

# Synaptic Vesicles Have Two Distinct Recycling Pathways

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**Abstract.** In this paper, evidence is presented that two distinct synaptic vesicle recycling pathways exist within a single terminal. One pathway emanates from the active zone, has a fast time course, involves no intermediate structures, and is blocked by exposure to high  $Mg^{2+}$ /low  $Ca^{2+}$  saline, while the second pathway emanates at sites away from the active zone, has a slower time course, involves an endosomal intermediate, and is not sensitive to high  $Mg^{2+}$ /low  $Ca^{2+}$ . To visualize these two recycling pathways, the temperature-sensitive *Drosophila* mutant, *shibire*, in which vesicle recycling is normal at 19°C but is blocked at 29°C, was used. With exposure to 29°C, complete vesicle depletion occurs as exocytosis proceeds while endocytosis is

blocked. When the temperature is lowered to 26°C, vesicle recycling membrane begins to accumulate as invaginations of the plasmalemma, but pinch-off is blocked. Under these experimental conditions, it was possible to distinguish the two separate pathways by electron microscopic analysis. These two pathways were further characterized by observing the normal recycling process at the permissive temperature, 19°C. It is suggested that the function of these two recycling pathways might be to produce two distinct vesicle populations: the active zone and nonactive zone populations. The possibility that these two populations have different release characteristics and functions is discussed.

THE most widely accepted explanation for transmitter release is the vesicle hypothesis, which proposes that transmitter is stored in synaptic vesicles and released by exocytosis (del Castillo and Katz, 1955). This process involves the fusion of the vesicle with the presynaptic membrane and the formation of a pore through which the transmitter is expelled. While most agree that transmitter release is achieved by exocytosis, controversy continues over the fate of the vesicle membrane once it has fused. One school of thought, pioneered by Heuser and Reese (1973), proposes that after the vesicle fuses and expels its contents, it collapses into the plasma membrane, mixing with it completely, and is then reinternalized via coated vesicles at sites away from the release site (for review see Heuser, 1989). Indeed, with exhaustive stimulation produced by such treatments as lanthanum ( $La^{3+}$ ) or  $\alpha$ -latrotoxin ( $\alpha$ -LTX)<sup>1</sup> in the absence of  $Ca^{2+}$ , membrane in the form of plasmalemmal invaginations, coated pits/vesicles, and cisternae accumulates in the terminal at sites away from the active zone (Ceccarelli et al., 1973; Heuser and Reese, 1973). Also, synaptic vesicle proteins become

detectable along the axolemma after stimulation with these substances, suggesting they have been inserted into the plasma membrane (von Wedel et al., 1981; Valtorta et al., 1988; Torri-Tarelli et al., 1990).

A second school of thought proposed by Ceccarelli et al. (1973) suggests that with milder, more physiological types of stimulation, the vesicle membrane does not collapse into the plasma membrane but rather is immediately reinternalized at the site of exocytosis (for reviews see Valtorta et al., 1990; Fesce et al., 1994). Thus, it was observed that under conditions of intense electrical stimulation (when exocytosis predominates) or under conditions of rest, immediately after stimulation (when endocytosis predominates), the distribution of dimples and protuberances (exo/endocytotic events) was the same, suggesting that both exocytosis and endocytosis occur at the active zone (Ceccarelli et al., 1979). Also, vesicular membrane proteins are not detected on the axolemma during conditions of intense recycling induced by extensive electrical stimulation or application of  $\alpha$ -LTX in presence of extracellular  $Ca^{2+}$ , suggesting that under more physiological conditions of stimulation, the vesicle contact with the axolemma is fleeting, and intermixing of vesicle and plasma membranes does not occur (von Wedel et al., 1981; Valtorta et al., 1988; Torri-Tarelli et al., 1990).

The above observations, taken together, suggest that there may be two different mechanisms for recycling in the terminal, with two different sites of reinternalization. With moderate stimulation (for example, produced by electrical stimulation of the nerve), the evidence suggests that recy-

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1. Abbreviations used in this paper: ACh, acetylcholine;  $\alpha$ -LTX,  $\alpha$ -latrotoxin; NMJ, neuromuscular junction; *shi*, *shibire*<sup>st</sup>; SLMV, synaptic-like microvesicles; TsTx, tityustoxin.

cling occurs rapidly at the site of exocytosis, while with excessive stimulation (produced by certain drugs or toxins), recycling also occurs at sites away from the release site. Recently, two different rates of endocytosis have been reported using capacitance measurements, a faster component resulting from weaker stimulation, and a slower component resulting from strong stimulation (von Gersdorff and Matthews, 1994). Thus, various lines of evidence point to the existence of two recycling mechanisms in the terminal.

## Materials and Methods

In this paper, 4-d-old adult female *Drosophila melanogaster* of the temperature-sensitive paralytic mutant, *shibire<sup>ts1</sup>* (*shi*), were used to observe the vesicle recycling pathway. The *shi* gene encodes the protein dynamin (Chen et al., 1991; van der Blik and Meyerowitz, 1991), which is involved in the process whereby vesicles/cisternae pinch off from the plasma membrane during the process of endocytosis (Kosaka and Ikeda, 1983a,b; Damke et al., 1994; Hinshaw and Schmid, 1995; Takei et al., 1995). In *shi* flies, the mutant dynamin molecule is functional at 19°C (permissive temperature) but becomes dysfunctional at 29°C (restrictive temperature), possibly because of a configurational change in the molecule resulting from the amino acid substitution. Thus, the process of endocytosis is very specifically blocked at the pinch-off stage at the restrictive temperature without any effects on other processes in the cell. In the neuron, exposure to 29°C results in vesicle depletion as exocytosis (transmitter release) proceeds normally while endocytosis (vesicle recycling) is blocked (Koenig et al., 1989). By lowering the temperature to 19°C after exposure to 29°C, the dynamin molecule once again becomes functional, and normal recycling proceeds in a synchronized manner, revealing the various intermediate steps in the process of vesicle reformation (Koenig and Ikeda, 1989).

The terminals of the photoreceptor cell, the retinula cell, were observed for these experiments because these cells have two distinct advantages for this work. One is that it is possible to stimulate the cell without dissecting, by simply exposing the eye to light. (Stimulation of the cell to cause exocytosis is necessary for vesicle depletion to occur at the restrictive temperature, 29°C.) A second advantage of using this cell for studies on synaptic transmission is the abundance and characteristic structure of the active zones, which makes them easy to locate and identify.

Individual *shi* flies were mounted in Tackiwax, ventral side up, over an opening in a plastic tube so that the spiracles on the thorax and abdomen remained exposed to the air in the tube. This allowed the fly to breathe after being immersed in saline (128 mM NaCl, 4.7 mM KCl, 1.8 mM CaCl<sub>2</sub>, 5 mM Tris aminomethane HCl, pH 7.4). The head was not embedded in wax, so that the two compound eyes were unobstructed, allowing stimulation by light. The light source, which was on during the entire experiment, was microscope illumination.

After the fly was embedded in wax, it was immersed in 19°C saline. Control *shi* and wild-type (Oregon-R strain) flies were allowed to remain in this condition during the course of the various experiments. For the depletion experiments, the 19°C saline was instantly replaced with 29°C saline. For the recovery experiments, the 19°C saline was replaced with 29°C saline to deplete the terminals, and then the 29°C saline was replaced with either 26 or 19°C saline, depending on the experiment. 26°C was chosen as the best temperature to demonstrate recycling membrane build-up after trying various temperatures. At 29°C, membrane build-up also occurs, but the difference in the time courses of the two different recycling pathways was not as apparent as at 26°C. Below 26°C, membrane build-up often did not occur. The temperature of the saline was maintained using a thermoelectric unit and was monitored by a thermistor placed in the bath.

After exposure to the various temperatures required for each experiment, the fly was fixed for EM. The saline was instantly exchanged for fixative (2% paraformaldehyde, 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4), the temperature of which was the same as that of the replaced saline (19, 26, or 29°C, depending on the experiment). The proboscis was then pulled out with a fine forceps, cut around its base, and removed, exposing the brain and optic lobes. This procedure takes about 5 s. To ensure immediate penetration of the fixative into the cartridge area of the lamina where the retinula cell terminals are located, a fine pipette was inserted into the dissected head at the opening created by removal of the proboscis, and fixative was perfused by pressure injection for 5 min. After 30 min in the aldehyde mixture, the fixative was replaced with 4% glutar-

aldehyde in 0.1 M phosphate buffer, pH 7.4, for 2 h. The fly was then post-fixed in 2% OsO<sub>4</sub> in 0.1 M cacodylate buffer, pH 7.4, block stained in 1% aqueous uranyl acetate, dehydrated in alcohol, and embedded in Spurr. Thin sections were stained with uranyl acetate and lead hydroxide and were observed on an electron microscope (model CM-10; Phillips Electronic Instrs., Co., Mahwah, NJ) and photographed.

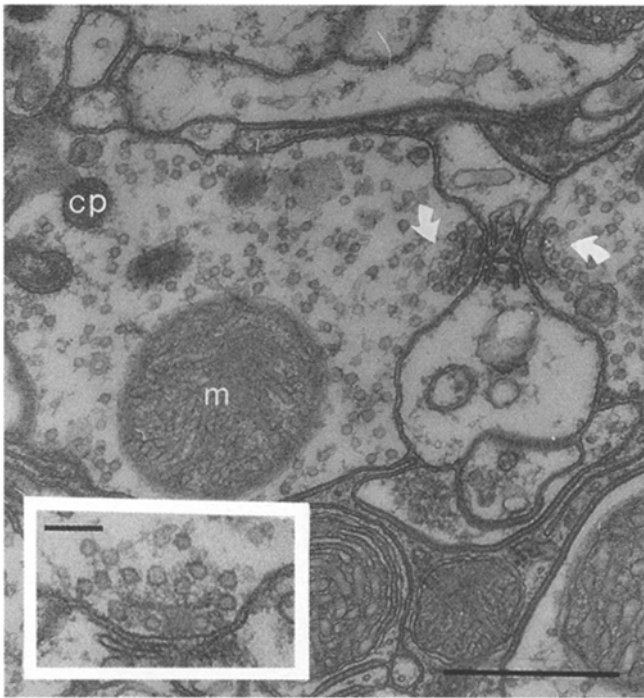
For experiments that required exposure of the terminals to high Mg<sup>2+</sup>/low Ca<sup>2+</sup> saline, the proboscis was removed as described above, and the normal saline was instantly replaced by saline containing 35 mM MgCl<sub>2</sub> and 1 mM CaCl<sub>2</sub>. The osmolarity was adjusted by reducing NaCl by an equiosmolar amount. A pipette was inserted into the opening created by removal of the proboscis, and the brain and optic lobes were perfused for the duration of the exposure (up to 30 min, depending on the experiment).

## Results

Terminals of the photoreceptor cell, the retinula cell, which synapses on interneurons of the first optic ganglion (lamina ganglionaris), were used for this study. These terminals contain many elongate synaptic vesicles dispersed throughout the cytoplasm, as well as glial capitate projections, mitochondria, and smooth cisternae (Fig. 1). The active zones are characterized presynaptically by possessing a dense body, which is made up of a round or oblong (depending on the plane of sectioning) electron-dense base capped by a filamentous structure that parallels the presynaptic membrane and eventually attaches to it. Readily releasable vesicles dock on either side of the electron-dense base under the filamentous cap. In addition, a cluster of vesicles hover above the cap, appearing to be attached to it by fine filaments (Fig. 1, *Inset*). (For a more detailed description of the dense body, see Koenig and Ikeda, 1989.)

### Vesicle Depletion at 29°C

At 19°C, wild-type and *shi* terminals are indistinguishable. However, after 30-s exposure to 29°C in the light adapted condition, *shi* retinula cells are completely depleted of synaptic vesicles. This depletion occurs because exocytosis proceeds normally at 29°C, but endocytosis is blocked (see Materials and Methods). It has been reported that when endocytosis is blocked by certain drugs or toxins, the synaptic vesicle membrane becomes permanently incorporated into the axolemma, causing an increase in the surface area of the terminal (Ceccarelli and Hurlbut, 1980; Haimann et al., 1985; Valtorta et al., 1988). However, when endocytosis was blocked using *shi*, no increase in membrane (in the form of evaginations, invaginations, coated pits/vesicles, or cisternae) suggesting that the vesicle membrane had been inserted into the plasma membrane, was observed in the very early stage of complete depletion. The entire length of the terminal was observed with both cross and longitudinal sections (Fig. 2). The reappearance of a substantial amount of membrane in the form of invaginations and cisternae was only observed if endocytosis was allowed to proceed by lowering the temperature, as will be demonstrated in the following sections. This observation is similar to our previously published results on the coxal neuromuscular junctions (NMJ's), in which a morphometric analysis designed specifically to measure the loss of vesicle membrane and increases in plasma membrane, coated pits/vesicles, and cisternae showed that no



**Figure 1.** Typical retinula cell terminals, containing mitochondria (*m*), capitate projections (*cp*), synaptic vesicles dispersed throughout cytoplasm, and a dense body surrounded by a clump of vesicles (*arrows*). (*Inset*) Active zone with dense body. Note the docked vesicles under the filamentous cap and the cluster of vesicles above the cap, some of which appear to be attached to it by fine filaments. Bars: 0.5  $\mu\text{m}$  (47,500 $\times$ ); *Inset*, 100 nm (76,000 $\times$ ).

increase in terminal plasma membrane equivalent to the lost vesicle membrane is observed in the early stage of complete vesicle depletion in *shi* (Koenig and Ikeda, 1989). The fate of vesicle membrane during depletion using *shi* is not addressed in the experiments presented here. However, the aforementioned paper can be consulted for a discussion of the implications of this observation.

### Two Sites of Recycling Membrane Build-up at 26°C

To observe the synaptic vesicle reformation process after depletion, *shi* flies were first exposed to 29°C for 30 s (to cause depletion) and then the temperature was lowered to 26°C. At this temperature, vesicle recycling membrane begins to accumulate, but the normal pinch-off process is inhibited, leading to a large accumulation of this membrane along the plasmalemma. This temperature was therefore used to determine the site(s) of reinternalization of membrane during recovery from depletion. After various lengths of exposure to 26°C, the *shi* flies were instantly fixed and processed for EM. In this way, the initial stage of the vesicle reformation process could be observed.

After 1 min at 26°C, membrane was observed to be emanating from either side of the dense body base, the location where vesicles normally dock and release (Fig. 3 A). This membrane formed a flat, cisterna-like structure that gradually enlarged with longer exposure to 26°C. This represents the first site of reinternalization of vesicle membrane. Of 376 active zones from eight different flies, 371

had membrane emanating from under the dense body within 1 min.

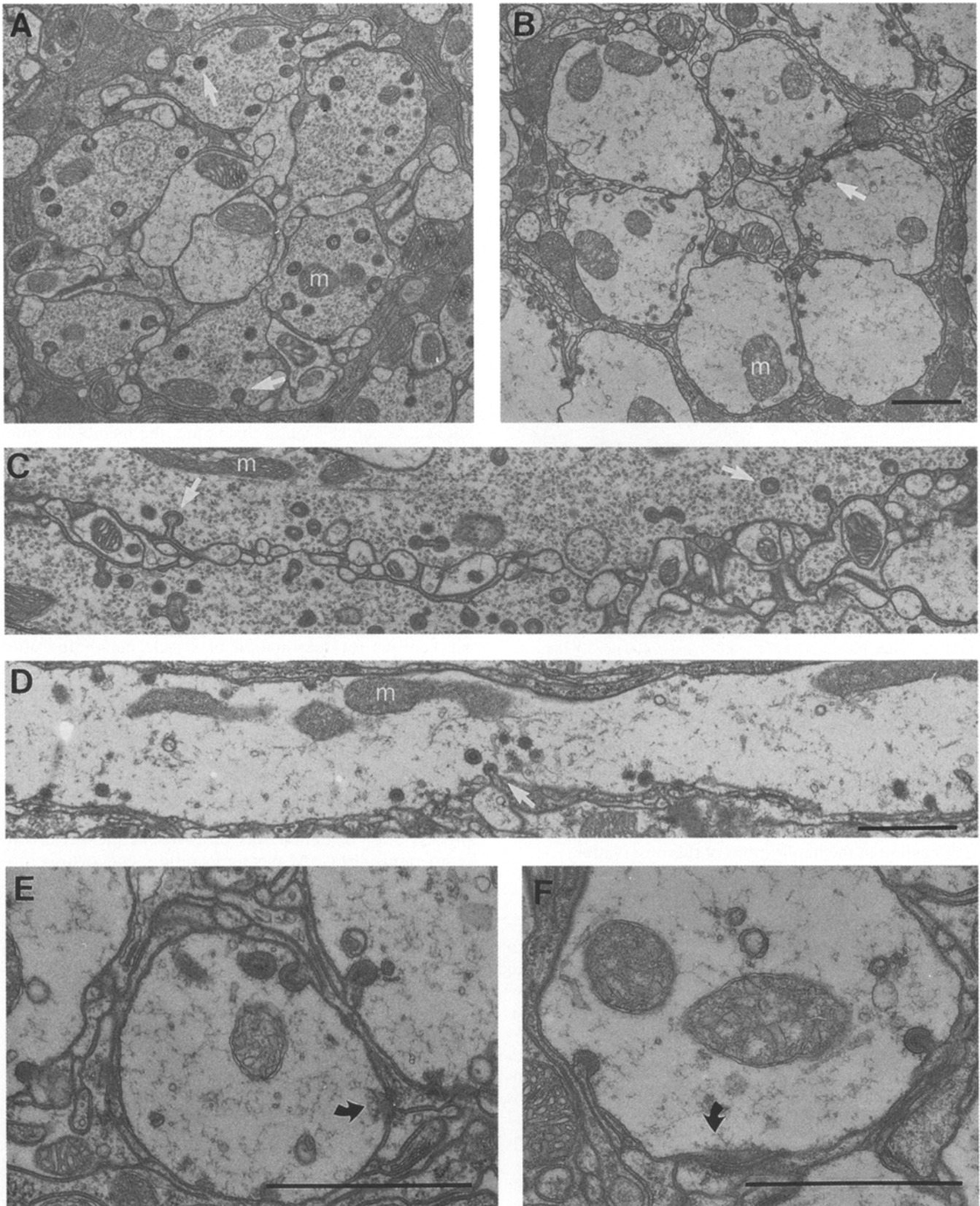
After 2 min at 26°C, many pits were also observed at sites along the plasma membrane away from the active zone (Fig. 3 B). These pits had electron dense material surrounding their neck portions, equivalent to the previously described “collars” observed around the neck portions of the pits at *shi* NMJ’s (Kosaka and Ikeda, 1983a). Occasionally, coated, collared pits were also seen. With longer exposure (5 min) to 26°C, the pits elongated into branching, tubule-like structures, which filled the terminal (Fig. 3 C). These tubule-like structures represent a second type of recycling membrane, which emanates at sites away from the active zone. All terminals observed possessed this type of recycling. In Fig. 3 C, both types of recycling are demonstrated.

The two types of membrane invaginations that are induced by allowing endocytosis to proceed without normal pinch-off are quite distinct structurally. In Fig. 4, further examples of the two types of membrane recycling are shown. As can be seen in this figure, the membrane emanating from either side of the dense body base forms a flat, cisterna-like structure with a cross-sectional diameter of about 20 nm (within any particular plane of sectioning). No branching of this structure was ever seen. The membrane emanating from the sites away from the dense body is tubule-like with a cross-sectional diameter of about 60 nm. Many branch points, each surrounded by an electron-dense collar, were apparent on these structures. Thus, the observations suggest that there may be two different mechanisms for reformation of vesicle membrane after exocytosis, one with a faster time course located at the active zone, and a second with a slower time course located away from the active zone.

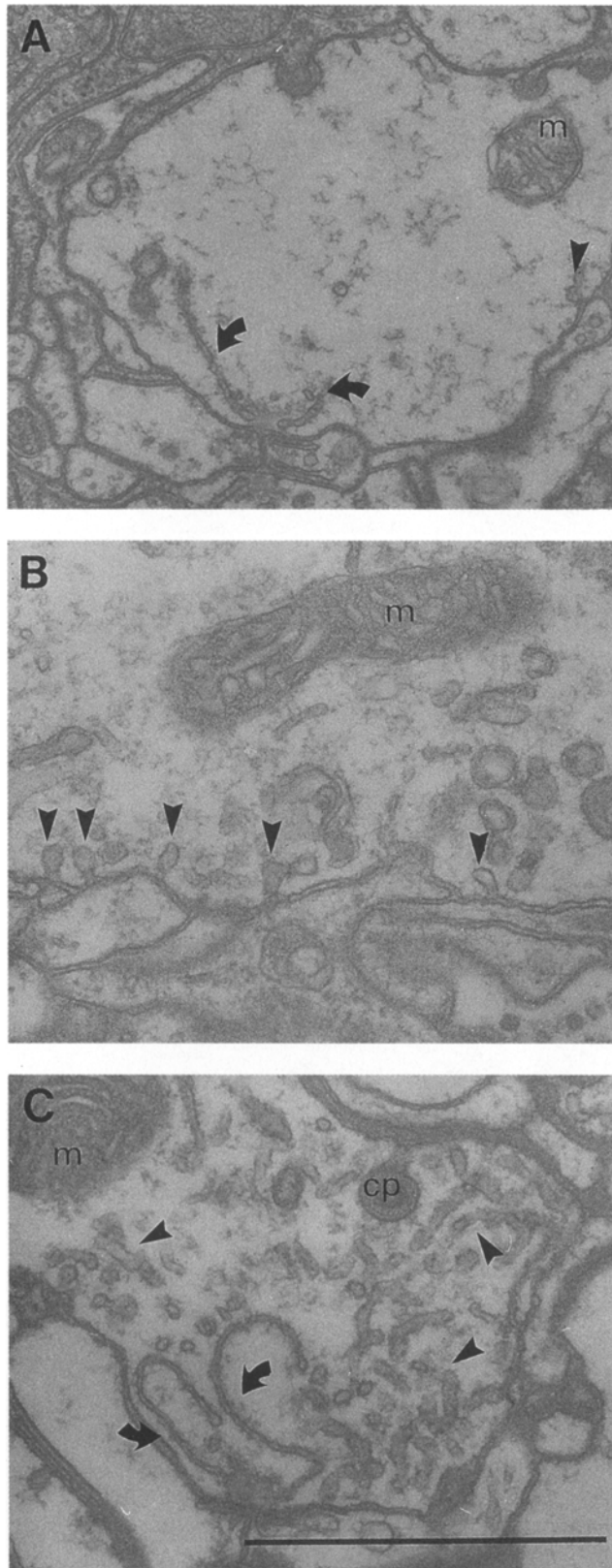
### Two Recycling Pathways after Depletion

To better characterize the two recycling pathways suggested by the observations at 26°C, the vesicle reformation process after depletion was observed at the permissive temperature, 19°C. At this temperature, endocytosis proceeds normally, allowing observation of the intermediate steps in the recycling pathway up to the point of total recovery of the vesicle population. *Shi* flies were exposed to 29°C for 30 s to deplete the synapses and then returned to 19°C. After various exposure times to 19°C, the flies were fixed and processed for EM.

Within 1 min at 19°C, at most of the release sites, a few vesicles were observed docked on either side of the dense body base under the filamentous cap (Fig. 5). At some terminals, a small cluster of vesicles was seen around the dense body. Of 357 active sites observed from six different flies, 312 had at least one vesicle under the dense body. (Possibly, all the active sites possessed vesicles, but in some cases they were out of the plane of sectioning.) As can be seen, the rest of the terminal is still depleted of vesicles at this early stage. During this initial stage of recovery at 19°C (0–1 min), examples of the flat, cisterna-like structures that were seen emanating from under the dense body at 26°C were not observed. This suggests that reformation of the vesicle population located at the release site does not involve intermediate structures such as coated pits/



**Figure 2.** Typical retinula cell terminals at 19°C and after depletion at 29°C. (A) Cross section of typical lamina cartridge of a *shi* fly at 19°C, made up of six retinula cell axons, surrounding first-order sensory neurons, L<sub>1</sub> and L<sub>2</sub>. Note the many vesicles in the retinula cell axons. (B) Cross section of typical *shi* cartridge at 29°C. Note the lack of vesicles in the retinula cell axons. (C) Longitudinal section of typical *shi* retinula cell axon at 19°C. (D) Longitudinal section of typical *shi* retinula cell axon at 29°C. Note loss of vesicles. No increase in membrane, in the form of invaginations, coated or uncoated pits/vesicles, or cisternae is observed in depleted *shi* terminals. (E and F) Examples of depleted *shi* retinula cells shown at higher magnification. Note depleted active zones (arrows). *m*, mitochondria; arrows in A–D, capitate projections. Bars: 1 μm (11,250×); (C and D) 1 μm (17,000×); (E) 1 μm (36,000×); 1 μm (38,500×).



**Figure 3.** Retinula cell terminals demonstrating two sites of recycling membrane. (A) Typical *shi* retinula cell terminal after 30-s exposure to 29°C, followed by 1-min exposure to 26°C. Note membrane (arrows) emanating from either side of the dense body at the active zone and the otherwise depleted terminal. One collared pit is seen to the far right (arrowhead) (B) *shi* retinula

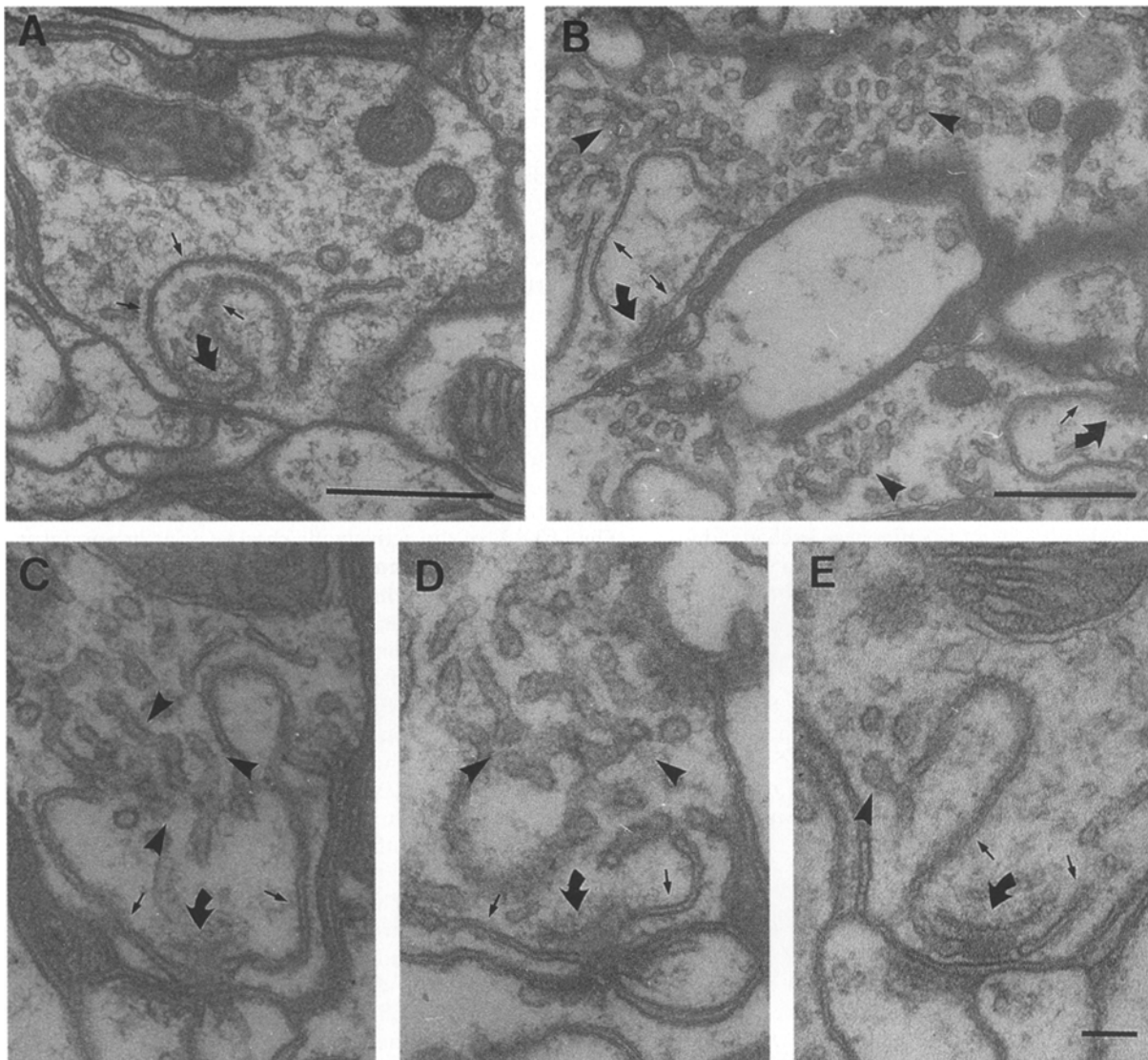
vesicles or cisternae, but rather is accomplished by direct pinch-off at the plasma membrane. Thus, the cisterna-like structures that emanate from under the dense body at 26°C do not normally occur, but rather are formed because membrane pinch-off is blocked by the *shi* gene product at this temperature, causing a build-up of the reforming vesicle membrane.

A second wave of recycling, which results in the reformation of the rest of the vesicle population located throughout the terminal, was observed to begin after ~1 min. This recycling pathway was similar to that described previously for the NMJ's of the coxal muscle, involving uncoated, collared pits that elongate and branch and eventually pinch off to form cisternae from which vesicles reform (Koenig and Ikeda, 1989). This process is described in detail in the aforementioned paper and is therefore presented here in more general terms in Figs. 6 and 7. As can be seen, collared pits first appear on the terminal membrane at locations away from the active zone (1–5 min) (Fig. 6 A). These pits gradually enlarge and branch, forming a complex of tubule-like structures. At the branch points, an electron-dense collar, similar to the one at the plasma membrane was observed (Fig. 6, B and C). This nonactive zone recycling membrane appeared at discrete sites along the terminal, rather than randomly along the terminal (5–10 min) (Fig. 6 D). These complexes seen in Fig. 6 D eventually pinch off from the plasma membrane and also pinch off at the branch points, forming numerous tubule-like cisternae (Fig. 7 A). Vesicles are observed associated with these cisternae and appear to bud from them (Fig. 7 B). Gradually, the cisternae are replaced with clusters of vesicles along the terminal (10–20 min). At this stage, small groups of vesicles appear to be connected to each other by an electron-dense material (Fig. 7 C). Note the docked vesicles under the two dense bodies in this figure, which represent a separate active zone population that was formed much earlier. Gradually, these vesicles disperse throughout the cytoplasm (20–25 min) (Fig. 7 D).

The time course of events leading to the replenishment of the nonactive zone population was somewhat variable and may reflect the general condition of the animal after experiencing total depletion. For example, in two specimens, vesicle clusters were apparent after 5 min, and in a third specimen, full recovery of the vesicle population had occurred within 15 min. Thus, the recycling process may proceed faster under normal conditions (without prior depletion).

The observations mentioned above concerning the normal vesicle reformation process at 19°C after depletion at 29°C give further evidence for the possibility that two distinct vesicle reformation processes exist in the same terminal. These two pathways apparently replenish two different populations of vesicles, a small population located

cell terminal after 30-s exposure to 29°C, followed by 2-min exposure to 26°C. Note collared pits emanating from the plasma membrane (arrowheads) (C) *shi* retinula cell terminal after 30 s exposure to 29°C, followed by 5 min at 26°C. Note many branching tubule-like structures throughout cytoplasm (arrowheads), and the long, unbranching cisternae emanating from either side of the dense body (arrows). *m*, mitochondria; *cp*, capitate projection. Bar, 1 μm (47,500×).



**Figure 4.** Examples of the two recycling pathways after 30-sec exposure to 29°C, followed by 5 min at 26°C. Note the long, unbranching cisterna (*small arrows*) emanating from under dense body at active zones (*curved arrows*). In some cases, the connection with the plasma membrane under the dense body is out of the plane of sectioning, as in (*E*). Also, the cisternae sometimes passes in and out of the plane of sectioning, giving a discontinuous appearance, as in *E*, right side. Note the many branching tubule-like structures (*arrowheads*) emanating away from active zones. Bars: (*A*) 0.5  $\mu\text{m}$  (44,000 $\times$ ); (*B*) 0.5  $\mu\text{m}$  (37,500 $\times$ ); (*C-E*) 100 nm (76,000 $\times$ ).

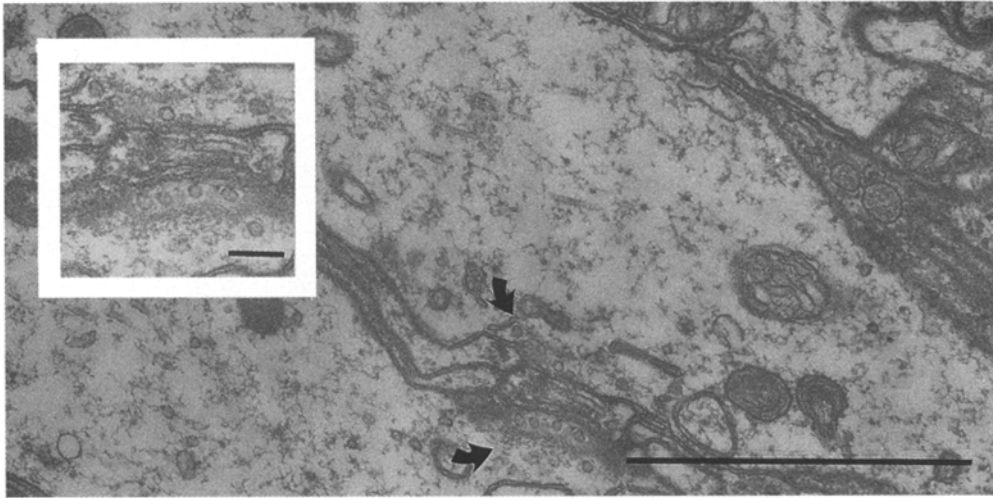
at the active zone and a larger population located throughout the cytoplasm away from the active zone. The population at the active zone is apparently reformed rapidly (within 1 min) without any intermediate structures, while the population dispersed throughout the cytoplasm is reformed more gradually (complete within about 30 min) by a pathway that includes collared pits and branching, tubule-like cisternae. For the remainder of this paper, these two pathways will be referred to as the active and nonactive zone pathways.

#### **Sensitivities of Pathways to $\text{Mg}^{2+}/\text{Ca}^{2+}$**

To further demonstrate the uniqueness of the two pathways described here, it would be advantageous to pharmacologically block one pathway while leaving the other un-

affected. This was attempted by exposing the terminals to high  $\text{Mg}^{2+}/\text{low Ca}^{2+}$  saline. The rationale for this approach stems from our previous observations on the coxal NMJ's that synapses exposed to saline with elevated  $\text{Ca}^{2+}$  demonstrate a marked increase in the number of docked vesicle/active site (Koenig et al., 1993), while synapses exposed to high  $\text{Mg}^{2+}/\text{low Ca}^{2+}$  saline demonstrate abnormal endo/exocytotic pits on the plasma membrane under the dense body cap where release occurs (Koenig, J.H., and K. Ikeda, unpublished results.) Since no abnormalities were observed at sites away from the active zone in the presence of these salines, these observations suggest that  $\text{Ca}^{2+}/\text{Mg}^{2+}$  may affect active zone recycling without affecting nonactive zone recycling.

*Shi* flies dissected to expose the optic lobes and perfused with normal saline were exposed to 29°C for 30 s to de-



**Figure 5.** Recovery of active zone vesicle population after depletion. Longitudinal section of a typical retinula cell terminal after 30-s exposure to 29°C, followed by 1 min at 19°C. Note two active zones (arrows) with a few vesicles in an otherwise depleted terminal. (Inset) Higher magnification of the two active zones. Bars: 1  $\mu\text{m}$  (45,000 $\times$ ); (Inset) 100 nm (70,000 $\times$ ).

plete the terminals, and then the saline was exchanged for 26°C saline containing 35 mM  $\text{Mg}^{2+}$ /1 mM  $\text{Ca}^{2+}$  (experimental flies) or normal saline (control flies). After 5 min at 26°C in the high  $\text{Mg}^{2+}$ /low  $\text{Ca}^{2+}$  saline or the normal saline, the flies were fixed and processed for EM.

In three *shi* control flies perfused with normal saline at 26°C for 5 min, the recycling membrane emanating from under the dense body was quite apparent in 118 of the 123 active sites observed. Also, the nonactive zone recycling membrane was very prevalent in all of the terminals observed (Fig. 8 A). However, in the terminals exposed to 26°C for 5 min in high  $\text{Mg}^{2+}$ /low  $\text{Ca}^{2+}$  saline, the recycling membrane emanating from under the dense body did not develop, although the recycling membrane emanating away from the active zone developed normally in all of the terminals (Fig. 8 B). At a few terminals, a small amount of membrane was observed under the dense body, as though the process of membrane internalization had been aborted (Fig. 8 C). Of 427 active zones from nine different flies, only 33 had a small amount of membrane emanating from under the dense body. The percentage of active zones with recycling membrane dropped from 95.9% in normal saline to 7.7% in high  $\text{Mg}^{2+}$ /low  $\text{Ca}^{2+}$  saline, while 100% of the terminals exhibited nonactive zone recycling in both salines. Thus, the amount of active zone recycling membrane is greatly reduced by exposure to high  $\text{Mg}^{2+}$ /low  $\text{Ca}^{2+}$  saline, while the nonactive zone recycling membrane is not. These observations suggest that high  $\text{Mg}^{2+}$ /low  $\text{Ca}^{2+}$  blocks active zone recycling, but not nonactive zone recycling.

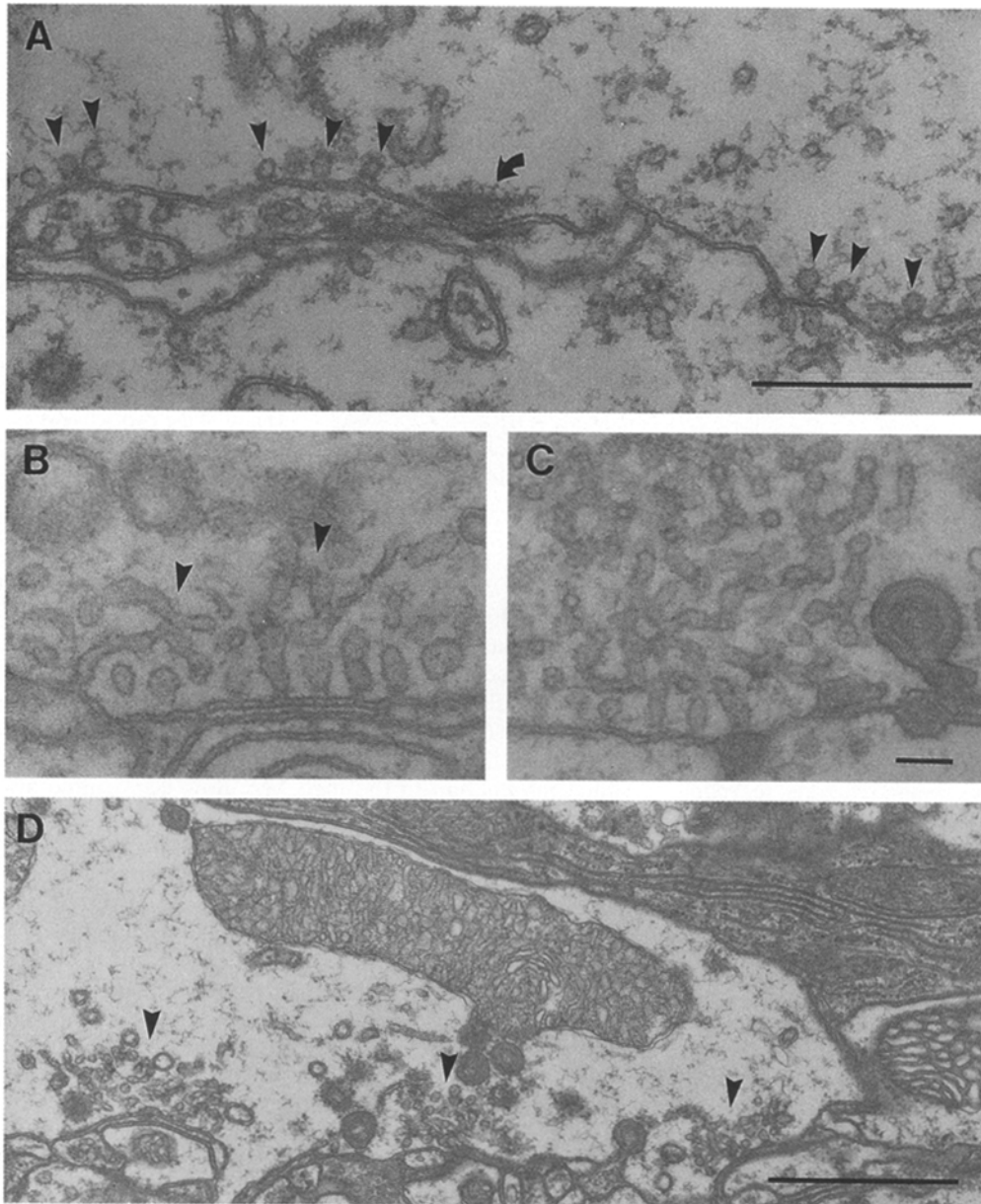
The effect of high  $\text{Mg}^{2+}$ /low  $\text{Ca}^{2+}$  saline on normal recycling at 19°C was also investigated. *Shi* flies were exposed to 29°C for 30 s while being perfused with normal saline to deplete the synapses. Then, the saline was replaced with high  $\text{Mg}^{2+}$ /low  $\text{Ca}^{2+}$  saline at 19°C. After 10 min at 19°C, the vesicle population seen at the dense body in normal saline was absent, while the recycling membrane away from the active zone was prominent. These terminals were similar to that shown in Fig. 8 B. Of 378 active zones from six different flies, only 14 had any vesicles under the dense body. In three control *shi* flies treated identically but perfused with normal saline, 83 of the 130 active zones observed possessed vesicles. Thus, only 3.7% of the active zones possessed vesicles in high  $\text{Mg}^{2+}$ /low  $\text{Ca}^{2+}$  saline,

while 63.8% of the active zones had vesicles in normal saline. These observations provide further evidence that high  $\text{Mg}^{2+}$ /low  $\text{Ca}^{2+}$  blocks active zone vesicle recycling but not nonactive zone recycling.

It was observed that full recovery of the nonactive zone vesicle population was rare under the conditions of dissection and perfusion necessary for experiments using  $\text{Mg}^{2+}$ . Control *shi* flies treated identically but exposed to normal saline rather than to the high  $\text{Mg}^{2+}$ /low  $\text{Ca}^{2+}$  saline also exhibited only partial recovery. An example of a terminal from a dissected and perfused control *shi* fly after 30 min at 19°C in normal saline is shown in Fig. 9. In addition to some normal-appearing vesicles, unusual cisternae and elongated vesicle-like structures are prevalent. Thus, the trauma of the dissection apparently influenced the ability of the terminals to recover their full complement of vesicles. Full recovery such as that shown in Fig. 7 was only obtainable in flies undissected until the time of fixation.

## Discussion

The data presented here give the first direct evidence that two distinct pathways for the reformation of synaptic vesicles after exocytosis exist within a single terminal. The evidence that these two pathways are truly distinct is: (a) obvious and consistent differences in the structure of the accumulated recycling membranes of the two pathways at 26°C; the active zone recycling membrane is flat, cisternae-like, unbranching, and  $\sim 20$  nm in cross-sectional diameter, while the nonactive zone recycling membrane is tubule-like, branching, and  $\sim 60$  nm in cross-sectional diameter; (b) consistent differences in time of appearance of the recycling membrane at 26°C; the active zone membrane appears between 0–30 s, while the nonactive zone membrane begins to appear after 2 min; (c) differences in recycling pathways at 19°C; the active zone pathway involves no intermediate structures in the reformation of its vesicle population, while the nonactive zone pathway involves tubules and cisternae in the reformation of its vesicle population; (d) differences in the time of reappearance of the two vesicle populations at 19°C after depletion; the active zone vesicles appear within 30 s, while the nonactive zone vesicles begin to appear after about 20 min; (e) differences in



**Figure 6.** Nonactive zone recycling pathway (2–5 min recovery at 19°C). (A) Longitudinal section of retinula cell axons after 30-s exposure to 29°C, followed by 2-min exposure to 19°C. Note the collared pits (arrowheads) located along the terminal plasma membrane away from active zone (arrow). Note the synaptic vesicle docked under the dense body. (B) Retinula cell terminal after 30-s exposure to 29°C, followed by 3-min exposure to 19°C. Note the enlargement of the collared pits and, in some cases, the elongation and branching (arrowheads). (C) Retinula cell terminal after 30-s exposure to 29°C, followed by 5 min at 19°C. Note the many branching, tubule-like structures. (D) Retinula cell axon after 30-s exposure to 29°C, followed by 5 min at 19°C. Note the three complexes of tubule-like structures at three discrete sites along the axon (arrowheads). Bars: (A) 0.5  $\mu\text{m}$  (63,000 $\times$ ); (B and C) 100 nm (76,000 $\times$ ); (D) 1  $\mu\text{m}$  (27,500 $\times$ ).

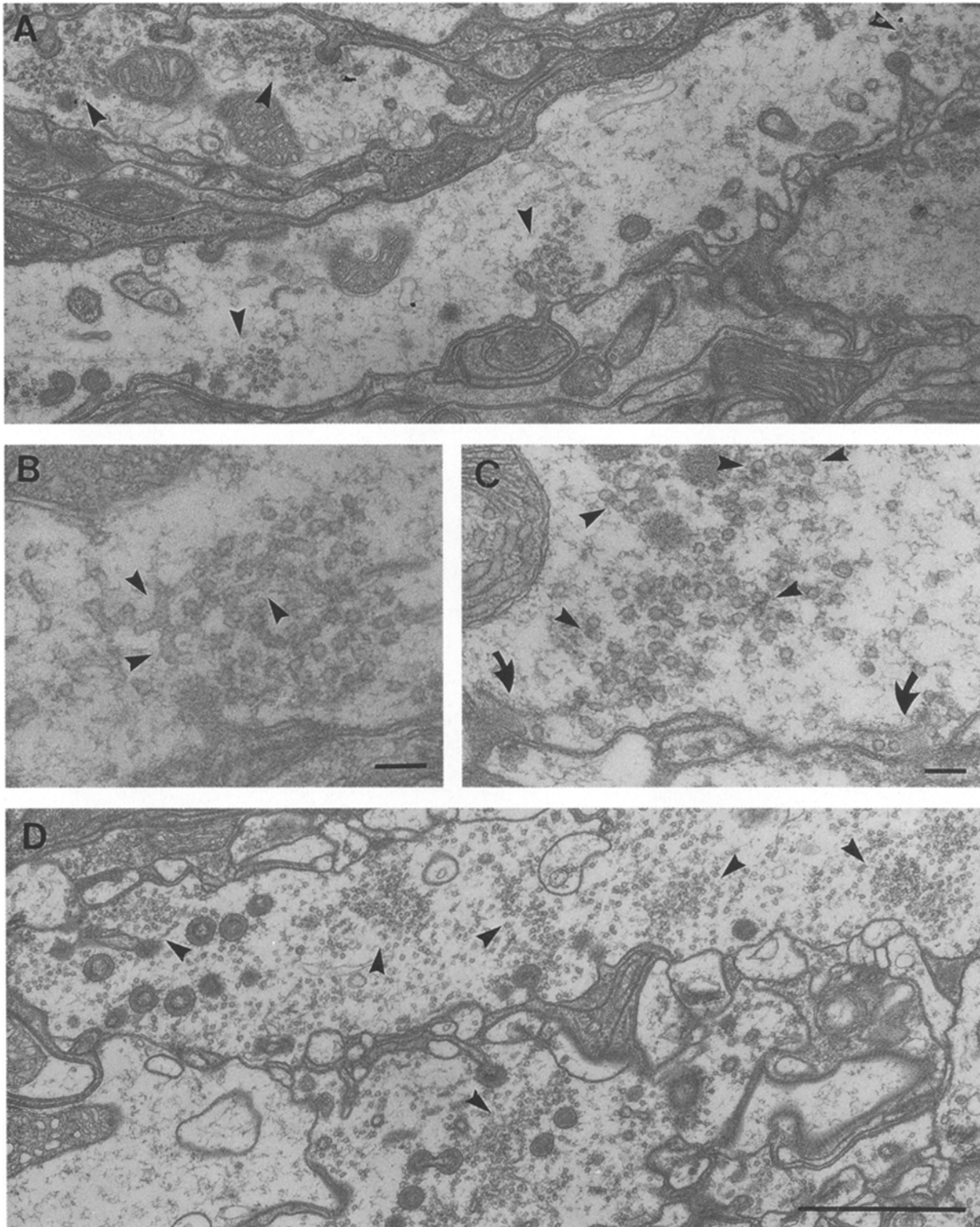
sensitivity to high  $\text{Mg}^{2+}$ /low  $\text{Ca}^{2+}$  saline; in this saline, active zone recycling is blocked, resulting in no accumulation of active zone recycling membrane at 26°C and no reformation of vesicles at 19°C after depletion. On the other hand, nonactive zone recycling is unaffected at both 26 and 19°C in this saline.

The two pathways described here are similar in various respects to the two proposed recycling pathways discussed in the Introduction. For example, the pathway postulated by Heuser and colleagues emanates away from the active site and involves intermediate structures including invaginations and cisternae, similar to the nonactive zone pathway described here. Although our data suggest that the cisternae are formed by pinching off of large uncoated invaginations, rather than coalescence of coated vesicles, the nonactive zone recycling pathway described here is, in the opinion of these authors, essentially equivalent to that postulated by Heuser and others. The pathway postulated by Ceccarelli and colleagues, which emanates from the active

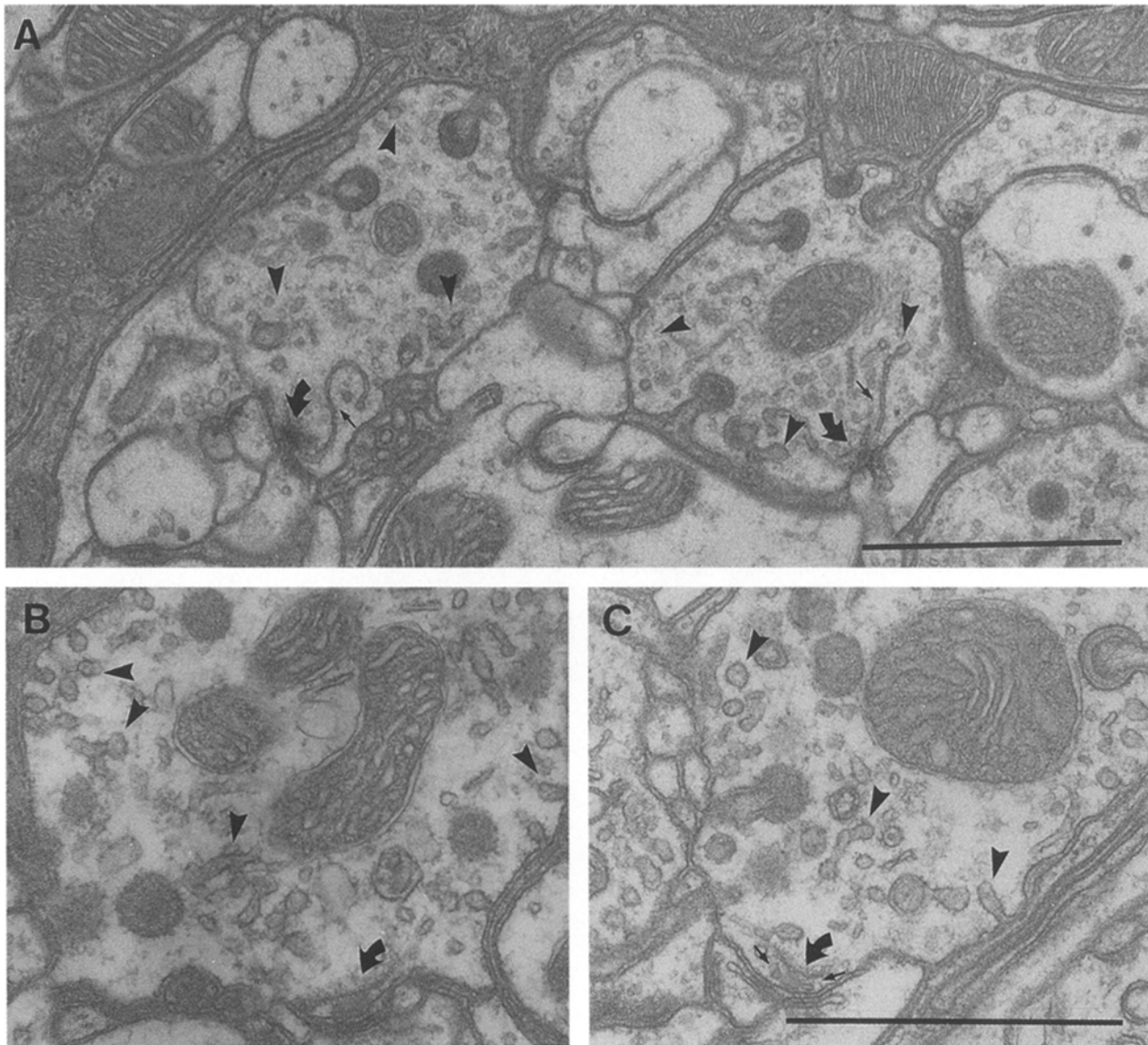
zone, does not involve intermediate structures such as coated vesicles or cisternae, and has a very rapid reformation process, fits well with our observations on the reformation process of the active zone vesicle population. Thus, the observations presented here, combined with the previously published observations suggesting two different recycling pathways (mentioned in the Introduction), provide strong evidence that two distinct pathways are used for vesicle reformation after exocytosis.

If two separate recycling pathways, with different sites of origin, morphologies, time courses, and sensitivities to  $\text{Ca}^{2+}/\text{Mg}^{2+}$ , exist in the same terminal, the question immediately arises: For what purpose does the cell have these two pathways? One possibility is that the two pathways exist in order to produce two distinct vesicle populations. The existence of two distinct synaptic vesicle populations, one more readily releasable than the other, has been proposed by a number of investigators, based on a variety of biochemical, electrophysiological, electron microscopical,





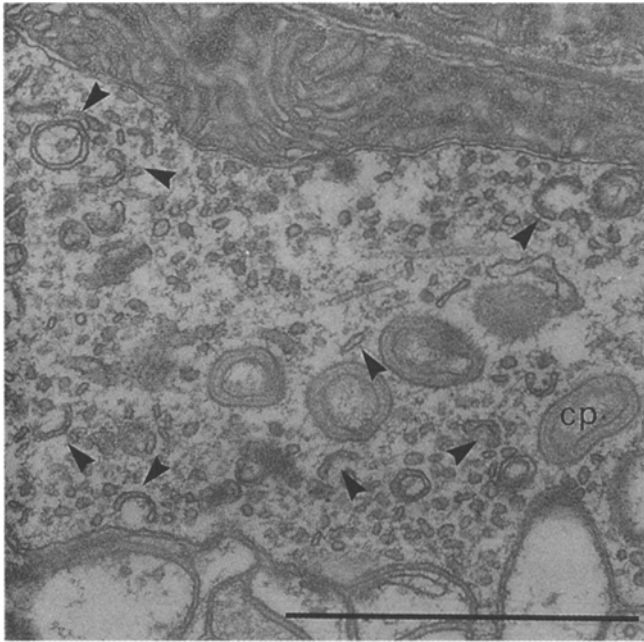
**Figure 7.** Nonactive zone recycling pathway (15–25-min recovery at 19°C). (A) Retinula cell axon after 30-s exposure to 29°C, followed by 15 min at 19°C. Note the clumps of vesicles/tubules at discrete sites along axon (*arrowheads*). (B) Higher magnification of clump of vesicles showing relationship to tubule-like structures. (C) Retinula cell terminal after 30-s exposure to 29°C, followed by 25 min at 19°C. Note the electron dense substance attached to some of the vesicles (*arrowheads*). Also note the two active zones (*arrows*) with vesicles docked under the filamentous cap of the dense body. (D) Retinula cell axon after 30-s exposure to 29°C, followed by 25 min at 19°C. Note clumps of vesicles (*arrowheads*) as well as vesicles dispersed throughout the cytoplasm. Bars: (A and D) 1  $\mu\text{m}$  (27,500 $\times$ ); (B) 100 nm (76,000 $\times$ ); (C) 100 nm (62,500 $\times$ ).



**Figure 8.** Blockage of active zone recycling at 26°C by high  $Mg^{2+}$ /low  $Ca^{2+}$  saline. (A) Two control *shi* retinula cells in a dissected preparation after 30-s exposure to 29°C followed by 5-min exposure to 26°C in normal saline. Note presence of two types of vesicle recycling. At the active zones (*large arrows*), long flat cisterna (*small arrows*) are visible emanating from under the dense bodies. Elsewhere in the cytoplasm, nonactive zone recycling is also apparent, some of which is pointed out by arrowheads. (B) Retinula cell terminal after 30-s exposure to 29°C, followed by 5-min exposure to 26°C in 35 mM  $Mg^{2+}$ /1 mM  $Ca^{2+}$  saline. Note the collared pits and tubule-like structures (*arrowheads*) away from active zone (*arrow*), which represents nonactive zone recycling. Note that no membrane is emanating from under the dense body, as occurs in normal saline. (C) Retinula cell terminal treated as described in 8 B. Note small amount of membrane (*small arrows*) emanating from under either side of the dense body at the active zone (*large arrow*). Bars: (A) 1  $\mu m$  (36,400 $\times$ ); (B and C) 1  $\mu m$  (47,000 $\times$ ).

and immunocytochemical observations. As early as 1961, Birks and MacIntosh postulated the existence of a readily releasable subpopulation of vesicles located near the pre-synaptic membrane based on observations of two subfractions of releasable acetylcholine (ACh), one smaller and more easily liberated than the other. Two populations of vesicles, recycling and reserve, have also been reported in the electric organ of *Torpedo* (Zimmerman and Denston, 1977; Zimmerman and Whittaker, 1977; Giompres et al., 1981) and the myenteric plexus of guinea pig ileum (Ágoston et al., 1985). The recycling population is made up of smaller-diameter, denser vesicles that preferentially take

up newly synthesized transmitter. Studies using vesamicol to block ACh reuptake into recycling vesicles have demonstrated two different ACh compartments, a nerve impulse-releasable and a tityustoxin (TsTx)-releasable compartment, in cat superior cervical ganglion (Prado et al., 1992). Studies using capillary electrophoresis suggest two vesicular compartments, an easily released compartment and a reserve compartment (Kristensen et al., 1994). Recently, two populations of synaptic vesicles were identified immunocytochemically, a population associated with synapsin located distally to the release site and a population devoid of the protein located more proximally to the re-



**Figure 9.** Incomplete recovery from depletion in dissected and perfused fly. Longitudinal section of retinula cell terminal after 30-s exposure to 29°C, followed by 30 min at 19°C. The fly was dissected and perfused with normal saline for 30 min. Note unusual cisternae and elongated vesicle-like structures (arrowheads). *cp*, capitate projection. Bar, 1  $\mu$ m (47,000 $\times$ ).

lease site. The distal population is brought into play during high-frequency release, but not low-frequency release (Pieribone et al., 1995).

The observations demonstrating two distinct recycling pathways, plus those demonstrating two vesicle populations with different release characteristics, suggest that there may be a small subpopulation of rapidly recycling, readily releasable vesicles located at the active zone, which is largely or solely responsible for transmitter release under conditions of low frequency release. The second, larger population appears to be mobilized as a result of more intense electrical stimulation or by such treatments as  $K^+$ ,  $La^{3+}$ ,  $\alpha$ -LTX, or TsTx, all of which cause massive asynchronous release resulting in depletion (i.e., loss of the larger vesicle population located away from the active zone). If indeed two distinct populations exist, the relationship between them comes into question. Does the nonactive zone population exist simply to replenish the readily releasable population under conditions of intense release, or might it have a completely separate function? It is interesting to speculate whether spontaneous release of miniature post-synaptic potentials (mpps) might represent a normal function of the nonactive zone population, since the treatments that access this population (and cause depletion) all greatly increase spontaneous (asynchronous) release.

Of possible relevance to the possibility of two distinct populations of vesicles is the recent identification of a population of synaptic-like microvesicles (SLMVs) in peptide-secreting endocrine cells (for review see Thomas-Reetz and De Camilli, 1994). In addition to being morphologi-

cally indistinguishable from synaptic vesicles, the membranes of these vesicles have been shown to possess all or some (depending on the cell type) of the presently identified synaptic proteins, including synaptophysin, synaptobrevin, synaptotagmin, SV2, protein 29, and rab 3A. Furthermore, these vesicles store neurotransmitter substances. For example, SLMVs of pancreatic B cells store  $\gamma$ -aminobutyric acid (GABA) (Reetz et al., 1991), while SLMVs of PC12 (a chromaffin cell-derived cell line) cells store ACh (Bauerfeind et al., 1993). Finally, from studies using synaptophysin antibodies, it appears that SLMVs have a similar recycling pathway to that of the nonactive zone pathway described here, in that they recycle through an endosomal intermediate (Clift-O'Grady et al., 1990; Cameron et al., 1991; Linstedt and Kelly, 1991; Regnier-Vigouroux et al., 1991). It is possible, therefore, that the population of vesicles reformed through the nonactive zone pathway, although morphologically indistinguishable from the active zone population, might actually represent a population equivalent to the SLMV population in endocrine cells.

Recent observations using fluorescent dyes to image synaptic vesicle populations during release and recycling do not suggest the existence of two populations, since the fluorescent spots, assumed to be vesicle clusters, brighten uniformly as if recycling vesicles were mixing randomly throughout the vesicle pool (Betz and Bewick, 1992). However, the present data would suggest that these results should be interpreted as representing the recycling activity of the larger, nonactive zone population exclusively since the active zone population would not be distinguishable at the light microscopic level. Attempts to correlate transmitter release with dye loss demonstrated that after about a minute, the rate of dye loss decreased relative to the rate of transmitter release except at high rates of stimulation (Betz and Bewick, 1993). This deviation could be due, at least in part, to a second, active zone population. Thus, if indeed two populations of vesicles with different release characteristics exist in the terminal, attempts to correlate electrophysiological observations on transmitter release with observations that detect only the larger, nonactive zone population (such as biochemical or light microscopic level observations) could be misleading.

In conclusion, the data presented here demonstrate two separate pathways for synaptic vesicle reformation. For many years, seemingly contradictory observations on vesicle membrane removal after exocytosis have existed, resulting in two different proposals on how this is accomplished. Also, a completely separate group of observations has accumulated over the years suggesting two separate transmitter compartments with different release characteristics. It is suggested here that these two sets of observations may be related; that is, two separate populations of vesicles with different release characteristics and different recycling pathways may exist in the same terminal. It is hoped that the data presented here will help to stimulate research designed to determine if indeed two distinct vesicle populations exist in the terminal and what their functions might be.

We thank Ms. Grace Hong and Ms. Cecilia Macias for their excellent technical help and Ms. Sharyn Webb for her excellent secretarial help.

This work was supported by U.S. Public Health Science National Insti-

tute of Health grant NS 18856 and National Science Foundation grant BNS-8415920.

Received for publication 13 June 1996 and in revised form 21 August 1996.

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