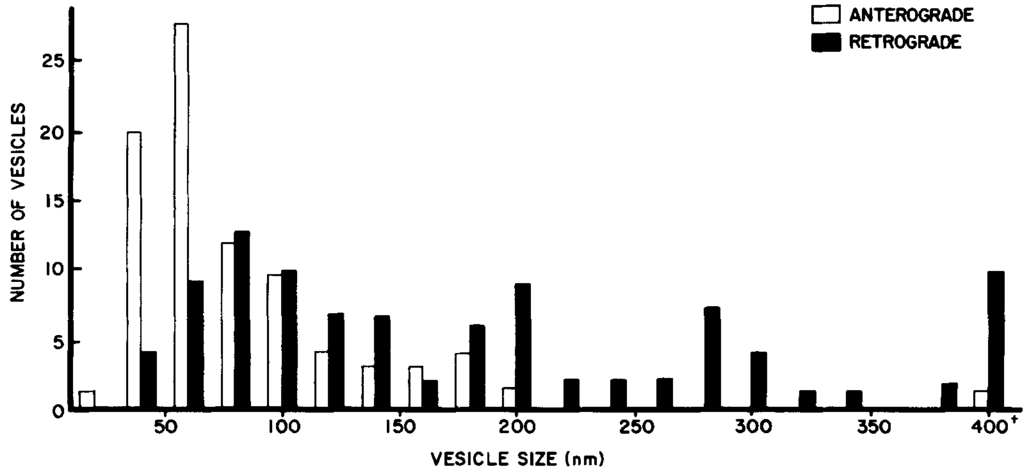


FIGURE 3 A histogram showing the relative sizes of the anterogradely and the retrogradely moving vesicles from preparations that had been cold blocked for 15 min and then re-warmed for 3 min before fixation as described in Fig. 1. Note that >60% of the anterogradely moving vesicles have a mean diameter of <80 nm. In contrast, >60% of the retrogradely moving vesicles are >80 nm. Furthermore, many of the retrogradely moving vesicles have a mean diameter of >200 nm. All histograms are based on measurements of 100 vesicles from each side of the cold block.

Microtubules Remain Polymerized after a Short-term Cold Block

Many microtubules depolymerize at 4°C, and after a long-term cold block many of the microtubules depolymerized in the region of the cold block (Tsukita and Ishikawa, 1980; Fahim et al., 1985). By contrast, we found that the microtubules remained polymerized after a brief cold block and that the structure of the axoplasm in the region of vesicle accumulation appeared normal. These results suggest that the rate of microtubule depolymerization in the cold-blocked axoplasm is a relatively slow process at 4°C.

Vesicles Accumulate within the Microtubule Domains of the Axoplasm

In the region of vesicle accumulation on both sides of the cold block, the transported organelles accumulated in linear arrays or "files." These linear arrays invariably occurred in the microtubule domains of the axoplasm (Fig. 1, *B* and *C*). Similar results have been obtained by Smith (1980). In his study, vesicle transport was blocked by compressing frog-myelinated axons and vesicles accumulated in files that were associated with the microtubule domains.

Fig. 2 shows that the accumulated vesicles were surrounded by a fretwork of short filamentous structures, some of which appeared to be attached to the microtubules. However, the vesicles made contact with so many filamentous structures in the interior of the axoplasm that it was not possible to distinguish those connections that might be important for the transport of vesicles along microtubules from those connections that were merely adventitious (Fig. 2).

Microtubules Separate from Other Cytomatrix Components at the Periphery of the Axoplasm

One of the advantages of extruded axoplasm is that there are no permeability barriers that could interfere with the rapid fixation of axoplasmic structures and fixation is most rapid at the very periphery of the axoplasm. Longitudinal sections through the periphery of the cold-blocked axoplasm showed that microtubules had partially emerged from the axoplasm (Fig. 4*A*). Although these microtubules were partially separated from the axoplasm, they retained their original orientation parallel to the long axis of the axon. Furthermore, many of these peripheral microtubules had regions that were effectively free from the matrix components that complicate structural analyses of the vesicle–microtubule interactions in the central axoplasm (compare Fig. 2 with Figs. 4 and 5).

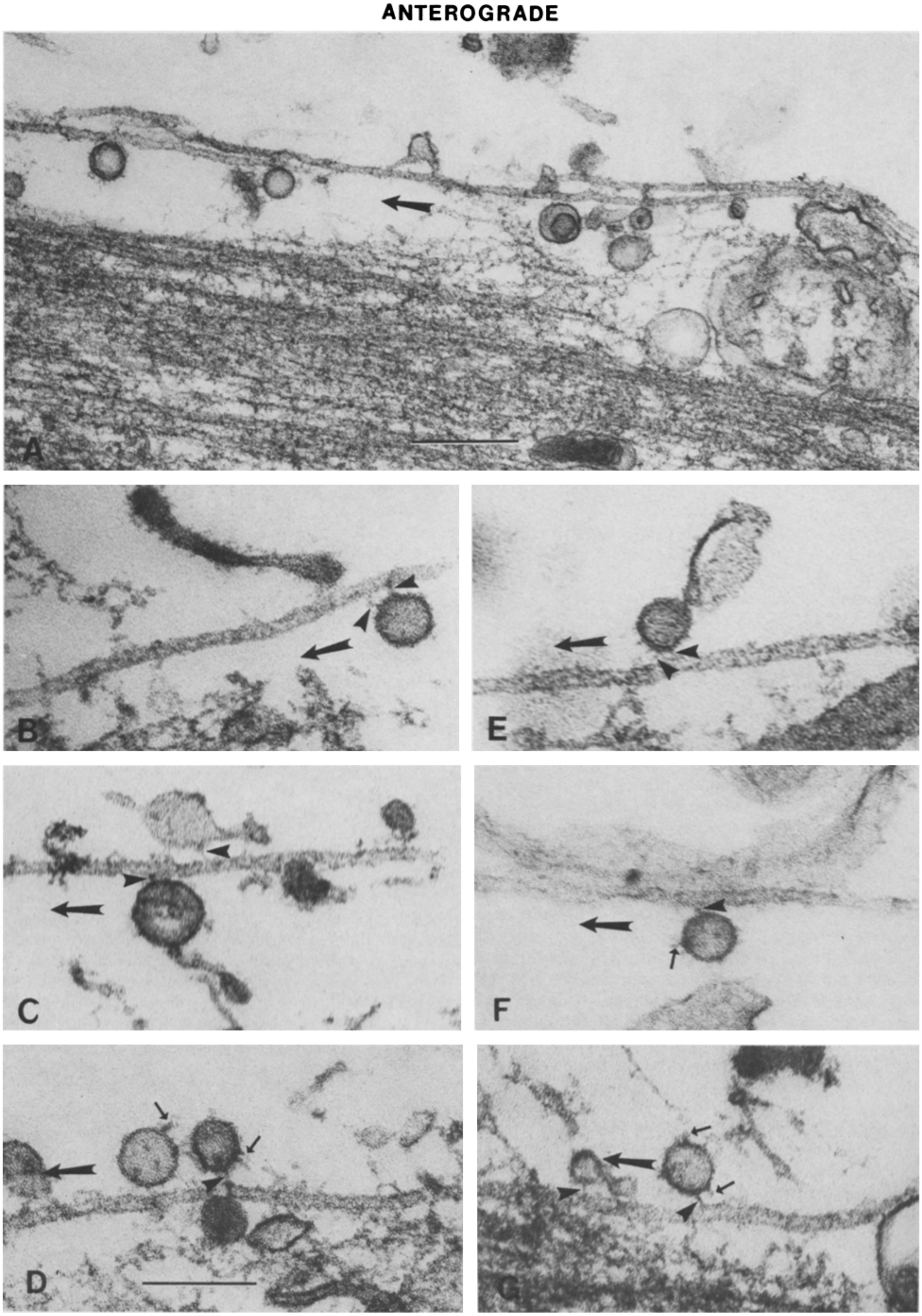
In the regions where the cytomatrix and other cytoskeletal structures were separated from the microtubules, the transported vesicles appeared to remain closely associated to these otherwise bare microtubules (Figs. 4 and 5). By contrast, vesicles were rarely closely associated with isolated neurofilaments. These results suggested that vesicles were associated preferentially with microtubules.

Transported Vesicles Are Preferentially Associated with Microtubules

To test the hypothesis that the transported vesicles are preferentially associated with microtubules at the periphery of the axoplasm, we measured the distance between the transported vesicles and the adjacent microtubules and then compared these distances with those between the vesicles and the adjacent neurofilaments. Neurofilaments were chosen for comparison, because, like the microtubules, they are long polymers with an extensive system of cross-bridges (Hirokowa, 1982). Furthermore, the neurofilaments outnumber the microtubules in the axoplasm. Thus, the neurofilaments can provide a measure of the background nonspecific binding of vesicles to polymers in the axoplasm.

If the vesicles are associated preferentially with the microtubules but not the neurofilaments, then the spatial relationships between the vesicles and the microtubules should differ from those between the vesicles and the neurofilaments. That is, the vesicle–neurofilament intervals should be more homogeneously distributed than the vesicle–microtubule intervals.

Fig. 6 compares the distributions of vesicle–microtubule intervals for 100 anterogradely transported vesicles and 100 retrogradely transported vesicles. The distributions of the


ANTEROGRADE
A
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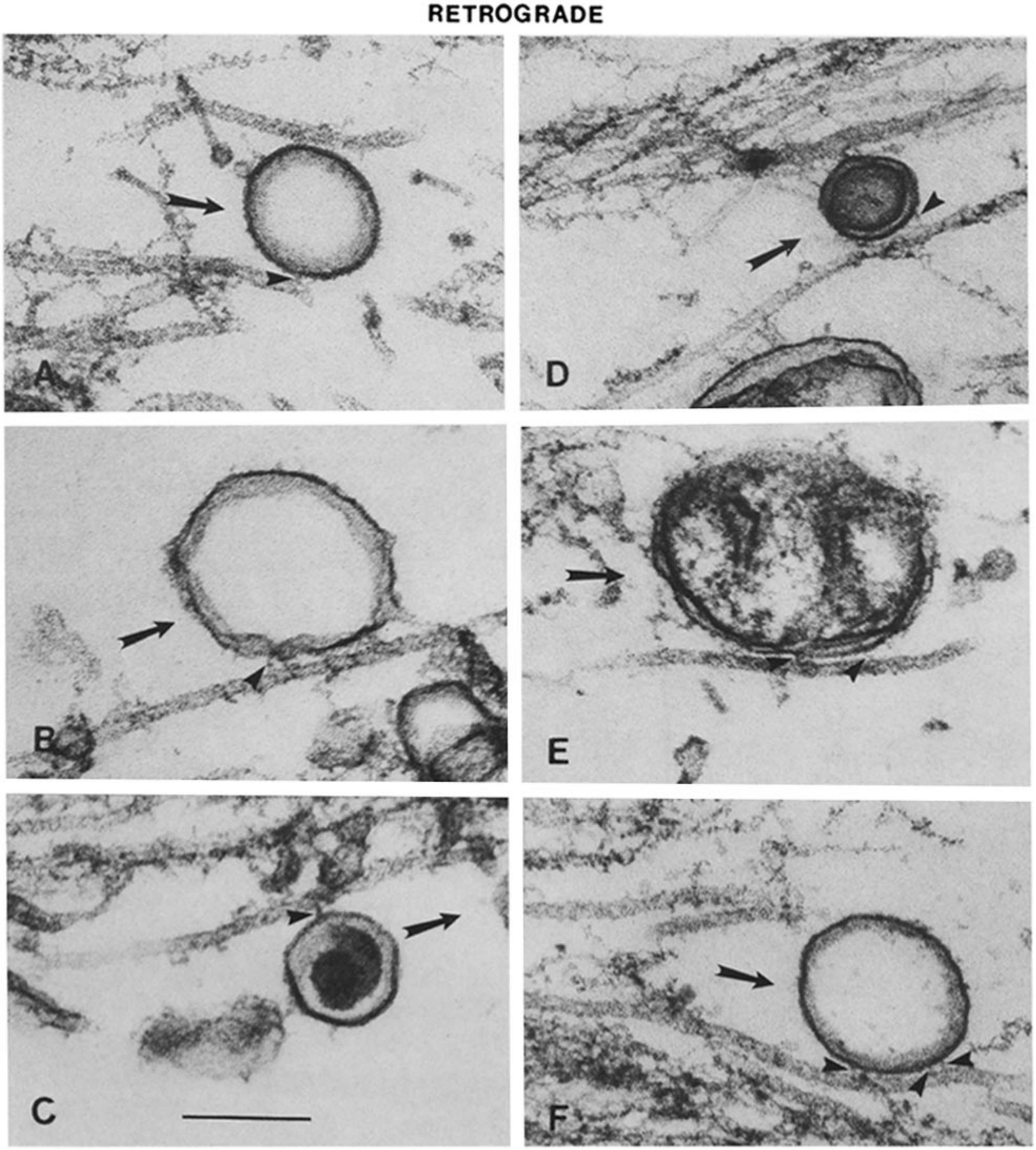

Figure 5 High magnification electron micrographs of retrogradely transported membranous organelles that are located at the periphery of cold-blocked axoplasm. Note that these vesicles are closely associated with one or more microtubules. Moreover, like the anterogradely transported vesicles (Fig. 4), cross-bridges connect some of these vesicles to the adjacent microtubules (arrowheads). Bar, 0.25 μm.

Figure 4 Electron micrographs of the peripheral region of axoplasm on the proximal side of the cold block. *A* is a low magnification micrograph and *B–G* are higher magnification micrographs). Large arrows indicate the probable direction of movement. (*A*) Note the three microtubules that have become partially separated from the rest of the axoplasm. The only structures that are directly attached to these microtubules are membranous organelles. The remainder of the axoplasm in this field consists primarily of neurofilaments that remain bundled together. (*B–G*) At higher magnification, note that vesicles are attached to microtubules by cross-bridges (arrowheads). Other structures that resemble these cross-bridges project from the surface of the vesicles (small arrows). However, these other structures are not attached to the adjacent microtubule. Bars, (*A*) 0.5 μm; (*B–G*) 0.25 μm.

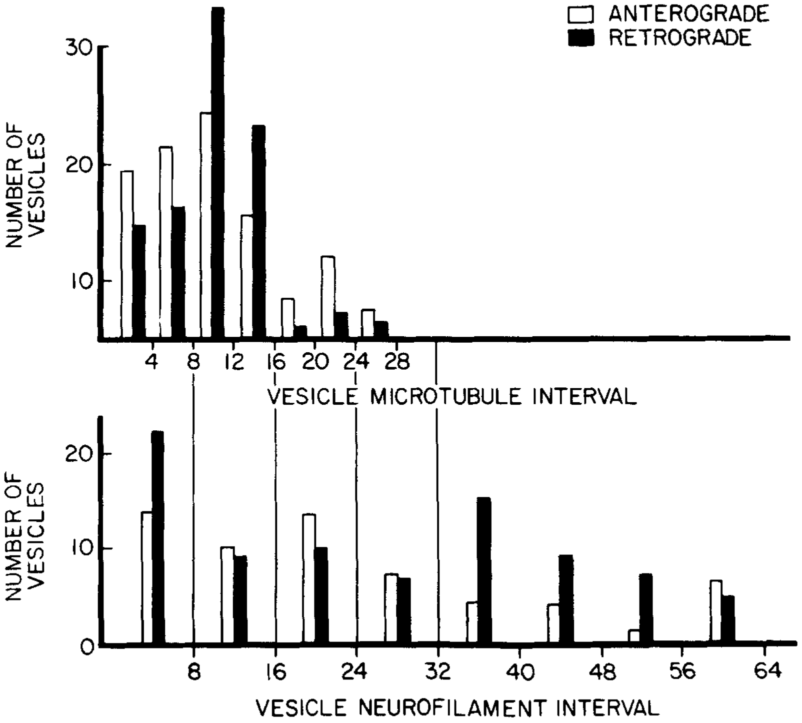

FIGURE 6 Histograms comparing the distance between individual vesicles either with their nearest microtubule or with their nearest neurofilament. The figure includes data from 100 anterogradely transported vesicles and 100 retrogradely transported vesicles. The vesicle-microtubule intervals for the anterograde vesicles were similar to those for the retrograde vesicles, and the vesicle-neurofilament intervals for the anterograde vesicles were also similar to those for the retrograde vesicles. However, the vesicle-microtubule intervals differed from the vesicle-neurofilament intervals. More than 80% of the vesicles were 16-18 nm from their nearest microtubule. In contrast, most of the vesicles were more than 18 nm from their nearest neurofilament. These results demonstrate that vesicles are preferentially associated with microtubules than with neurofilaments.

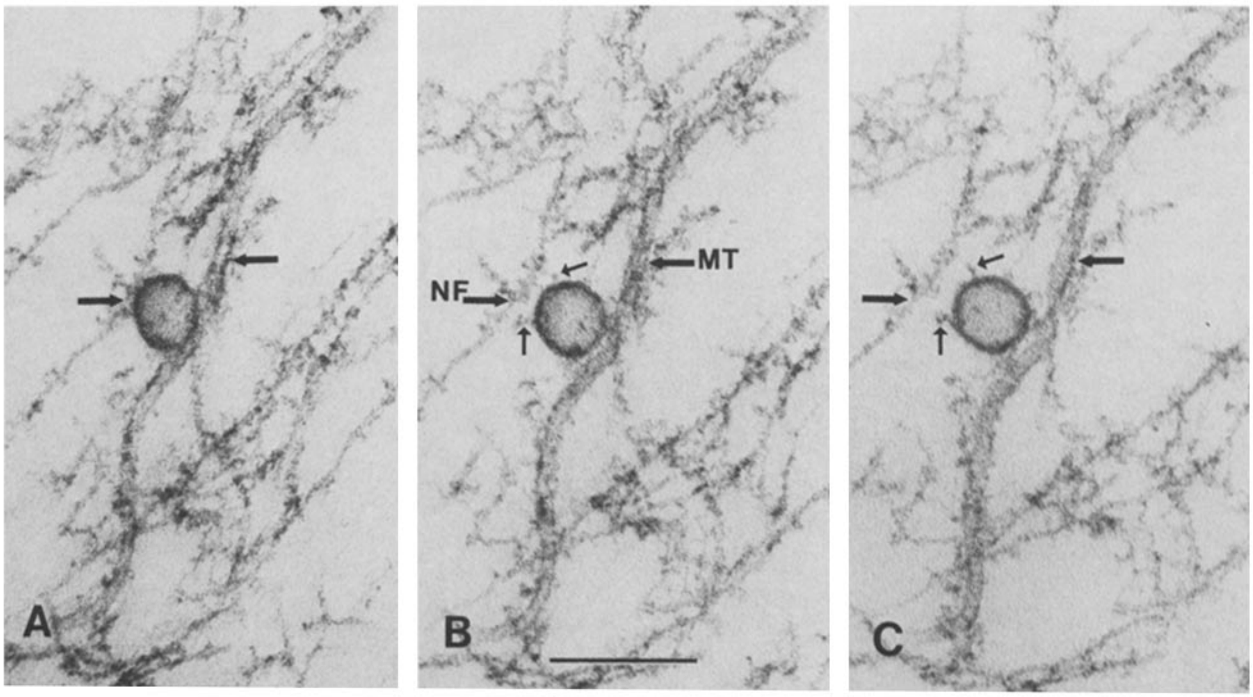

FIGURE 7 Electron micrographs taken at different angles of tilt showing a vesicle surrounded by cytoskeletal elements. The specimen was tilted around an axis that is parallel to the axis of the microtubule and neurofilament which appear to contact the vesicle (*A*). As the angle of tilt was varied, the vesicle remained attached to the microtubule (*MT*) but separated from the neurofilament (*NF*) (*B* and *C*). Structures resembling the vesicle transport cross-bridges are attached to the vesicle surface in regions where the vesicle is not apposed to a microtubule (small arrows). (*A*) −20°; (*B*) 0°; (*C*) +20°. Bar, 0.25 μm.

anterogradely and retrogradely transported vesicles were indistinguishable and ranged from 0 to 28 nm, and 70% of the transported vesicles were within 16 nm of the nearest microtubules.

By contrast, the vesicle-neurofilament intervals started at 0 and ranged beyond 64 nm, which was the cut-off point for the analysis. The distribution of the vesicle-neurofilament intervals correspond to that expected for two sets of structures that are present in the same volume and that have no specific spatial relationship with each other (i.e., homogeneously distributed). These results indicate that transported vesicles have a greater affinity for the microtubules than they do for the neurofilaments.

Superimposition of Vesicles and Microtubules

The distribution of the vesicle-microtubule intervals suggests that vesicles are separated from the microtubules by a distance that is usually ≤16 nm. However, it is possible that those vesicle–microtubule intervals that are <16 nm are an artifact of image overlap in the preparation. That is, in the 80–110-nm sections used here, an 80-nm vesicle separated by 16 nm from a 25-nm microtubule can be disposed in the section so that the vesicle is superimposed over the microtubule. In these cases, the vesicles may appear to be closer to the microtubule than they actually are. In fact, some of these vesicles will appear to directly contact the microtubule (Fig. 7*A*).

To evaluate this possibility, we examined vesicle-microtubule complexes while tilting the stage of the electron microscope and rotating the specimen around the axis of the microtubule. Comparison of the spatial relationship between a single vesicle and its adjacent microtubule at three angles of tilt (Fig. 7, *A–C*) demonstrates that this procedure overcomes the problem of superimposition and provides a measure of the maximum distance separating the vesicle and microtubule. Ten vesicles were analyzed by this method and the maximum vesicle–microtubule interval ranged between 14 and 22 nm with an average of 17 nm (SEM ± 2 nm). These results indicate that the vesicle–microtubule interval for both anterogradely and retrogradely transported vesicles is 17 nm and that the smaller intervals that we observed were artifacts of the superimposition of vesicles upon adjacent microtubules.

Cross-bridges Separate Transported Vesicles from Microtubules

Detailed examination of the space between the transported vesicles and their closest microtubules showed that >80% of the vesicles were connected to the microtubules by one or more filamentous structure(s). These structures form cross-bridges that link both the anterogradely transported vesicles (Fig. 4) and the retrogradely transported vesicles to their substrate microtubule (Fig. 5). Most of these cross-bridges were straight, unbranched, and had a uniform diameter of ~8 nm. The cross-bridges of the anterogradely transported vesicles were morphologically similar to those of the retrogradely transported vesicles.

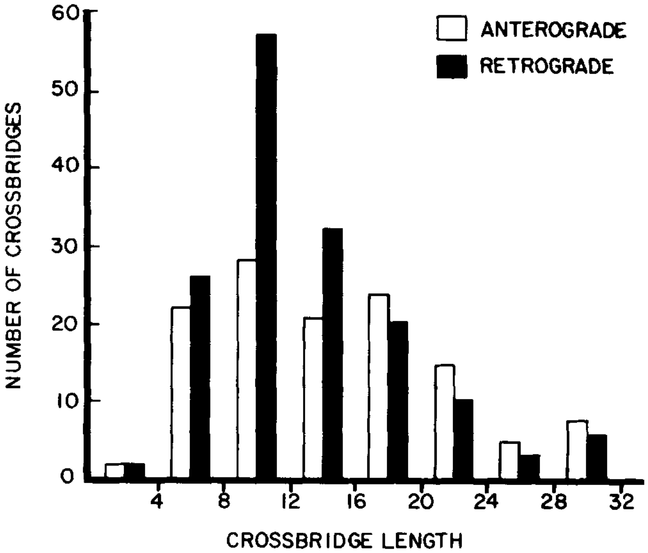

FIGURE 8 A histogram showing the distribution of the lengths of the cross-bridges found between vesicles and the nearest microtubule. Note that both the anterogradely and retrogradely moving vesicles have cross-bridges of the same length.

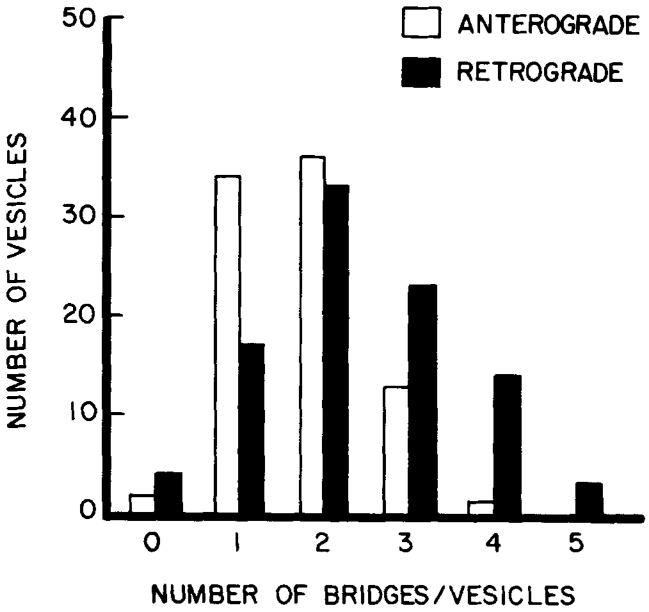

FIGURE 9 A histogram showing the number of cross-bridges that connect each anterogradely or retrogradely transported vesicles to its neighboring microtubule. Note that 70% of the anterogradely transported vesicles have up to two cross-bridges. In contrast, 70% of the retrogradely moving vesicles are connected by two or more cross-bridges to their nearest microtubule.

Cross-bridge Length

To more precisely determine the geometry of the cross-bridges, we measured the lengths of cross-bridges that attached microtubules to either anterogradely or retrogradely transported vesicles (Fig. 8). When plotted as a histogram, the cross-bridge lengths form a distribution that is skewed toward the shorter lengths. The assymetry of the distribution is probably due to the effects of the superimposition artifact discussed above.

Superimposition of vesicles upon microtubules can lead to an inappropriately low estimate of cross-bridge length but can not increase the apparent length of a cross-bridge. Thus, the longer cross-bridges provide a useful estimate of the cross-bridge lengths in our preparations. Moreover, if the cross-bridges determine the distance between the transported vesicles and the microtubules, then the cross-bridge length must correspond to the 17-nm vesicle–microtubule interval. 85% of the cross-bridges were <18 nm in length. The longer cross-bridges ranged between 16 and 22 nm and coincided with the upper range of the vesicle–microtubule intervals. These results indicate that the cross-bridges determine the size of the interval between the vesicles and microtubules.

How Many Cross-bridges Connect Transported Vesicles to Microtubules?

We counted the number of cross-bridges connecting either the anterogradely or retrogradely transported vesicles to their substrate microtubules. Preparations were scored for the presence of cross-bridges only if they had a microtubule–vesicle interval <28 nm. More than 95% of the vesicles had cross-bridges. Fig. 9 shows that 70% of the anterogradely transported vesicles had one or two cross-bridges and none of them had more than four cross-bridges. By contrast, 65% of the retrogradely transported vesicles had two or more cross-bridges, and some of them had as many as five cross-bridges.

Cross-bridges Are Preferentially Associated with Transported Vesicles

Most of the transported vesicles had structures resembling the 16–18-nm cross-bridges on regions of their surface that were not closely apposed to a microtubule (small arrows in Figs. 4 and 7). The number and the morphology of these cross-bridge–like structures that were found on the surface of the vesicles depended on the orientation of the structures in the section. Fig. 7 shows that at some angles of tilt the structures are not clearly visible (Fig. 7*A*). By contrast, when the same preparation is viewed at a different angle of tilt, these structures are distinct (small arrows in Fig. 7, *B* and *C*). Therefore, there are many more of these structures attached to the surface of the vesicles than are easily visualized in a single micrograph.

These structures that protruded from the surface of the vesicles were similar to the cross-bridges connecting the vesicles to the microtubules (arrowhead and small arrows in Figs. 4, 5, and 7). That is, they were straight, appeared unbranched, and were approximately the same length as the cross-bridges. These observations suggest that the protruding structures are the 16–18-nm vesicle transport cross-bridges and that these cross-bridges are located on regions of the vesicle surface that do not directly appose a microtubule.

We rarely found structures that resembled the 16–18-nm cross-bridges on the surface of isolated microtubules. However, other fine filamentous projections were located on some regions of isolated microtubules (Figs. 4 and 5). These fine filamentous projections may be similar to the MAPs that have been found more generally on neuronal microtubules. These MAPs differ in their length from the vesicle transport cross-bridges. Whereas the cross-bridges are 16–18 nm in length, MAPs are 20–25 nm in length (Kim et al., 1979; Suprenant and Dentler, 1982).

Together, these results suggest that the vesicle transport cross-bridges are preferentially located on the vesicle surface whether or not the vesicle contacts a microtubule. By contrast, the cross-bridges apparently remain attached to a microtubule only if they are already bound to a vesicle.

DISCUSSION

F. O. Schmitt (1968) was the first to formally set forth a cross-bridge model of fast axonal transport (i.e., vesicle transport). In his model, cross-bridges containing an ATPase are located on the surface of rapidly transported particles and these cross-bridges generate the force for the translocation of the particles along microtubules. This model is implicit in many of the notions that have subsequently been set forth for axonal transport and vesicle transport more generally (Pratt, 1980; for review see Schliwa, 1984).

Smith (1971) provided some support for the cross-bridge model in his electron microscopic analysis of synaptic vesicles in larval lamprey axons. He found that synaptic vesicles in these axons are attached to microtubules by cross-bridges. Although his evidence is consistent with a cross-bridge model for vesicle transport, other interpretations are possible. The vesicles that were examined in Smith's (1971) and subsequent studies (Ellisman and Porter, 1980; Schnapp and Reese, 1982; Hirokowa, 1982) may have been stationary in the axon. Thus, the cross-bridges between the vesicles and other structures may not have been directly involved in vesicle motility. Instead, some or all of these cross-bridges may be relatively stationary components of the extensive cross-linking system that characterizes the cytoskeleton of axons (Ellisman and Porter, 1980; Schnapp and Reese, 1982; Hirokowa, 1982).

Cold Blocking and Re-warming Extruded Axoplasm Generates Moving Vesicles for Electron Microscopic Analysis

If the cross-bridge model of vesicle transport is valid, then electron microscopic examination of vesicles moving along microtubules should reveal cross-bridges that link the moving vesicle to its substrate microtubule. To test this prediction, we have developed an experimental paradigm that specifically concentrates large numbers of moving vesicles for electron microscopic analysis.

In brief, our method involves the following steps. Focal cold-blocking axoplasm for 15 min concentrates transported vesicles in a small region of the axoplasm and selectively separates anterogradely transported vesicles from retrogradely transported vesicles. Electron microscopic analyses of the regions containing the accumulated vesicles indicate that the brief cold block used here does not produce any significant alterations of cytoskeletal organization in the region containing the accumulated vesicles.

Re-warming the axoplasm to 18°C for 3 min reactivated the accumulated vesicles and our observations with AVEC-DIC microscopy demonstrate that these vesicles moved normally. That is, the vesicles that had accumulated on the proximal side of the cold block moved primarily anterogradely and the vesicles on the distal side moved primarily retrogradely. At the periphery of the axoplasm, we found that individual microtubules partially separate from the other cytoskeletal structures of the axoplasm but that they retain their original orientation with respect to the long axis of the axon. Thus, this experimental paradigm generates large numbers of either anterogradely or retrogradely transported vesicles, and many of these vesicles can be rapidly fixed for electron microscopy while they are moving in a defined direction along individual microtubules at the periphery of the axoplasm.

Cross-bridges Participate Directly in the Active Translocation of Vesicles along Microtubules

Our results show that anterogradely and retrogradely transported vesicles are separated from their nearest microtubule by a relatively constant distance of 17 nm (Fig. 6). This distance corresponds to the length of the cross-bridges that we found between the transported vesicles and the microtubules (Fig. 8). Together, these results demonstrate that 16–18-nm cross-bridges connect both anterogradely and retrogradely moving vesicles to their substrate microtubule. We propose that these 16–18-nm cross-bridges are essential for the active translocation of vesicles along microtubules.

This proposal is supported by the observation that cross-bridges link vesicles and microtubules in a reconstituted vesicle transport system (Gilbert et al., 1985). When vesicles prepared from squid axoplasm are combined with demembranated, salt-extracted axonemal microtubules and ATP is added, the vesicles move along the axonemal microtubules. Electron microscopy of this reconstituted system shows that the vesicles are attached to the axonemal microtubules by cross-bridges that resemble those which we have described in the squid axoplasm.

Kinesin and the Vesicle Transport Cross-bridge

The proposal that vesicle transport is mediated by cross-bridges requires that the vesicle transport complex consists of at least three basic components—a microtubule, a cross-bridge and a vesicle. The existence of a three-component vesicle transport system is supported by the demonstration that a soluble component can be separated from both the vesicles and microtubules of axoplasm (Vale et al., 1985*b*, *c*). This soluble component is a protein that has been named kinesin. Brady (1985) has found a similar protein in chick brain. Vale et al. (1985*c*) proposed that one of the functions of kinesin is to bind to organelles and move them along microtubules. This proposal taken together with our observation that vesicle transport is mediated by cross-bridges raises the possibility that kinesin is a component of the cross-bridge system that mediates vesicle transport.

Analogies Between Vesicle Transport and Other Forms of Cross-bridge–mediated Motility

VESICLE TRANSPORT CROSS-BRIDGES HAVE A RELATIVELY STABLE ASSOCIATION WITH VESICLES AND A DYNAMIC ASSOCIATION WITH MICROTUBULES: The observation that cross-bridges participate directly in vesicle transport suggests that this process may have some fundamental similarities to other forms of cross-bridge-mediated motility. Both the actomyosin system of muscle and the dynein–microtubule system of axonemes use an ATP-dependent cross-bridge cycle to generate movement.

A basic feature of the organization of the cross-bridges in muscle and axonemes is that one end of the cross-bridge is anchored firmly to a component of the motile system (Huxley, 1976; Gibbons, 1981; Satir et al., 1981) and that the other end of the cross-bridge has a dynamic association with binding sites on an appropriate polymer and this association is modulated by ATP. In muscle, the stable end of the myosin cross-bridge is firmly attached to the thick filaments. In axonemes, the dynein cross-bridge has a stable attachment to the A-subfibril. The other ends of the myosin and dynein cross-bridges have a dynamic association with actin thin filaments (Eisenberg and Greene, 1980) and the B-subfibril (Gibbons, 1981; Satir et al., 1981), respectively.

We propose that the attachment of the vesicle transport cross-bridge to the vesicle is relatively stable, and that the attachment of the cross-bridge to the microtubule is much more dynamic. This proposal is supported by the observation that structures resembling the cross-bridges were found on those regions of the vesicle surface that were not directly apposed to a microtubule (Figs. 4, 5, and 7). In contrast, the microtubules rarely had any structures on their surfaces that resembled the vesicle transport cross-bridges except in those regions where they were apposed to a vesicle. In addition, the results of Gilbert et al. (1985) indicate that the cross-bridges remain attached to vesicles isolated from axoplasm.

Although the association of the cross-bridge with the vesicles may be more stable than its association with the microtubules, cross-bridges may also be able to dissociate from the vesicles under some conditions. For example, if kinesin is the vesicle transport cross-bridge, then the cross-bridges must be able to dissociate from the vesicles in the experimental system used by Vale et al. (1985*b*, *c*).

THE VESICLE TRANSPORT CROSS-BRIDGE MAY CONTAIN AN ATPASE: In the cross-bridge cycles of muscle and axonemes, ATP provides the energy for movement. Both myosin and dynein contain an ATPase that generates the energy for a conformational change in these cross-bridges (Eisenberg and Greene, 1980; Gibbons, 1981). This conformational change in the cross-bridge produces the motile force in muscle and axonemes. The vesicle transport system also requires ATP and apparently contains an ATPase (for review see Forman et al., 1983; Brady et al., 1985; Brady, 1985). We propose that this ATPase is part of the vesicle transport cross-bridge. Like the ATPase in myosin and dynein, the ATPase in the vesicle transport cross-bridge also may generate the energy for a conformational change that produces the force for vesicle transport.

ATP MAY MODULATE THE AFFINITY OF THE VESICLE TRANSPORT CROSS-BRIDGE FOR ITS SUBSTRATE MICROTUBULE DURING THE CROSS-BRIDGE CYCLE: For a cross-bridge mechanism to generate directional movement of a vesicle along a substrate microtubule, the cross-bridge must attach stereospecifically to the microtubule so that the conformational change in the cross-bridge will generate directional movement along the axis of the microtubule. Moreover, the cross-bridge must detach from the microtubule after the force generating conformational change is completed so that the vesicle can translocate along the microtubule. That is, a coordinated cross-bridge cycle is required for continuous vesicle movement along a microtubule.

In muscle and axonemes, the cross-bridge cycle is coordinated by the ATPase on the cross-bridge (Eisenberg and Greene, 1980; Gibbons, 1981; Satir, 1981). By acting through the ATPase, ATP both generates the energy for movement of the cross-bridge and it alters the affinity of the cross-bridge for its motile partner. In muscle and axonemes, ATP reduces the affinity of the myosin cross-bridge for its substrate polymer (Eisenberg and Greene, 1980; Gibbons, 1981; Satir, 1981). These effects of ATP on the affinity of the cross-bridge for its substrate polymer can be studied separately from ATP hydrolysis by using nonhydrolyzable nucleotide analogues of ATP.

The nonhydrolyzable nucleotide analogue AMPPNP has proved particularly useful for obtaining transient intermediates that are formed before hydrolysis of ATP in the motility cycles of muscle, axonemes and vesicle transport (Yount, 1971). In the vesicle transport system, AMPPNP facilitates the attachment of individual vesicles to microtubules (Lasek and Brady, 1985). This observation and others (Brady et al., 1985) have led Lasek and Brady (1985) to propose that ATP promotes the formation of a vesicle–cross-bridge–microtubule complex through its effects on the ATPase of the vesicle transport cross-bridge. Furthermore, we propose that hydrolysis of the ATP by the ATPase may be an important step in the release of the cross-bridge from the microtubule.

A Cross-bridge Model for Vesicle Transport

The observations discussed above lead us to propose a cross-bridge model for vesicle transport. The cross-bridge contains an ATPase. This ATPase produces energy that drives a conformational change in the cross-bridge. This conformational change provides the force that is required for vesicle movement. To move the vesicle along the microtubule, the conformational change must be incorporated into a motility cycle. We propose that this cycle involves the following steps—a cross-bridge on the surface of the vesicle first attaches to a microtubule stereospecifically; the cross-bridge changes its conformation moving the vesicle; finally, the cross-bridge detaches from the microtubule so that the cross-bridge cycle can be repeated. Although this cross-bridge cycle is conceptually similar to the motility cycles that have been proposed for myosin and dynein, the enzymatic properties of the vesicle transport ATPase suggest that the vesicle transport cycle has some unique features (Lasek and Brady, 1985; Brady, 1985; Vale et al., 1985*c*).

The Cross-bridge Model and Directionality of Vesicle Transport in Axons

BINDING SITES ON THE SURFACE OF ANTEROGRADE AND RETROGRADE VESICLES DETERMINE THEIR DIRECTION OF MOVEMENT: One of the most distinctive aspects of vesicle transport in neurons is its specific directionality. Vesicles and mitochondria are assembled from newly synthesized proteins in the nerve cell body and then they move primarily anterogradely in the axon toward the axon terminal (Grafstein and Forman, 1980). In the axon terminal, the vesicles participate in the process of neurotransmission and contribute to the renewal of the synaptic plasma membrane. The membrane components are recovered from the synaptic plasma membrane by endocytosis and are incorporated into multivesicular bodies which carry these components retrogradely from the axon terminal to the nerve cell body (LaVail and LaVail, 1974).

The direction in which vesicles are transported in the axon may be specified by determinants on their cytoplasmic surfaces. That is, the anterogradely transported vesicles may have a set of surface determinants that ensure anterograde movement, and these surface determinants may be altered in the axon terminal revealing the retrograde determinants that ensure retrograde movement (Fahim et al., 1985).

This proposal is supported by the following observations. Anterogradely transported vesicles are altered when they are converted into retrogradely transported vesicles in the axon terminal (Tsukita and Ishikawa, 1980; Smith, 1980; Fahim et al., 1985; see also Figs. 1–3). Moreover, when polystyrene beads and purified synaptic vesicles are injected into axons they move preferentially in one direction (Adams and Bray, 1983; Shroer et al., 1985). These observations indicate that the direction of transport is determined by the surface chemistry of the structures that are transported by the vesicle transport machinery.

THE CROSS-BRIDGES AND THE MICROTUBULES CONTRIBUTE TO THE DIRECTION OF VESICLE TRANSPORT IN AXONS: The microtubules and cross-bridges must also play an important part in defining the directionality of vesicle transport. Microtubules are the stable nondiffusible coordinate system for vesicle transport in axons and microtubules have an intrinsic polarity. By convention, one end of the microtubule is the plus end and the other end is the minus end. Analyses of microtubule polarity in vertebrate axons

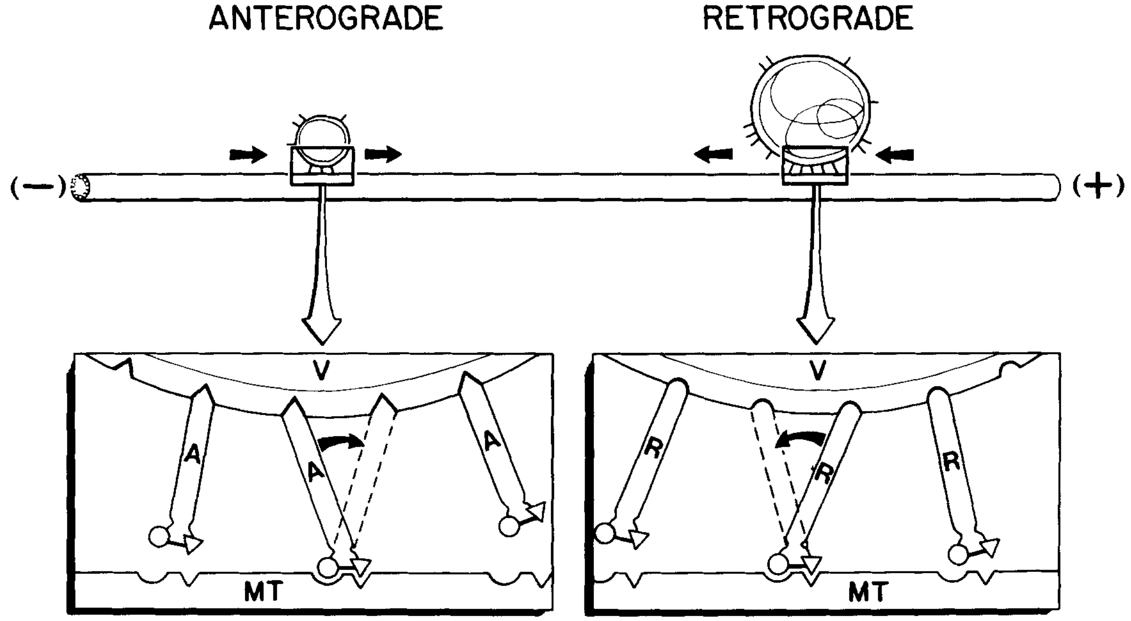

FIGURE 10 A schematic model for cross-bridge-mediated anterograde and retrograde vesicle transport in axons. In this model, all the microtubules have their plus ends toward the axon terminal. The axon contains anterograde (*A*) and retrograde (*R*) cross-bridges. The surfaces of vesicles have receptors that are specific for either the anterograde cross-bridges or the retrograde cross-bridges. The two forms of cross-bridges bind to the same site on the microtubule but they differ with regard to the direction of their conformational change. Binding of the cross-bridge to the vesicle is relatively stable. In contrast, the end of the cross-bridge that binds to the microtubule undergoes an ATP-dependant cycle of attachment and detachment to the microtubule that is coordinated with the confirmational change. (For details regarding the role of ATP in the cross-bridge cycle, see text; see also Lasek and Brady, 1985).

demonstrate that the microtubules have their plus ends toward the axon terminal (Heidemann et al., 1981). This observation coupled with the fact that vesicles move primarily either anterogradely or retrogradely in axons indicates that anterograde vesicles move toward the plus ends of microtubules and that retrograde vesicles move toward the minus ends.

For a cross-bridge to generate movement in one direction along a microtubule, the cross-bridge must bind stereospecifically to the microtubule and the conformational change in the cross-bridge must be oriented in the direction of movement. If axons contain only one type of vesicle cross-bridge, then this cross-bridge must be able to generate either an anterogradely or a retrogradely directed conformational change relative to the surface lattice of the microtubule. Alternatively, axons may contain two forms of vesicle transport cross-bridge: one form that attaches selectively to anterograde vesicles and generates force directed toward the plus end of the microtubule, and another form that attaches selectively to retrograde vesicles and generates force in the opposite direction.

We did not observe any morphological difference between the cross-bridges on anterograde and retrograde vesicles. However, our methods cannot distinguish subtle differences in the cross-bridges that would be related either to the direction of their conformational change or to their affinity for a particular vesicle surface. For example, if there are separate anterograde and retrograde cross-bridges, these may differ in some aspect of protein structure or with regard to associated modulatory proteins (Vale et al., 1985*c*). Such differences would be extremely difficult to detect with the electron microscope.

Conclusion

Based on the information discussed above, it is possible to propose a limited number of cross-bridge models for bidirectional vesicle transport. The model that we have chosen for illustration in Fig. 10 contains two forms of the vesicle transport cross-bridge: one that binds specifically to anterograde vesicles and drives the vesicle toward the plus end of the microtubule and another form that binds specifically to retrograde vesicles and generates force in the opposite direction. Although other variants of this model are possible, the model that we have proposed provides a reasonable framework for future discussion of cross-bridge–mediated vesicle transport in axons.

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