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ATP-dependent directional movement of rat synaptic vesicles injected into the presynaptic terminal of squid giant synapse

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ABSTRACT **The question as to whether synaptic vesicles prepared from vertebrate brain can be transported to the active zones of the squid giant synapse was studied by using a combined optical and electrophysiological approach. In order to visualize the behavior of the vertebrate synaptic vesicles *in situ*, synaptic vesicles isolated from rat brain were labeled with a fluorescent dye (Texas red) and injected into the presynaptic terminal of the squid giant synapse. The pattern of fluorescence that would result from passive diffusion was determined by coinjection of an unconjugated fluorescent dye (fluorescein). The patterns obtained with fluorescent synaptic vesicles were strikingly different from that obtained by simple diffusion of fluorescein. Although the fluorescein diffused freely in both directions, the vesicles moved preferentially into the terminal—i.e., toward the release sites—at a rate of 0.5 μm/sec. The final distribution of the injected fluorescent synaptic vesicles displayed a discrete localization that suggested a distribution coincident with the active zones of the presynaptic terminal. Like fast axonal transport, but unlike fluorescein movements in the terminal, the vesicle movement was energy dependent, since the addition of 2,4-dinitrophenol blocked the redistribution of vesicles completely. In addition, reduction of extracellular calcium concentration reversibly blocked vesicular movement as well. In conclusion, mammalian synaptic vesicles retain the cytoplasmic surface components necessary for translocation, sorting, and targeting to the proper locations by the native machinery of the squid giant synapse.**

The movement and disposition of synaptic vesicles (SVs) in presynaptic terminals have been areas of intensive research in recent years. Several different lines of investigation have led to the conclusion that SVs or their precursors are delivered to the presynaptic terminal by fast axonal transport (for review, see refs. 1 and 2). Many questions remain, however, about the fate of these vesicles during and after transport to the presynaptic terminal. For example, how are these vesicles targeted to the terminal rather than to the axonal plasmalemma? What occurs once the SV enters the presynaptic terminal: Do they proceed directly to the junctional areas or are they distributed stochastically? Moreover, what effect does activity have on these processes? Are the same molecular motors involved in moving vesicles in the terminal and in the axoplasm? Answers to most of these questions have been difficult or impossible to obtain because of limitations in the preparations and tools available to researchers. A combination of microinjection and intracellular recording with video microscopy techniques has now provided us with the means of addressing these fundamental questions in the presynaptic terminal of the squid giant synapse.

Several methods are now available for preparation of highly purified SVs from vertebrate neural tissue (3–6). These homogeneous fractions can be stripped of peripheral proteins and labeled with fluorescent probes without losing biological activity (neurotransmitter content and enzymatic activities). Previous studies with labeled SVs from vertebrate neural tissue have demonstrated that once they are introduced into isolated axoplasm from squid, the labeled SVs can interact with the motile machinery of fast axonal transport present in the axoplasm and can then be transported along the endogenous organelles (7). When the surface properties of the SVs were modified prior to injection into the axoplasm, the ability of the SVs to move and the directionality of movement were affected (refs. 1 and 7; S.T.B. and L. Yamasaki, unpublished observations). The principle of using a heterologous system that combined SVs from vertebrate sources with molluscan giant neurons and manipulating these constituent elements independently was thereby established as feasible. However, this type of preparation was primarily suited for studying the molecular mechanisms of fast axonal transport, particularly with regard to questions about putative molecular motors (i.e., kinesin or cytoplasmic dynein), and could not readily address questions about targeting or function in the terminal.

Extensive electrophysiological studies of the giant synapse have been conducted to characterize the physiological properties of synaptic transmission in the squid (8). In most respects, the squid giant synapse is a prototypical chemical synapse with the characteristic features of many vertebrate synapses such as the vertebrate neuromuscular junction. However, the large size and relative accessibility of the giant synapse of the squid permit intracellular recording within the terminal itself as well as microinjection of materials directly into the presynaptic terminal and adjacent axonal regions (9). As a result, the presynaptic terminal of the giant synapse is uniquely amenable to experimental manipulations for exploration of the molecular mechanisms underlying synaptic transmission. The ability to microinject materials into this presynaptic terminal has been particularly useful for examining possible functions for specific proteins associated with the terminal. For example, the injection of synapsin I has allowed the study of phosphorylation in the modulation of transmitter availability (9), and the injection of calcium indicators (10–12) enabled the visualization of the distribution of inward calcium current during synaptic release. A logical extension of these two approaches is to inject intact organelles into the terminal instead of proteins.

Microinjection of fluorescently labeled vertebrate SVs into the presynaptic terminal represents a powerful paradigm for understanding synaptic function. The ability to modify independently the SV components permits analysis of those vesicle components involved in the movement of organelles in the terminal, in the targeting of organelles to the junctional regions, and in the release of neurotransmitter. The electrophysiological properties of the giant synapse can be readily evaluated and continuously monitored, so the effects of an

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Abbreviations: SV, synaptic vesicle; TRSV, Texas red SV; DNP, 2,4-dinitrophenol.

experimental manipulation can be quantitatively analyzed, while simultaneously evaluating movements of the previously injected fluorescent SVs. We describe here the ATP-dependent, directional movement of vertebrate SVs in the presynaptic terminal of the squid giant synapse. The present set of experiments demonstrates that vesicles injected into a functional synapse are treated as intrinsic vesicles and are carried to the site of synaptic release in the presynaptic terminal.

MATERIAL AND METHODS

The following work was performed at the Marine Biology Laboratory in Woods Hole, MA. Reagents were obtained from Sigma unless otherwise stated. The squid giant synapse in the stellate ganglion of *Loligo pealeii* was removed from squid mantle under running seawater following standard protocol (13). Typically, squid with mantle lengths between 7 and 10 cm were utilized. Following removal of the stellate ganglion, it was placed in the recording chamber, and connective tissue was removed to allow presynaptic and postsynaptic recording and to facilitate visualization of the presynaptic terminal. The design of the experiment requires a clear visualization of the complete synaptic terminals, so only those stellate ganglia having superficial presynaptic terminals and simple structures were chosen. A synapse with simple structure was defined as having presynaptic terminals that ran longitudinally over the postsynaptic axons as opposed to those that either dive into the ganglion and contact the lower surface or those that wrap around the postsynaptic axon. In these cases, visualization of vesicular movement is very much impaired, although electrophysiological recordings could still be feasible. Preparations were perfused with oxygenated, running artificial seawater to maintain viability. In some experiments, Ca^{2+}-free artificial seawater, prepared by replacement of $CaCl_2$ with $MgCl_2$, was employed to eliminate activity.

Electrophysiological Techniques. Methods for electrophysiological studies were essentially as described previously (13). The presynaptic terminal was stimulated with extracellular electrodes located in the presynaptic bundle, and intracellular recordings were obtained at the presynaptic terminal near the point of entry of the presynaptic axon into the ganglion, prior to its ramification into the presynaptic digits. Intracellular recordings were required to characterize the presynaptic terminal, to assess its viability, and to check its ability to maintain a proper resting potential during the injection procedure. The identification of the presynaptic terminal could also be obtained optically as the injection of presynaptic dye immediately outlined the shape of the preterminal.

Preparation and Injection of Fluorescent Vertebrate SVs. Synaptosomal fractions were prepared from rat cortex, and SVs were purified from these synaptosomes by using a minor modification of the method of Huttner *et al.* (5). Following purification, 33-μg samples of SVs were resuspended in 200 μl of precleared Texas red (Molecular Probes) in labeling buffer (0.67 mg of Texas red per ml/0.4 M NaCl/10 mM Hepes, pH 7.4/10 mM EGTA) and incubated for 4 hr at 4°C. This step removes peripheral proteins and labels integral membrane proteins of the vesicle fraction. Following labeling, the Texas red SVs (TRSVs) were pelleted by a 15-min spin at 260,000 × g in a Beckman TL-100 ultracentrifuge and washed several times by resuspension into labeling buffer without Texas red. TRSVs could then be stored as aliquots either at −80°C for 4–8 wk or on ice for up to 10 days. Prior to use, TRSVs were pelleted, washed, and resuspended in injection buffer (0.1 M potassium aspartate/10 mM Hepes, pH 7.2). Previous studies had shown that this treatment does not interfere with the ability of TRSVs to move in fast axonal transport (7).

Unconjugated fluorescein was added to the injection fluid along with the TRSVs so that two fluorescent dyes were simultaneously injected. The fluorescein served several important functions. It was utilized as a control for the diffusion of any nonvesicular components and defined the morphology of the terminals and adjacent axon. In addition, the fluorescein provided a measure of the relative thickness in different regions of the presynaptic terminal and axon. The choice of unconjugated fluorescein as the second dye was made on the basis of two criteria. (*i*) Fluorescein and Texas red have sufficiently different excitation and emission wavelengths so that the two dye distributions could be compared at all times by switching filters and that observation at one wavelength would not contribute to bleaching for the other fluorochrome. (*ii*) Fluorescein did not significantly interact with other structures of the presynaptic terminal (SVs, plasmalemma, cytoskeletal structures, etc.).

Injection of TRSVs and fluorescein solutions into the presynaptic terminal were accomplished by using carefully beveled micropipettes. The beveling step was crucial as vesicular material was difficult to inject if the micropipette opening was not large enough. Conversely, a very large micropipette opening invariably leads to presynaptic terminal death, due to the influx of seawater, with a consequent disruption of synaptic organization and paralysis of vesicular mobility. Two important parameters were taken into consideration: (*i*) to bevel the electrode with an angle sufficiently steep in order to combine a low resistance (<1 MΩ) with a diameter sufficiently small so as to prevent injury to the presynaptic terminal and (*ii*) to ensure good suspension of SVs, as they tend to aggregate and form large masses that could prevent injection by blockage of the electrode. If injected, these aggregated vesicular masses exhibited a general lack of mobility. The actual injection was performed by use of pressure pulses. These pulses had a duration of 100–150 msec and the pressure varied from 20–35 psi (1 psi = 6.89 kDa).

Microscopic Techniques. The fluorescence was imaged by using a ×10 objective with an optical aperture of 0.22, which provided images to a double microchannel plate image intensifier coupled to a videcon camera (C1966-20 VIM System; Hammamatsu Photonics). This system allowed the visualization of fluorescence at extremely low light levels. Though fine structures could not be resolved by using this optic, this system gave excellent images of the bulk of the injected material in the interior of the presynaptic terminal. The image was then fed to an image processor (C1966; Hammamatsu Photonics), which allowed a variety of image enhancements, including a running average of fluorescence, the subtraction of background, and greyscale or pseudocolor manipulations. Fluorescent images were also recorded directly on video tape using a conventional VHS-type videocassette recorder, and the taped image was used for further analysis after the experiment. The fluorescent images were then displayed either as grey level images or using pseudocolor to emphasize fluorescent profile of injected materials.

RESULTS

Movement of TRSVs in the Presynaptic Terminal of the Squid Giant Synapse. After the pressure injection of presynaptic vesicles, SVs—i.e., Texas red fluorescence—were eventually distributed throughout the terminal area and outlined the complete structure of the synapse (Fig. 1). Similar results were obtained in four additional experiments. The image in Fig. 1 was taken 50 min after the initial injection. The injection site was located at the base of the upward pointing digit (arrow). It is clear from the illustration that the vesicles

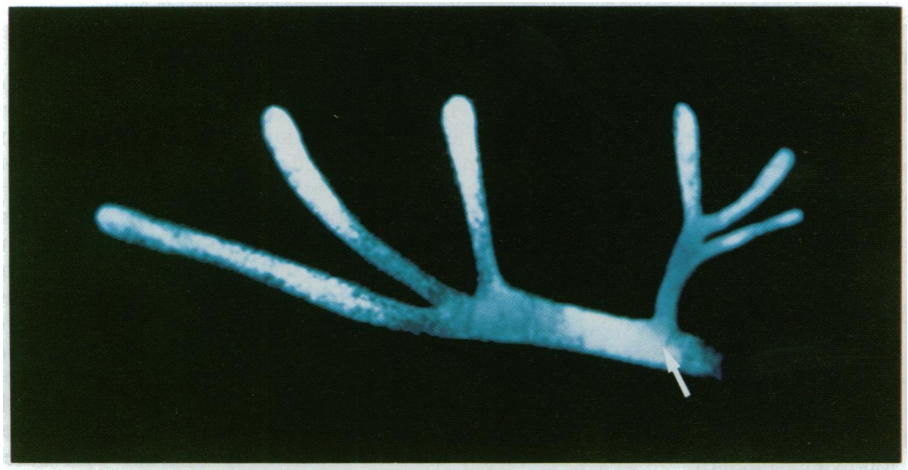

FIG. 1. Distribution of SVs in the squid giant synapse. This image highlights the distribution of Texas red fluorescence—i.e., the SVs—50 min after the initial TRSV and fluorescein coinjection. The density of the fluorescence is not uniform over the entire synapse. Instead, the distal portions of the digits have higher density and indicate a higher concentration of the TRSVs. The injection site was located at the base of the upward pointing digit (arrow), which contained three branchlettes. By contrast, coinjected fluorescein never concentrated in any portions of the presynaptic terminal.

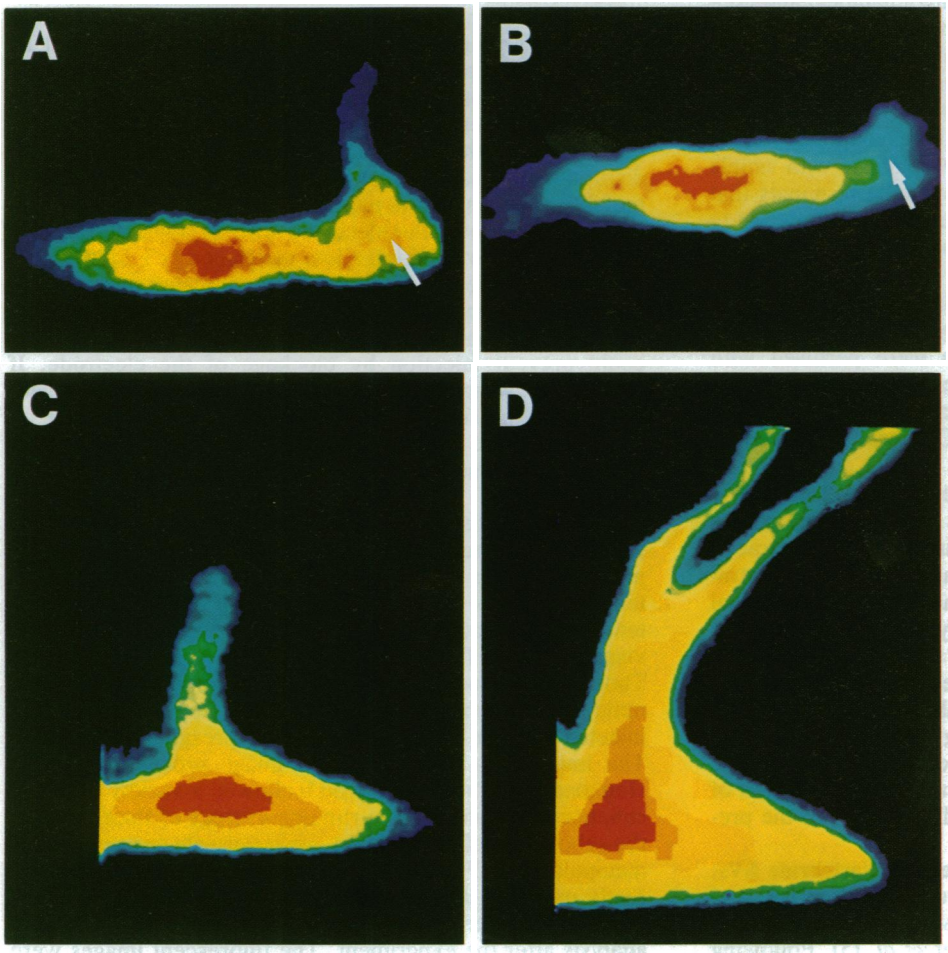

FIG. 2. Distribution of fluorescein and TRSV at different times after the initial injection. These images were obtained from the same synapse as that in Fig. 1 and have the same orientations. The fluorescein distribution 10 min after the injection (*A*) was mostly in the main trunk of the synapse and the axon. The latter corresponds to the area toward the right of the upward pointing digit. The TRSV distribution (*B*) has a similar distribution except that it is more restricted. The injection sites are indicated by arrows. A clear difference in the distribution of TRSV and fluorescein was observed 27 min after the initial injection (*C* and *D*). These images were taken under a higher magnification than that shown in *A* and *B*, and the terminal region to the left is not included. Due to prolonged diffusion, the fluorescein density is relatively low (*C*) such that its profile is smaller than the actual size of the synapse, which is better delineated by the distribution of TRSVs (*D*). In contrast to the distribution of fluorescein, TRSVs move along the terminal digit "purposefully" and penetrate deep into the branches (*D*). The distance that TRSVs travel along the digit is far longer than that on the retrograde direction—i.e., to the right.

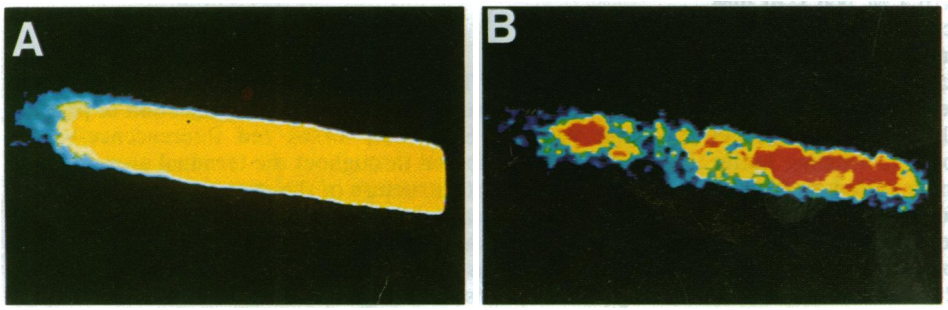

FIG. 4. Vesicular movement is energy dependent. In the presence of 0.66 mM DNP, the distribution of TRSV remained unchanged 35 min after injection (*B*), whereas fluorescein faded away rapidly after the initial injection. Terminal digits pointing toward the left in this preparation were not invaded by TRSVs. The picture in *A* was taken shortly after a second injection to highlight the initial distribution of fluorescein.

only moved anterogradely as there was virtually no fluorescence in the axon to the right. Furthermore, the SVs were preferentially concentrated in the areas corresponding to synaptic junctions—i.e., the last few hundred microns of the digits. In the case of the terminal digit, the horizontal one on the left, the vesicular material was seen to cover a large portion of the digit but was less concentrated near its base. The density of fluorescence in this digit is not entirely uniform, not unlike that observed from direct measurement of calcium entry using Fura II (12), and suggests a correlation with the localized distribution of the active zones in the terminal.

In order to provide a chronological description of the vesicular movement in the presynaptic arbor illustrated in Fig. 1, the distribution of fluorescence observed at earlier times after the injection is illustrated in Fig. 2. The initial distributions of the fluorescein (Fig. 2*A*) and the Texas red (Fig. 2*B*) fluorescence provide a direct measure of the location of the injected presynaptic vesicles within the terminal. This image was taken 10 min after the injection of a 10-pl volume of fluid containing TRSVs (0.44 mg/ml), fluorescein (0.14 mg/ml), and ATP (14 mM) in injection buffer. Following the initial injection, the presynaptic electrode was removed and the movement of vesicles was monitored by using the epifluorescence microscopy system.

As illustrated in Fig. 2*B*, the TRSVs were still largely confined to the general area of injection at 10 min, while the fluorescein had clearly diffused beyond the original injection site, moving both proximally and distally. After a period of 27 min, much of the fluorescence associated with the injected fluorescein had diffused away and consequently outlined an image smaller than the actual size of the synapse (Fig. 2*C*). In contrast, movement of TRSVs during the last 17 min covered a distance of ≈500 μm, measured from injection site to the tip of right branch (Fig. 2*D*). This measurement provides a transport rate of 0.5 μm/sec. By comparison, fast axonal transport in the isolated axoplasm has an average rate of 1.9 μm/sec in the anterograde direction and 1.3 μm/sec in the retrograde direction. Instead of measuring the rate of fluorescent vesicular movement by direct observation of individual vesicles (7), our imaging system only allowed us to estimate the transport rate by the movement of fluorescence boundary. This approach provided a lower limit of the transport rate because the criterion for defining the boundary was to find a line where there was an abrupt change in the fluorescence density. By this criterion, there was clearly weak fluorescence beyond the line but change in fluorescence density was too gradual to provide a consistent boundary definition. A quantitative description of TRSV movement, measured from the same synapse as that of Figs. 1 and 2, is illustrated in Fig. 3, where the movements to the terminal digit (square) and to the upward-pointing digit (circle) were plotted separately. The linear fits provide a transport rate of 0.5 and 0.4 μm/sec, respectively.

Movement of TRSVs was preferential in the anterograde direction. In fact, TRSVs were always observed to advance into the digits and to concentrate in the distal portions of the presynaptic terminals, there being essentially no detectable fluorescence in the retrograde direction. A clear demonstration of this point can be seen in Fig. 2*D*, where the distance of Texas red distribution into the digit is far longer than that into the larger proximal part of the axon (toward the right).

In contrast, injected fluorescein diffused quickly both forward into the presynaptic terminal and back into the proximal portion of the presynaptic axon. As a result, the fluorescein signal soon became widely distributed and thus attenuated. In fact, qualitatively the largest movement of fluorescein occurred in the retrograde direction, reflecting the larger sink for diffusion.

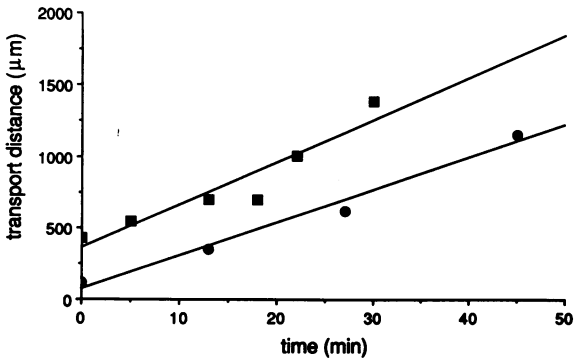

FIG. 3. Rate of vesicular movement. The measurements were obtained from the same synapse as that in Fig. 1. TRSV fluorescence movement in the horizontal direction (■)—i.e., toward the terminal digit to the left—and in vertical direction (●)—i.e., into the pointing digit—are plotted in the same graph. The distances were measured upward from the injection site to the boundary of fluorescence (see text). The straight lines are the linear regression fits for each direction. The data points do not start at zero distance because the fluorescence distribution covered a significant area instantaneously; presumably it was forced by the pressure applied through the injection electrode.

Distribution of Labeled SVs Following Inhibition of ATP Synthesis. In order to determine whether the movement required ATP, 2,4-dinitrophenol (DNP) (0.66 mM) was added to the extracellular fluid in place of the ATP. This has been shown to produce a total blockage of axonal transport (14, 15). If the movement of presynaptic vesicles in the terminals were to be an active process, dependent on ATP, then inhibition of ATP synthesis should block the redistribution of TRSVs as it blocks fast axonal transport. Coinjection of TRSVs and fluorescein with DNP demonstrated that although distribution of material due to passive diffusion (fluorescein) was not modified by the treatment, the redistribution of SVs did not occur following inhibition of axonal transport (see Fig. 4). Specifically, the fluorescein fluorescence typically faded away rapidly and required multiple injections to outline the profile of the synapse (Fig. 4*A*), whereas TRSV distribution remained stationary even after multiple injections (Fig. 4*B*). These experiments, which were repeated three times, demonstrated no movement of vesicles for periods up to 40 min after injection.

Distribution of Labeled SVs Following the Removal of Extracellular Calcium. Extracellular calcium was removed from the bathing medium to evaluate its role on the movement of TRSVs in a series of experiments similar to those described in previous sections. In this case, the extracellular calcium was removed from the bathing medium from the time of fine dissection in the recording chambers for a period of at least 30 min prior to injections of TRSVs and fluorescein. After this treatment, the extracellular calcium concentration was sufficiently low to prevent transmitter release, although presynaptic action potentials still exhibited a normal amplitude. As a result of this treatment, repeated in four synapses, no movement of TRSVs away from the initial site of injection could be detected. The movement of TRSVs under these conditions of low extracellular calcium resembled those following blockage of axonal transport by DNP. Furthermore, in three of these four experiments, partial or complete movement of the TRSVs could be recovered when the extracellular calcium concentration was raised to 10 mM. By contrast, removal of intracellular calcium in the axoplasm by perfusion with buffers containing as much as 10–20 mM EGTA had no detectable effect on the transport of organelles in fast axonal transport (14–16).

DISCUSSION

The results described above indicate that mammalian SVs moved when injected into the axoplasm of the squid giant synapse. Furthermore, the vesicles were specifically transported to the site of synaptic release. The rate and directionality of this movement are consistent with the view that movement occurs via the normal transport systems of the presynaptic terminal. In some respects, this movement bears a certain resemblance to vesicle movement due to fast axonal transport obtained in isolated axoplasm (1, 14, 15).

As in the isolated axoplasm, TRSVs in the presynaptic terminal move preferentially in the anterograde direction and in an ATP-dependent fashion. When the injection electrode was located near the base of the distal-most digit, SVs were seen to move toward that digit alone, again suggesting that vesicular movement is preferentially in an anterograde direction. There are, however, some differences. For example, the average velocity of movement for fluorescent vesicles in the terminal is only 0.5 μm/sec as compared to 1.9 μm/sec for fast axonal transport in the anterograde direction. Several mechanisms might be invoked to explain all or part of this apparent reduction in velocity. These include differences in the methods used for rate measurement in the two preparations (i.e., movement of large groups of organelles as opposed to movement of individual organelles), differences between the cytoskeleton of the terminal regions and the axoplasm, and multiple mechanisms for movement in the terminal. Another difference is that the reduction of extracellular calcium seems to completely block the movement of TRSVs in the presynaptic terminal, but fast axonal transport appears unaffected by low calcium levels (14–16). Some investigators have reported inhibition of fast axonal transport following application of a calcium channel blocker (17) or inhibitors of calmodulin (18). Such differences may reflect either an effect of the extracellular calcium on a regulatory process in the terminal (and possibly the axon) or the fact that cytoskeletal organization is primarily actin microfilaments in the presynaptic terminal as opposed to microtubules and neurofilaments in the axon. The extent to which vesicle movements in the terminal regions are homologous to fast axonal transport remains to be determined, but study of the movements of vertebrate SVs in the squid giant presynaptic terminal should permit elucidation of the underlying molecular mechanisms of vesicle movements in the terminal.

A striking feature of the redistribution of TRSVs in these studies is the specificity of targeting. No gross unevenness of vesicular distribution among the terminal digits was observed, suggesting that the base of the terminal arborization is a strategic site for injection that allows vesicular distribution to all presynaptic digits. The mechanisms for sorting and targeting of endogenous vesicles in the presynaptic terminal appear to function equally well on the injected vertebrate TRSVs. Apparently the information required for sorting and targeting must be present on the vesicles themselves and is sufficiently well conserved to be operational even when mammalian SVs are directed by the molluscan presynaptic terminal. Systematic modification of the vesicle surface prior to injection should permit identification of the vesicle surface features that determine the targeting and sorting of SVs in the presynaptic terminal. Similar studies have already determined that certain protein components of the vesicles affect the directionality of transport in axonal transport (7).

The fact that agents known to block axonal transport or to interfere with synaptic release were also capable of modifying vesicular movement indicates that the movement of TRSVs is well correlated with vesicular movement in the presynaptic terminal *per se*. SVs appear to move only when the preterminal is fully functional and capable of releasing transmitter. This is to be expected if the vesicle movements do relate to the mechanisms of transport, sorting, and release. Moreover, it is probable that the final distribution of vesicular material in these experiments concerns not only the actual accumulation of vesicles but also the apposition of vesicles onto the presynaptic axolemma. These experiments not only demonstrate that presynaptic vesicles move in the proper direction and accumulate in appropriate locations but also suggest that the system may in fact allow the vesicles to fuse with the membrane of the presynaptic terminal.

The small size and inaccessibility of most presynaptic terminals have hindered studies on the molecular mechanism of synaptic release and associated phenomena. A similar impasse had been reached in the study of molecular mechanisms of fast axonal transport prior to the application of video microscopy imaging methods to the study of fast axonal transport in the isolated axoplasm (14, 15). The result was the discovery of a new class of mechanochemical enzymes, the kinesins (19, 20). Analyses of the movements of vertebrate SVs in the presynaptic terminal of the squid giant axon using a combination of electrophysiological and video microscopic methods promise to provide comparable insights into the molecular mechanisms of synaptic release and transport of vesicles in the presynaptic terminal.

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