MEMBRANE RECYCLING IN THE CONE CELL ENDINGS OF THE TURTLE RETINA

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ABSTRACT

The ultrastructural effects of dark, light, and low temperature were investigated in the cone cell endings of the red-eared turtle (*Pseudemys scripta elegans*). Thin sections revealed that in dark-adapted retinas maintained at 22°C, the neural processes which contact the cone cells at the invaginating synapses penetrated deeply into the photoreceptor endings. When dark-adapted retinas were illuminated for 1 h at 22°C, the invaginating processes were apparently extruded from the synaptic endings. On the other hand, 1-h exposure to a temperature of 4°C in the dark caused the invaginating processes to become much more strikingly inserted than at room temperature. A morphometric analysis showed that the ratio between the synaptic surface density of the endings and their total surface density decreased in the light and increased in the dark and cold. Freeze-fracturing documented fusion of synaptic vesicles with the presynaptic membrane in all conditions tested. These observations suggest that the changes in configuration of the pedicles in the light, dark, and cold reflect a different balance between addition and retrieval of synaptic vesicle membrane from the plasmalemma; in the dark, the rate of vesicle fusion is increased, whereas in the cold, membrane retrieval is blocked.

When the eyecups were warmed up and illuminated for 30-45 min after cold exposure, a striking number of vacuoles and cisterns appeared in the cytoplasm and coated vesicles were commonly seen budding from the plasmalemma. 60-90 min after returning to room temperature, the endings had reverted to their normal configuration, and the vast majority of vacuoles, cisterns, and coated vesicles had disappeared. When horseradish peroxidase was included in the incubation medium, very few synaptic vesicles were labeled at the end of the period of cold exposure. 30-45 min after returning to 22°C, vacuoles and cisterns contained peroxidase, whereas most synaptic vesicles were devoid of reaction product. 2 h after returning to 22°C, coated vesicles, vacuoles, and cisterns had disappeared and a number of synaptic vesicles were labeled. These experiments suggest that vacuoles, cisterns, and coated vesicles mediate the retrieval of the synaptic vesicle membrane that has been added to the plasmalemma during cold exposure.

The vesicle hypothesis of transmitter release states that quanta of chemical transmitter are stored in synaptic vesicles and that they are released into the synaptic cleft upon vesicle interaction with the presynaptic membrane (7, 18–20, 48, 57). Convincing evidence exists that transmitter release is a secretory event, mediated by fusion of the synaptic vesicles with the cell membrane. This process is in fact documented by numerous observations of continuity of the vesicular membrane with the plasmalemma in thin-sectioned specimens (12, 15, 16, 29, 68); by images of exocytosis at the active zone in replicas of freeze-fractured specimens (30, 50); and by the results of experimental studies which demonstrate that electrical or chemical stimulation causes a decrease in number of synaptic vesicles and a parallel increase in the surface of the endings (6, 12, 13, 15, 28, 29, 35, 39, 45, 49, 52, 71). Thus, once transmitter has been released, a mechanism must exist for replenishment of the supply of vesicles. In addition to synthesis of new vesicles in the cell body and their subsequent transport along the axon (48), it has been suggested that the complement of vesicles in the synaptic endings is reutilized locally many times (12, 13, 29, 55, 58, 59, 63). In the frog neuromuscular junction, two main hypotheses have been proposed for vesicle turnover during synaptic activity: (a) vesicles transiently fuse with the presynaptic membrane, discharge their contents, and then bounce back into the cytoplasm where they are refilled with transmitter (12); (b) the vesicular membrane is incorporated into the plasmalemma. upon exocytosis and is subsequently retrieved by coated vesicles. These fuse to form cisterns which, in turn, give rise to synaptic vesicles (29).

The present paper describes the membrane events which accompany synaptic activity in photoreceptor cells. The retina of the red-eared turtle (*Pseudemys scripta elegans*) was chosen for several reasons: first, the majority of the photoreceptor cells in this reptilian species are cones, and their endings are easily distinguished from rod pedicles. Second, the responses to illumination of photoreceptor, horizontal, and bipolar cells in the turtle are well-documented by electrophysiological studies (2–5, 21–23, 54, 61, 62). Finally, in a cold-blooded vertebrate, the isolated retina remains viable for many hours at 22°C, and this permits experimental manipulation of the synaptic endings in vitro.

In a complex organ such as the retina, rapid structural changes in the photoreceptor endings are difficult to investigate because tracers or fixative fluids must diffuse long distances before reaching the outer plexiform layer. Thus, movement of synaptic vesicle membrane has to be blocked or slowed down. For this purpose we used low temperature, which on many occasions helped to dissect out specific biological mechanisms. Three morphological methods were applied: (a) electron microscopy of thin-sectioned specimens; (b) freeze-fracturing; and (c) ultrastructural tracer technique. Thin sections were used to investigate whether the fine structure of the pedicles changes upon exposure to dark, light, and cold. Freeze-fracturing was used to document exocytosis of synaptic vesicles. Finally, membrane recycling was followed by permeation of the extracellular space with horseradish peroxidase. A preliminary account of the results of this study has been published elsewhere (60).

MATERIALS AND METHODS

Thin-Sectioned Specimens

Red-eared turtles were dark-adapted overnight; they were decapitated and their eye globes were enucleated under very dim light. Each eye globe was opened with an equatorial incision and the vitreous body was removed from the posterior segment with a swab applicator. The eyecup was equilibrated in the dark for 5 min in a physiological solution (14) resembling turtle cerebrospinal fluid in its composition (105.0 mM NaCl; 2.6 mM KCl; 20.0 mM NaHCO$_3$; 1.0 mM CaCl$_2$; 1.0 mM MgCl$_2$; 3.0 mM glucose). The solution was saturated with a 95% O$_2$-5% CO$_2$ mixture; the final pH was 7.7. The eyecup was then mounted with agar in a conical depression in the bottom of a Lucite chamber. Throughout the length of the experiment, the incubating medium was perfused over the retina at the rate of 5 ml/min; its average temperature was 22°C.

The effects of light were tested by maintaining the retina for 1 h at 22°C under continuous illumination with white light from an 80-W tungsten quartz-iodine lamp delivered by an 11-80 Fiber Optic Illuminator (American Optical Corp., Southbridge, Mass.). Irradiance was measured by a calibrated silicon photodiode (model 40 A Opto-meter, United Detector Technology Inc., Santa Monica, Calif.) placed at the same distance from the source as the retina. The irradiance transmitted was 1.5 × 10^5 μW cm^{-2}; assuming that all of the light had a wavelength of 500 nm, the flux of the source was calculated to be approximately 7.5 × 10^4 erg cm^{-2} sec^{-1}, which saturates the response of turtle cone cells when applied as a 10-ms flash (2). However, some energy was lost as light passed through the layer of fluid covering the retina.

The effects of low temperature were tested by keeping the preparation in the cold room at 4°–6°C. The retina was warmed up by transferring it to physiological solution at 22°C.

Eyecups in which the retina appeared detached from the pigment epithelium were discarded because a variable portion of the photoreceptor cells had lost their outer segments.

At the end of the experiment, the physiological solution was replaced with fixative fluid at the same temperature. 2% glutaraldehyde in 0.1 M Sörenson phosphate buffer at pH 7.4 (41) was routinely used as a primary fixative. 30 min later, the eyecups were transferred to specimen vials and fixation was continued for 12–24 h at 4°C. The sclera was then removed, and the retina was trimmed into small pieces and postfixed for 1 h at 4°C in a solution of 1% osmium tetroxide in Sörenson phosphate buffer at pH 7.4. The specimens were subsequently rinsed, stained en bloc with uranyl acetate (36), and finally embedded in Epon-Araldite. In one experiment, the eyecups were fixed in 2% osmium tetroxide in Millonig phosphate buffer pH 7.0 (44) at 22°C.

Freeze-Fracturing

The retinas were fixed for 20–30 min with 2% glutaraldehyde in 0.1 M Sörenson phosphate buffer at pH 7.4, perpendicularly sectioned at 150 μm with a Smith and Farquhar tissue chopper (DuPont Instruments-Sorvall, Newtown, Conn.), and equilibrated for 2 h with 20% glycerol in phosphate buffer. The sections were subsequently mounted on gold specimen carriers and rapidly frozen in the liquid phase of partially solidified Freon 22 (monochlorodifluoromethane) cooled with liquid nitrogen. Finally, specimens were fractured and replicated with carbon-platinum without etching in a Balzers 360 M apparatus (Balzers High Vacuum Corp., Santa Anna, Calif.) fitted with an electron beam gun for platinum shadowing and a quartz crystal monitor for standardizing replica thickness.

Tracer Experiments

The eyecup was placed in the center of a small organ culture dish filled with 0.7% horseradish peroxidase (Worthington Chemical Co., Freehold, N. J.) in physiological solution. The incubation medium was intermittently bubbled with 95% O_2–5% CO_2 and replaced at 1-h intervals.

At the termination of the experiment, the peroxidase was removed and a solution of 2% glutaraldehyde in Sörenson phosphate buffer, pH 7.4, was added directly to the culture dish. 15 min later, the eyecups were transferred to specimen vials and maintained in the fixative solution for an additional 4 h. The sclera was subsequently removed and the retina was perpendicularly sectioned at 150 μm with the tissue chopper. The

sections were rinsed in both phosphate buffer and 0.5 M Tris-HCl buffer at pH 7.6 and preincubated in the dark at 4°C for 30 min in a 0.05% solution of diaminobenzidine-tetrahydrochloride (DAB) in Tris-HCl buffer at pH 7.6. They were subsequently incubated in the dark for 30 min in a 0.05% solution of DAB in Tris-HCl, containing 0.01% hydrogen peroxide (24). The sections were rinsed in both Tris-HCl buffer and phosphate buffer and postfixed for 1 h at 4°C in 1% osmium tetroxide, 1.5% potassium ferrocyanide in distilled water (37). The sections were finally dehydrated and stacked in small bundles in an Epon-Araldite mixture.

Quantitative Procedures

Experimental changes in the surface density of the endings were estimated by comparing ratios between the portion of the pedicle surface which is occupied by synaptic specializations (synaptic surface) and total surface density of the pedicle. Synaptic surface was defined as the region of the vitreal surface of the endings which is occupied by invaginating and basal synapses and by intervening segments of unspecialized plasmalemma. The total surface of the endings encompassed both their vitreal and lateral aspects; sclerally, the fiber was excluded from the measurements in those rare instances in which the endings were continuous with the cell body. Tangentially cut endings, in which no synaptic membrane was included in the plane of section, were discarded. The density of the synaptic surface and the total surface density of the endings were determined by counting the intersections (I) of a linear probe with the cell membrane and dividing twice the number of intersections by the total length of the test line (L_T) according to Tomkeieff (66):

$$S_v = \frac{2I}{L_T}.$$

The ratio of the synaptic surface density (S_{v_s}) to the total surface density of the endings (S_{v_e}) was obtained by dividing the intersections of the synaptic surface (I_s) by the intersections of the total surface of each ending (I_e) (9). In fact,

$$S_{v_s} = \frac{2I_s}{L_T} \quad S_{v_e} = \frac{2I_e}{L_T} \quad \text{and} \quad \frac{S_{v_s}}{S_{v_e}} = \frac{\frac{2I_s}{L_T}}{\frac{2I_e}{L_T}} = \frac{I_s}{I_e}.$$

Surface area ratios were compared in thin sections of plastic embedded eyecups which had been exposed to the following experimental conditions: a) incubation for 1 h at 22°C in the dark, b) incubation for 1 h at 22°C under continuous illumination at 1.5×10^5 μW cm^{-2}, and c) incubation for 1 h at 4°C in the dark.

Five tissue blocks from each experiment were sectioned perpendicular to the retinal layers; electron micrographs were taken of all cone cell endings present in

each set of sections at a constant magnification of 4,000. Negatives were printed at a photographic enlargement of 2.3, and during exposure a test system containing 111 lines intersecting at 5-mm intervals was superimposed on each print. The significance of the effects of the experimental procedures on the surface ratios was evaluated by t test.

RESULTS

In the retina of *Pseudemys*, cone cells predominate and are characterized by a pyramidal synaptic ending or pedicle connected by a narrow fiber to the cell body (Fig. 1). The synaptic endings of the few rod cells are easily recognized because they are continuous with the cell body without an intervening fiber. Cone pedicles make both invaginating and basal synapses with the processes of horizontal, bipolar, and other photoreceptor cells (41). Each invaginating synapse encloses the extremity of either two (dyad) or three (triad) processes; in both dyads and triads, two deeply inserted processes lie on either side of a wedge-shaped projection of the pedicle, called the synaptic ridge, which is bisected by a synaptic ribbon. In triads, a third process lies centrally and less deeply inserted (Fig. 2).

Effects of Dark, Light, and Cold

When the eyecup of dark-adapted turtles was maintained in vitro for 1 h at 22°C in the dark, the vitreal surface of the synaptic endings of cone cells was gently concave; dyads and triads[1] were deeply invaginated into the endings and were partially or totally surrounded by pedicle cytoplasm (Fig. 2). Mesodyads and mesotriads were short and ran a relatively straight course; most of the vitreal surface of the pedicles was occupied by basal junctions.

The cytoplasm of the dark-adapted pedicles contained synaptic ribbons, numerous 40- to 50-nm vesicles, randomly dispersed vacuoles up to 500 nm in diameter, and small clusters of coated

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[1] Generally, the processes of dyads and triads were obliquely or cross-cut and thus appeared as profiles isolated from the parent neuron and enclosed by a deep invagination of the pedicle surface. In oblique sections, the invagination appeared tethered to the basal aspects of the ending by two closely apposed segments of the pedicle membrane which resembled the mesaxon of peripheral nerve fibers (Figs. 4, 5, and 16) and, thus, will be referred to as "mesodyad" or "mesotriad" for the remainder of this paper.

vesicles (Fig. 2); at the periphery of the endings, microtubules and profiles of agranular reticulum were also seen. A few coated invaginations of the plasmalemma were visible in these pedicles, budding from the valleys on either side of the synaptic ridges as well as from the regions of unspecialized membrane which intervene between the basal junctions. When the period of exposure to dark was increased up to 24 h, the morphology of the endings was unchanged.

When the dark-adapted retina was exposed to continuous illumination for 1 h with white light irradiating 1.5×10^5 μW cm^{-2}, the base of most pedicles appeared flat (Fig. 3). Dyads and triads barely indented the surface of the endings and were not longer surrounded by pedicle cytoplasm. Thus, mesodyads and mesotriads were either absent or very short. Upon illumination, the population of vacuoles was somewhat larger than in the dark. Also, coated vesicles seemed to be more numerous toward the vitreal surface of the pedicles and in the region surrounding the invaginating synapses. No further change was observed when the period of illumination was increased beyond 1 h.

When the retina was maintained for 1 h in the dark at 4°C, dyads and triads penetrated very deeply into the pedicles. Mesodyads and mesotriads increased strikingly in length and assumed a tortuous course in the interior of the endings (Fig. 4). The vitreal surface of the pedicles was elaborately infolded and basal junctions appeared widely separated from one another. In the cytoplasm, fewer coated vesicles were apparent than in dark-adapted retinas maintained at room temperature, whereas coated invaginations of the plasmalemma were present along the length of mesodyads and mesotriads.

The effects of low temperature were progressive. When the retina was maintained in the dark at 4°C for 12 h the invaginating processes were so deeply inserted into the endings that the whole pedicle cytoplasm was permeated with meandering mesodyads and mesotriads which had acquired a characteristic undulating appearance (Fig. 16). In many places, territories of pedicle cytoplasm were seen which were completely surrounded by a double membrane. These images most likely represented tangential sections through curving mesodyads and mesotriads, for the cleft intervening between their limiting membranes was filled with reaction product when horseradish peroxidase was added to the incubating medium (see
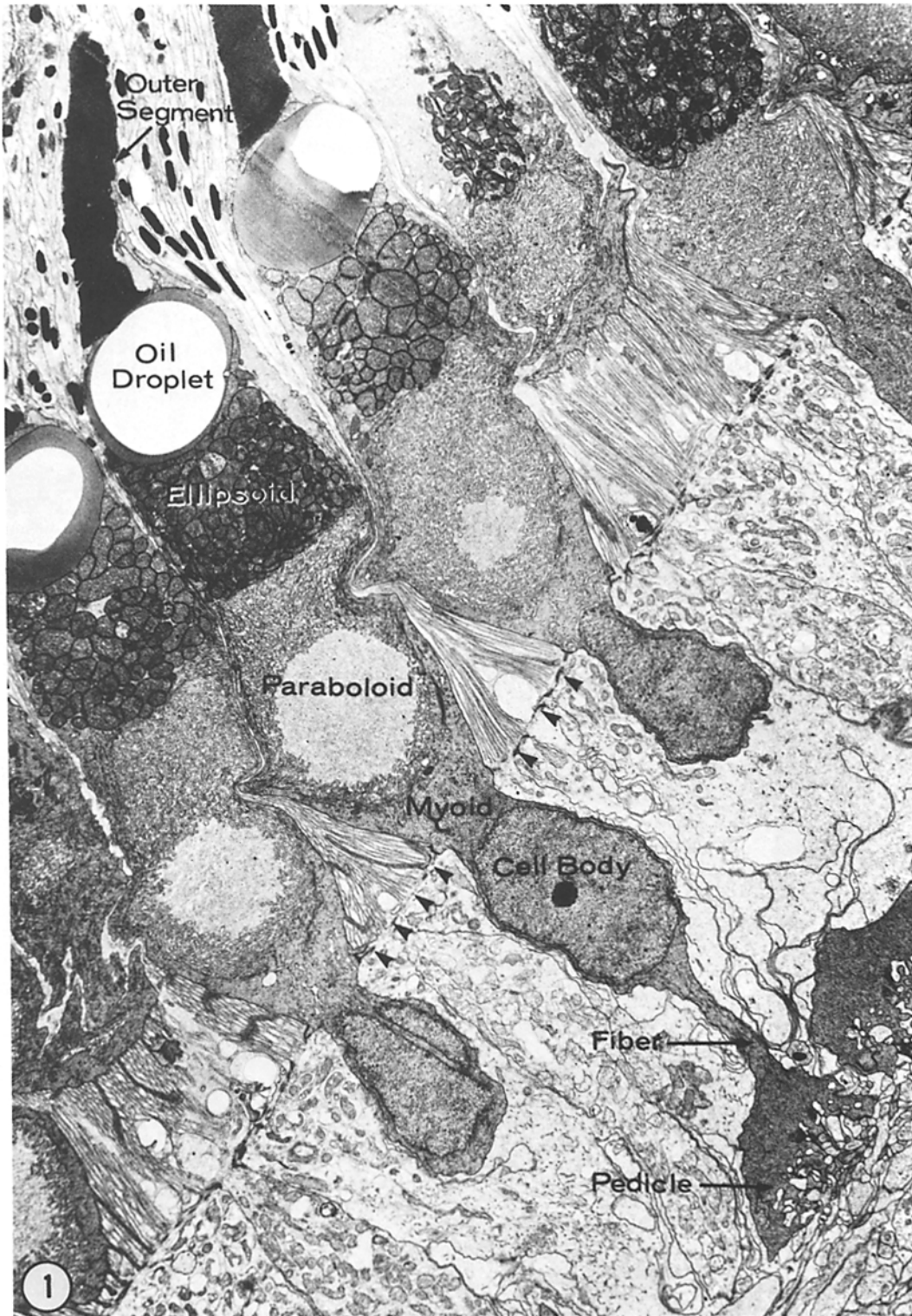
FIGURE 1   Cone cell of the turtle, *Pseudemys scripta elegans*. The outer segment is relatively short and gently tapering. The inner segment consists of a spherical fat globule or oil droplet; the ellipsoid, filled with mitochondria; the paraboloid, a glycogen body surrounded by agranular reticulum; and the myoid, characterized by profiles of granular and agranular reticulum, free ribosomes, and the Golgi apparatus. At the outer limiting membrane (arrowheads), the photoreceptor proper is continuous with the cell body, which contains the nucleus. A narrow fiber connects the nuclear region to a pyramidal synaptic ending or pedicle. × 2,900.

below). There was no doubt that the pedicle cytoplasm contained fewer synaptic vesicles than at 22°C (Fig. 16); furthermore, coated depressions of the plasmalemma were in places very numerous at the pedicle surface (Fig. 4, inset). Similar results (Fig. 5) were obtained when the intact animal was maintained at 4°C and in the dark for 24 h, thus proving that low temperatures are also effective in vivo.

There are two interpretations for the effects of light, dark, and low temperature on the cone cell endings: (a) dyads and triads actively move into the pedicles in the dark or cold and actively recede upon illumination; (b) the apparent movement of the invaginating processes reflects variations in the amount of presynaptic membrane, and, in the dark, the pedicles accommodate the excess plasmalemma by flowing along the length of the incoming processes.

To distinguish between these alternatives, the ratio of the synaptic surface density to the total surface density of the endings of 70 cone cells was compared in three experimental conditions: 1 h light at 22°C, 1 h dark at 22°C, and 1 hr dark at 4°C. The values of I_s/I_e (mean ± S.E.) were:

1 h light at 22°C:

$$\frac{I_s}{I_e} = 0.278 \pm 0.011;$$

1 h dark at 22°C:

$$\frac{I_s}{I_e} = 0.500 \pm 0.011;$$

1 h dark at 4°C:

$$\frac{I_s}{I_e} = 0.682 \pm 0.012.$$

t-statistic evaluation showed that the mean surface ratio in the dark at 22°C was significantly higher than in the light at 22°C ($P < 0.01$) and significantly lower than in the dark at 4°C ($P < 0.01$). Thus, the quantitative estimates demonstrate that the relative amount of synaptic surface decreased upon illumination and increased in the cold. Fig. 6 illustrates the percentage of the total surface of the endings which is occupied by synaptic surface in the light, dark, and cold.

Recovery from Light, Dark, and Cold

To establish whether light stimulation caused permanent alterations in cone cells, the response of the illuminated endings to dark and to low temperature was tested. When the retina was illuminated at 22°C for 1 h and subsequently maintained in the dark at 22°C for 1 h dyads and triads were deeply invaginated in all but a few pedicles. When the retina was illuminated at 22°C for 1 h and subsequently maintained in the dark at 4°C for 1 h, all dyads and triads assumed the strikingly invaginated configuration which is typically caused by cold and dark alone.

To test the reversibility of the cold effect, the retina was incubated at 4°C for 1 h in the dark and subsequently illuminated for 1 h at 22°C. As a result, the endings shifted to their light configuration. Clearly, exposure to light at 22°C caused internalization of all the membrane that had been added to the base of the endings in the dark and cold.

To identify the mechanism responsible for this retrieval of pedicle membrane, eyecups were incubated in the dark at 4°C for 1-4 h, rapidly warmed up by transferring them into physiological solution at 22°C, and illuminated for variable time intervals (10, 30, 45, 60, and 90 min). 30-45 min after illumination at 22°C, the cytoplasm of the endings contained a striking number of vacuoles and curved cisterns (Fig. 7). Vacuoles were spherical profiles 50-500 mm in diameter with transparent contents. In places, their surface was provided with coated and uncoated hemispherical or tubular evaginations (Fig. 7, inset). Curved cisterns, on the other hand, were either dilated and enclosed an empty lumen, or flattened. In many instances, small vacuoles and cisterns were included in the halo of synaptic vesicles that are bound to the synaptic ribbons. Coated invaginations of the plasmalemma were numerous at the base of the endings and on either side of the synaptic ridges, but cytoplasmic coated vesicles were increased only slightly. Tubular profiles of the agranular reticulum were still present at the periphery of the endings; they could be easily distinguished from vacuoles and cisterns because they branched and were associated with bundles of microtubules.

When osmium tetroxide was used as a primary fixative, the distinction between vacuoles, cisterns, and tubular profiles of the agranular reticulum was no longer possible, for all these structures appeared as irregular membranous compartments with a wide lumen (Fig. 8).

After 60-90 min illumination at 22°C, the endings had reacquired their usual complement of
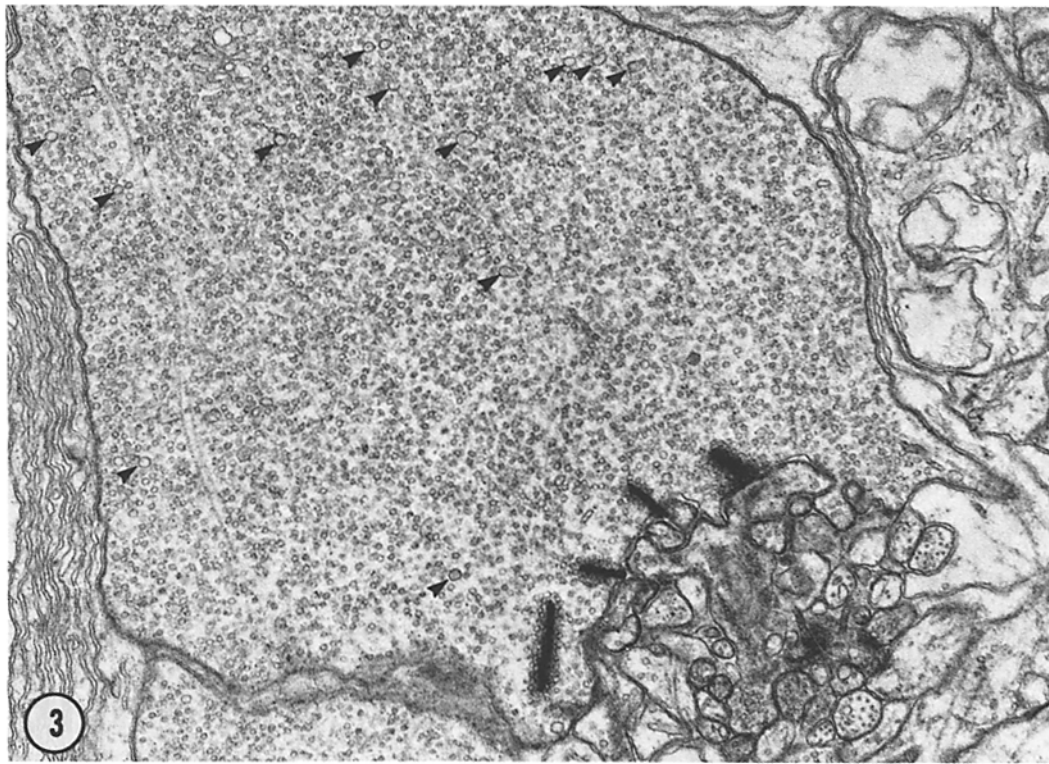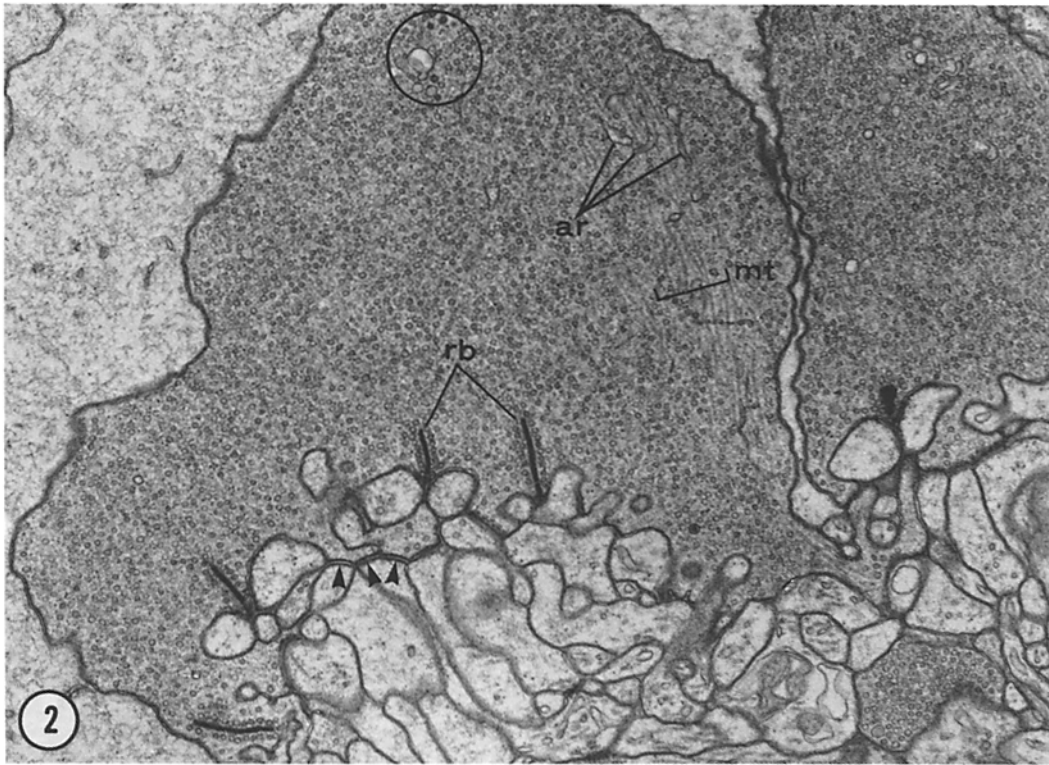
cytoplasmic organelles. Vacuoles and cisterns were few in number and only exceptionally were they associated with the synaptic ribbons.

Lysosomal equivalents were exceedingly rare at all stages of recovery from cold exposure: only two multivesicular bodies were seen in the several hundred pedicles examined and they were totally absent in the fiber and cell body.

When the retina was incubated at 4°C for 1 h in the dark and subsequently maintained at 22°C in the dark for variable time intervals, the endings went through a similar sequence of cytological events but vacuoles and cisterns were less numerous.

To test the effects of light at low temperature, the retina was maintained in the dark at 4°C for 1 h and subsequently illuminated at 4°C for 1 h. At the end of this experiment, dyads and triads were remarkably invaginated into the cone pedicles. Fig. 9 summarizes the effects of light, dark, and cold on cone cell endings.

Vesicle Exocytosis in Dark, Light, and Cold

The aim of the experiments with the freeze-fracturing technique was to establish whether the changes in synaptic surface of the pedicles caused by light, dark, and cold were accompanied by exocytosis of synaptic vesicles. In the photoreceptor endings, interaction of synaptic vesicles with the plasmalemma takes place on the slopes of the synaptic ridges (53) and it is characterized by a spectrum of plasmalemmal deformations called synaptic vesicles sites (30). These consist of a pleiomorphic population of protrusions on the outer membrane leaflet and complementary depressions on the inner leaflet (50).

A variable number of synaptic vesicle sites was found on the slopes of the synaptic ridges in all conditions tested; namely (a) when the eyecup was maintained in the dark at 22°C for 1 h and subsequently fixed in the dark (Fig. 10); (b) when the eyecup was illuminated at 22°C for 1 h and subsequently fixed in the light (Fig. 11); (c) when the eyecup was maintained in the dark at 4°C for 1 h and subsequently fixed in the cold (Fig. 12) or when the intact turtle was maintained at 4°C for 24 h.

It could be ruled out that the process of fixation caused the appearance of vesicle sites, because in all experimental conditions ridges were consistently found in which synaptic vesicle sites were absent (Fig. 13).

Uptake of Horseradish Peroxidase

The aim of the tracer experiments was to investigate the uptake of horseradish peroxidase by the cone cell endings in the dark, light, and cold and to obtain further evidence that vacuoles, cisterns, and coated vesicles mediate the process of membrane internalization which takes place during recovery from cold exposure.

DARK AT 22°C: When the retina was incubated with horseradish peroxidase in the dark at 22°C for 1 h, a large proportion of the synaptic vesicles in the cone cell endings were filled with dense reaction product (Fig. 14); labeled vesicles were randomly distributed throughout the cytoplasm, and both labeled and unlabeled vesicles were associated with the ribbons (Fig. 14, inset A). Tracer-containing vesicles were not merely confined to the synaptic ending; they also occurred as far sclerad as the cell body, although they were consistently absent from the myoid region.

Small groups of labeled cytoplasmic vacuoles were occasionally present in the pedicles; typically, the reaction product was represented by a

FIGURE 2   1 h dark 22°C, in vitro. Cone pedicles are pyramidal in shape and establish both invaginating and basal synapses. At invaginating synapses, either three (triad) or two processes (dyad) make specialized contact with a wedge-shaped projection of the pedicle (synaptic ridge) which is bisected by a synaptic ribbon (rb). Basal junctions (arrowheads) are specialized contacts established by the vitreal surface of the pedicle with one variety of bipolar cell. Besides ribbons, the cytoplasmic constituents of the pedicle are represented by numerous synaptic vesicles, randomly dispersed vacuoles surrounded by coated vesicles (circle) and profiles of the agranular reticulum (ar) which are associated with bundles of microtubules (mt). When the dark-adapted retina is maintained for 1 h in the dark at 22°C, the basal surface of the pedicles is gently concave, and dyads and triads are deeply invaginated into the ending. × 18,300.

FIGURE 3   1 h light 22°C, in vitro. The base of the pedicle is flat and dyads and triads barely ident the surface of the ending. Mesodyads and mesotriads are absent. A number of round vacuoles (arrowheads) are dispersed throughout the cytoplasm of the ending. × 13,100.
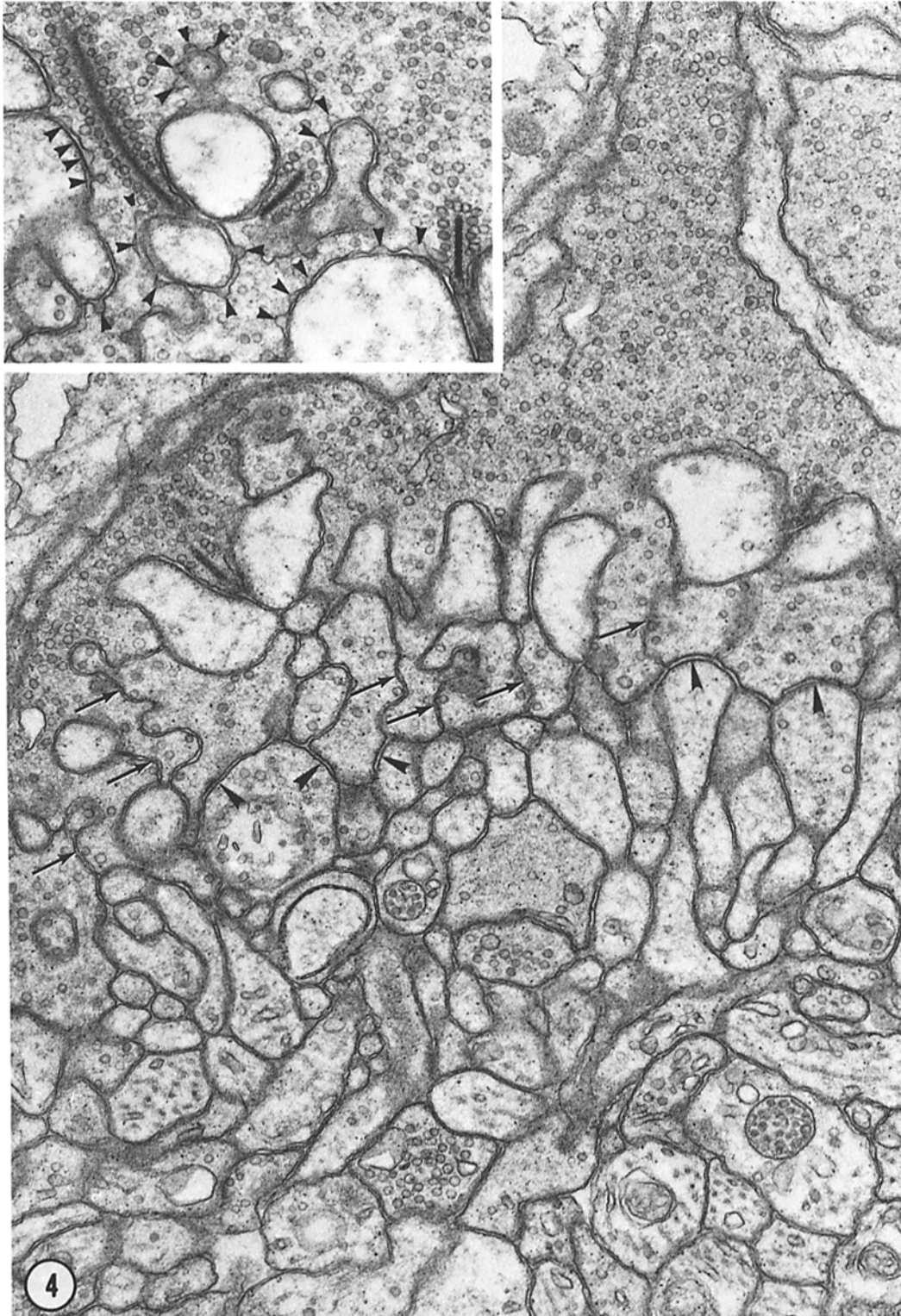
FIGURE 4 1 h dark 4°C, in vitro. Dyads and triads penetrate very deeply into the pedicle. Mesodyads and mesotriads (arrows) have increased strikingly in length and assume a tortuous course in the interior of the ending. The basal surface of the pedicle is elaborately infolded and basal junctions appear widely separated from one another (arrowheads). *Inset:* 12 h dark 4°C, in vitro. In places, a striking number of coated invaginations of the plasmalemma (arrowheads) occur on the pedicle surface. × 34,200; *inset* × 39,500.
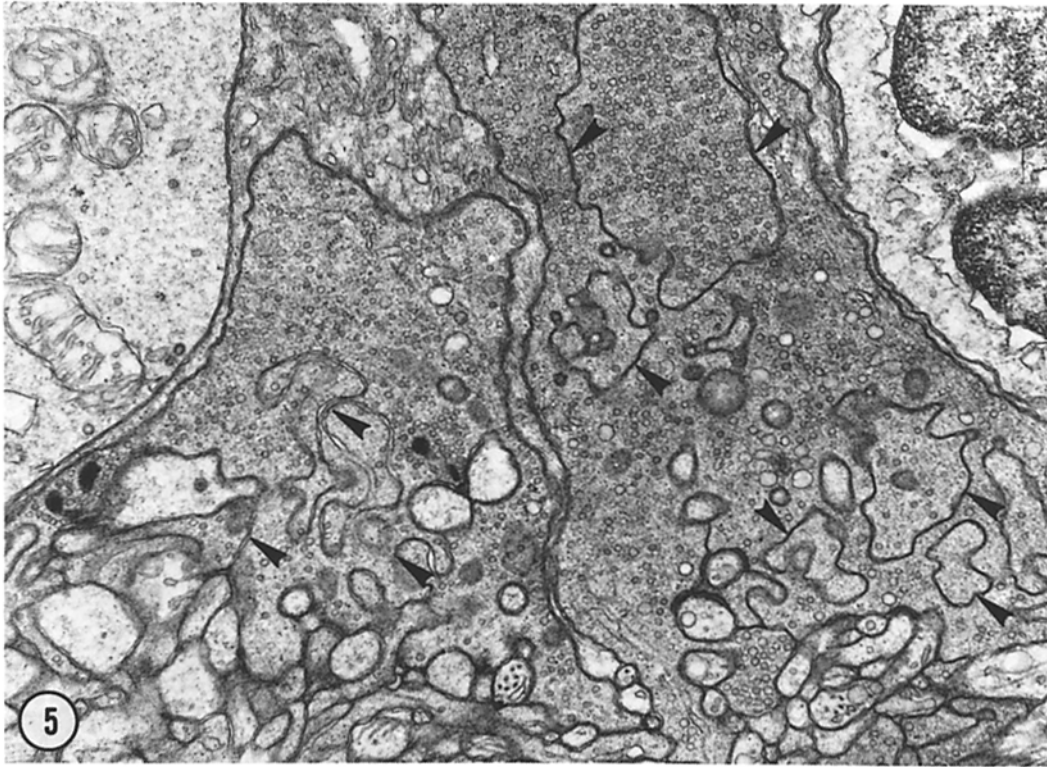
FIGURE 5   24 h dark 4°C, in vivo. Dyads and triads are deeply inserted into the endings and the cytoplasm is filled with tortuous mesodyads and mesotriads (arrowheads). The ending on the right belongs to a rod cell. × 26,200.

layer of dense material on the inner aspect of their limiting membrane, whereas the center of the vacuoles appeared unstained (Fig. 14, inset B).

The sites of coated vesicle formation, that is, the valleys on either side of the synaptic ridges and the unspecialized regions of the pedicle base, were often marked by flask-shaped invaginations of the plasmalemma containing dense reaction product.

The tubular profiles of agranular reticulum associated with the peripheral bundles of microtubules did not contain horseradish peroxidase (Fig. 14). Occasionally, labeled synaptic vesicles were superimposed on these profiles in the thickness of the section, but images of fusion between labeled vesicles and agranular reticulum were never observed.

LIGHT AT 22°C: When the retina was incubated with horseradish peroxidase in the light at 22°C for 1 h, labeled synaptic vesicles were very few and appeared randomly distributed throughout the pedicles (Fig. 15); again, tracer-containing

vesicles were not bound preferentially to the synaptic ribbons. Most of the round vacuoles were unlabeled. Forming coated vesicles, filled with reaction product, were abundant in these specimens, particularly in the valleys on either side of the synaptic ridges (Fig. 15, inset). The tubules of agranular reticulum were unlabeled.

DARK AT 4°C: When the retina was incubated with horseradish peroxidase in the dark at 4°C for 1 h, the concentration of the reaction product in the intercellular spaces of the outer plexiform layer was low, probably because the diffusion of the enzyme along the narrow intercellular clefts of the retina was slower at low temperature. Therefore, the eyecup was exposed to horseradish peroxidase for various periods of time up to 12 h in the dark and cold. In all instances, very few synaptic vesicles were labeled (Fig. 16). Along the length of the tortuous mesodyads and mesotriads, forming coated vesicles were filled with reaction product.
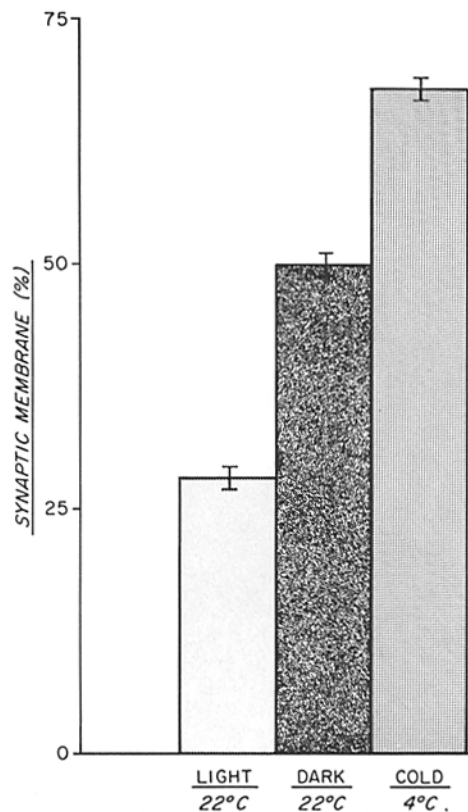
DARK AT 4°C FOLLOWED BY LIGHT AT

FIGURE 6 Percentage of total surface of the endings which is occupied by synaptic surface in light, dark, and cold (see Materials and Methods for the details of the procedure).

2 2 °C: When the retina was incubated with horse-radish peroxidase in the dark at 4°C for 1–4 h, rapidly warmed up to 22°C and illuminated for 30 or 45 min, the majority of the vacuoles and cisterns contained reaction product, whereas only

a few synaptic vesicles were labeled (Fig. 17). Coated vesicles filled with peroxidase were seen either budding from the plasmalemma or scattered throughout the cytoplasm; in addition, heavily labeled coated vesicles were observed in the process of fusing with or budding from vacuoles which were either lightly labeled or totally devoid of reaction product (Fig. 17, inset).

When the retina was: (a) exposed to horserad-ish peroxidase for 4 h at 4°C, (b) illuminated at 22°C for 1 h in the presence of the enzyme, and (c) finally maintained in a peroxidase-free me-dium in the dark at 22°C for 1 h, dense reaction product was still present in the intercellular spaces of the outer plexiform layer. In the pedicles, vacuoles and cisterns had disappeared and a pro-portion of the synaptic vesicles were labeled (Fig. 18). Tracer-containing lysosomes were occasion-ally found in the myoid region (Fig. 19).
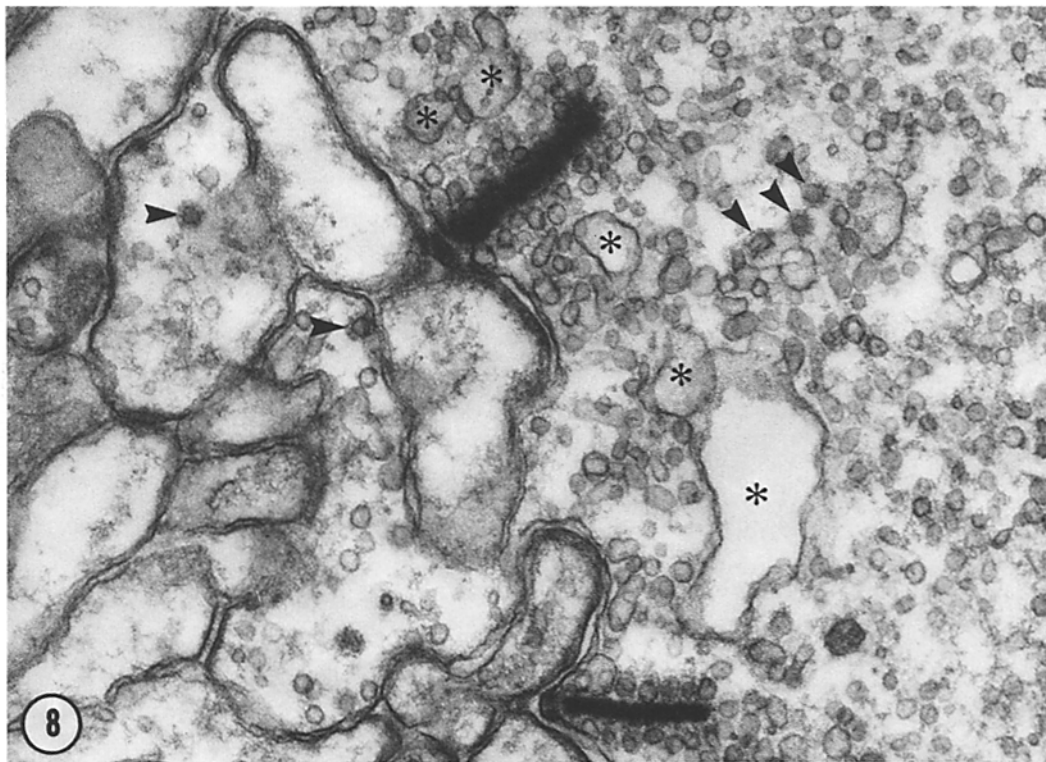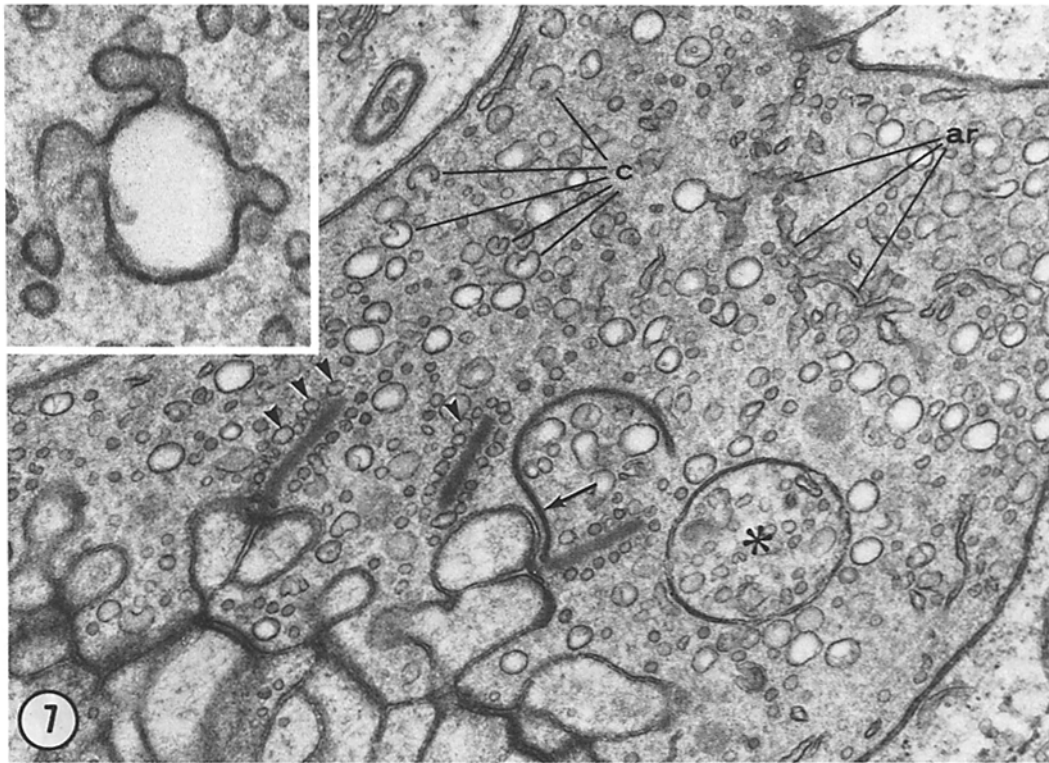
DISCUSSION

Effects of Light, Dark, and Cold on the Fine Structure of the Photoreceptor Endings

The present study demonstrates that the synap-tic endings of cone cells have a different configu-ration when the dark-adapted turtle retina is main-tained in vitro for 1 h in the dark, light, and cold. In the dark at 22°C, dyads and triads are deeply invaginated into the pedicles. Upon illumination, they are apparently extruded from the photore-ceptor cell and barely indent the basal surface of the endings. In the dark and cold, dyads and triads become remarkably invaginated and the whole ending is permeated by tortuous mesodyads and mesotriads. The effects of both illumination and

FIGURE 7 4 h dark 4°C, followed by 45 min light 22°C, in vitro. The cytoplasm of the ending contains a striking number of vacuoles and curved cisterns, whereas synaptic vesicles are few. Cisterns are either small and dilated (c) or large and flat; in this latter instance, they may be cut tangentially, and thus appear as circular profiles which completely encircle a cytoplasmic territory (asterisk). Note that vacuoles (arrowheads) and a cistern (arrow) are bound to the ribbons. Branching tubules of the agranular reticulum (ar) are present in the ending; they can be distinguished from vacuoles and cisterns on account of their elongated shape and irregular lumen. Inset: A vacuole is provided with uncoated evaginations which may represent budding synaptic vesicles. × 41,300.

FIGURE 8 1 h dark 4°C, followed by 45 min light 22°C, in vitro, primary fixation with osmium tetroxide. When osmium tetroxide is used as a primary fixative, the distinction between vacuoles, cisterns, and tubules of the agranular reticulum is no longer possible, for all these structures appear as irregular membranous compartments with a dilated lumen (asterisk). The basal region of the pedicle is rich in coated vesicles (arrowheads). × 55,500.

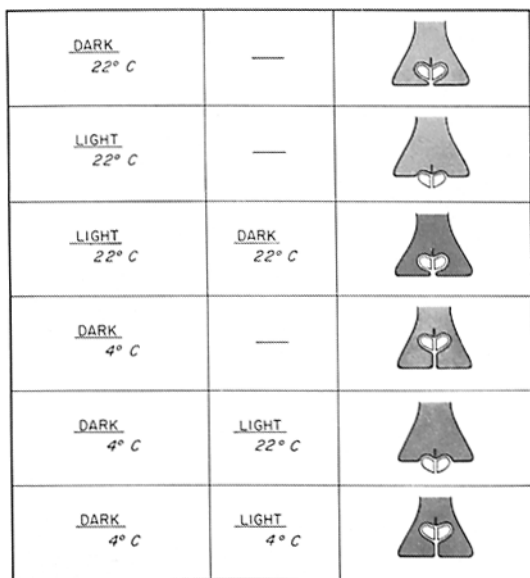|		
DARK 22° C	—	
LIGHT 22° C	—	
LIGHT 22° C	DARK 22° C	
DARK 4° C	—	
DARK 4° C	LIGHT 22° C	
DARK 4° C	LIGHT 4° C	

FIGURE 9 Effects of darkness, light, and temperature on cone cell endings.

low temperature are reversible and this excludes the possibility that the morphological changes reflect impairment of the viability of the photoreceptor cells.

Because the effects of light, dark, and cold on the shape of cone cell pedicles were restricted to their base, which is occupied by synaptic specializations, experimental changes in the surface density of the endings were estimated by comparing ratios between synaptic surface density and total surface density. This stratagem permits the mea-surement of variations in the amount of synaptic surface without determining the thickness of the sections or the volume of the endings, provided (a) the shape of the measured objects, and (b) their dimension relative to section thickness remain constant. The results of this quantitative analysis demonstrated that the relative amount of synaptic surface was decreased in the light and increased in the cold as compared to the dark-adapted state at room temperature. There are two interpretations for these findings: either membrane is redistributed between the synaptic and non-synaptic regions of the pedicle surface, or it is exchanged between cell surface and cytoplasmic compartment. The fact that a relative decrease in synaptic surface is consistently accompanied by an increase in cytoplasmic vacuoles and cisterns strongly supports the second interpretation. It is therefore unlikely that, in the three experimental conditions tested, the invaginating processes actively move in and out of the endings, but rather the pedicles compensate for the excess plasmalemma by flowing along the length of the incoming processes. These observations are in agreement with the results of an earlier study by Cragg (17) who noted that the rod cell endings in the rat were larger when the animal was exposed to dark for a long period.

The changes in configuration of the pedicles probably represent the net result of a different balance between addition and retrieval of plasma membrane in the dark, light, and cold. Because at 22°C the light and dark configurations of the

FIGURE 10   1 h dark 22°C, in vitro, freeze-fracturing. The plasmalemma of a synaptic ridge (SR) is identified by the presence of a particle aggregate in the interior of the membrane of an adjoining horizontal cell process (HP). The pleiomorphic deformations of the outer leaflet of the pedicle plasmalemma (arrowheads) represent sites of vesicle interaction with the cell membrane: they appear as minute protrusions, hemispherical bumps, or volcano-like openings and probably correspond to different phases of the process of vesicle exocytosis. × 104,200.

FIGURE 11   1 h light 22°C, in vitro, freeze-fracturing. En face view of the outer leaflet of the plasmalemma of a synaptic ridge (SR): sites of synaptic vesicle exocytosis (arrowheads) also occur at the end of 1-h illumination. × 77,800.

FIGURE 12   1 h dark 4°C, in vitro, freeze-fracturing. Low temperature does not prevent vesicle exocytosis, for synaptic vesicle sites (arrowheads) are present on both the outer (OL) and inner (IL) leaflets of the cell membrane of a synaptic ridge. HP, particle aggregate in the interior of the plasmalemma of an adjoining horizontal cell process. × 101,000.

FIGURE 13   1 h dark 22°C, in vitro, freeze-fracturing. In all experimental conditions tested, synaptic ridges (SR) are consistently found in which sites of vesicle exocytosis are absent. HP, particle aggregate in the interior of the plasmalemma of an adjoining horizontal cell process. × 89,600.
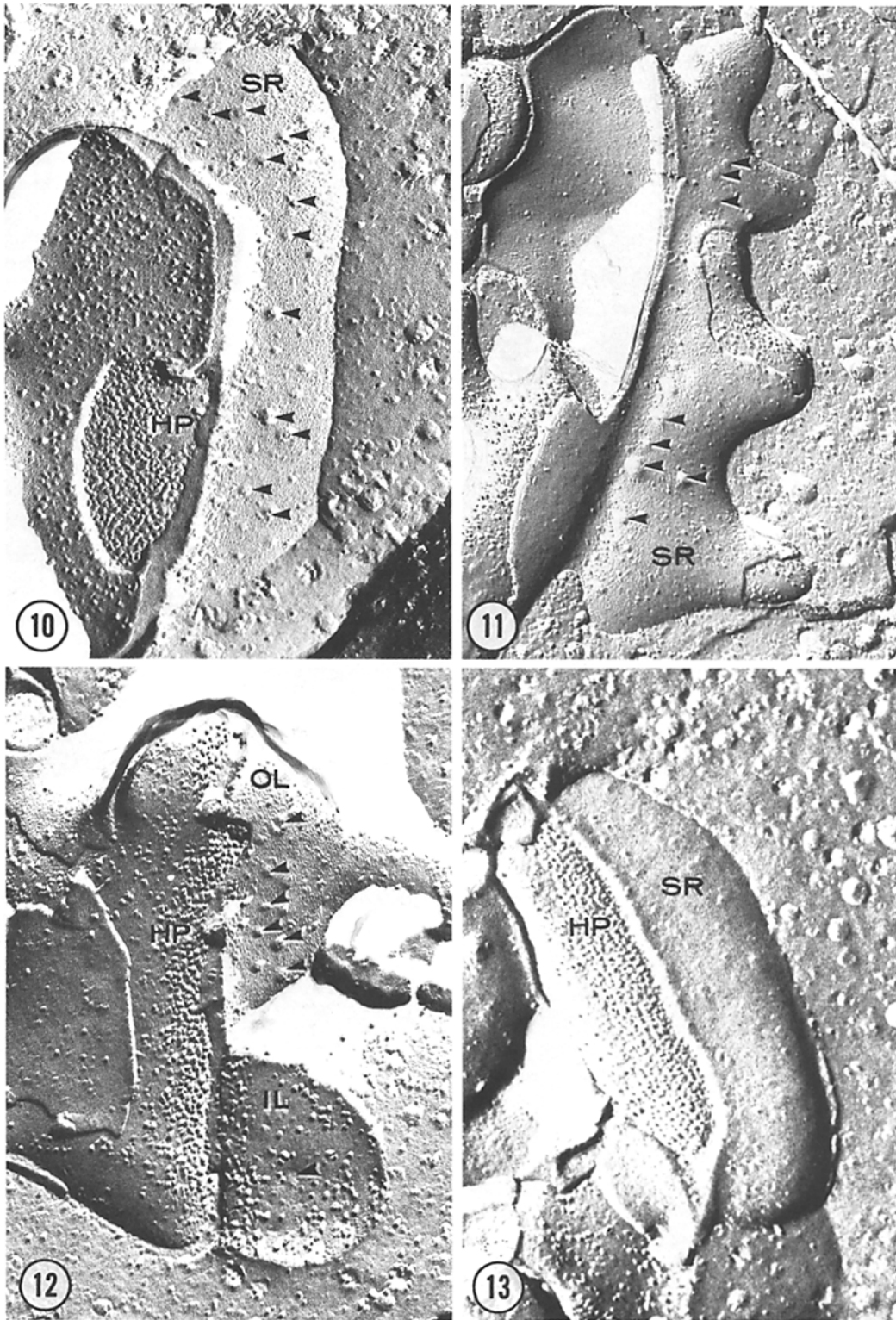
FIGURE 14 1 h dark 22°C, horseradish peroxidase. A large proportion of the synaptic vesicles in the cone pedicle contain reaction product: labeled vesicles are randomly distributed throughout the cytoplasm, and both labeled and unlabeled vesicles are associated with the synaptic ribbon (*inset A*). Tracer-containing vesicles also occur in the fiber. The profiles of agranular reticulum (arrowheads), which are associated with the peripheral bundles of microtubules, do not contain horseradish peroxidase. *Inset B:* Small groups of vacuoles (*v*) are occasionally present in the pedicles; typically, the reaction product is represented by a layer of dense material on the inner aspect of their limiting membrane, whereas the center of the vacuoles appears unstained. × 31,700; *inset A* × 53,500; *inset B* × 37,200.
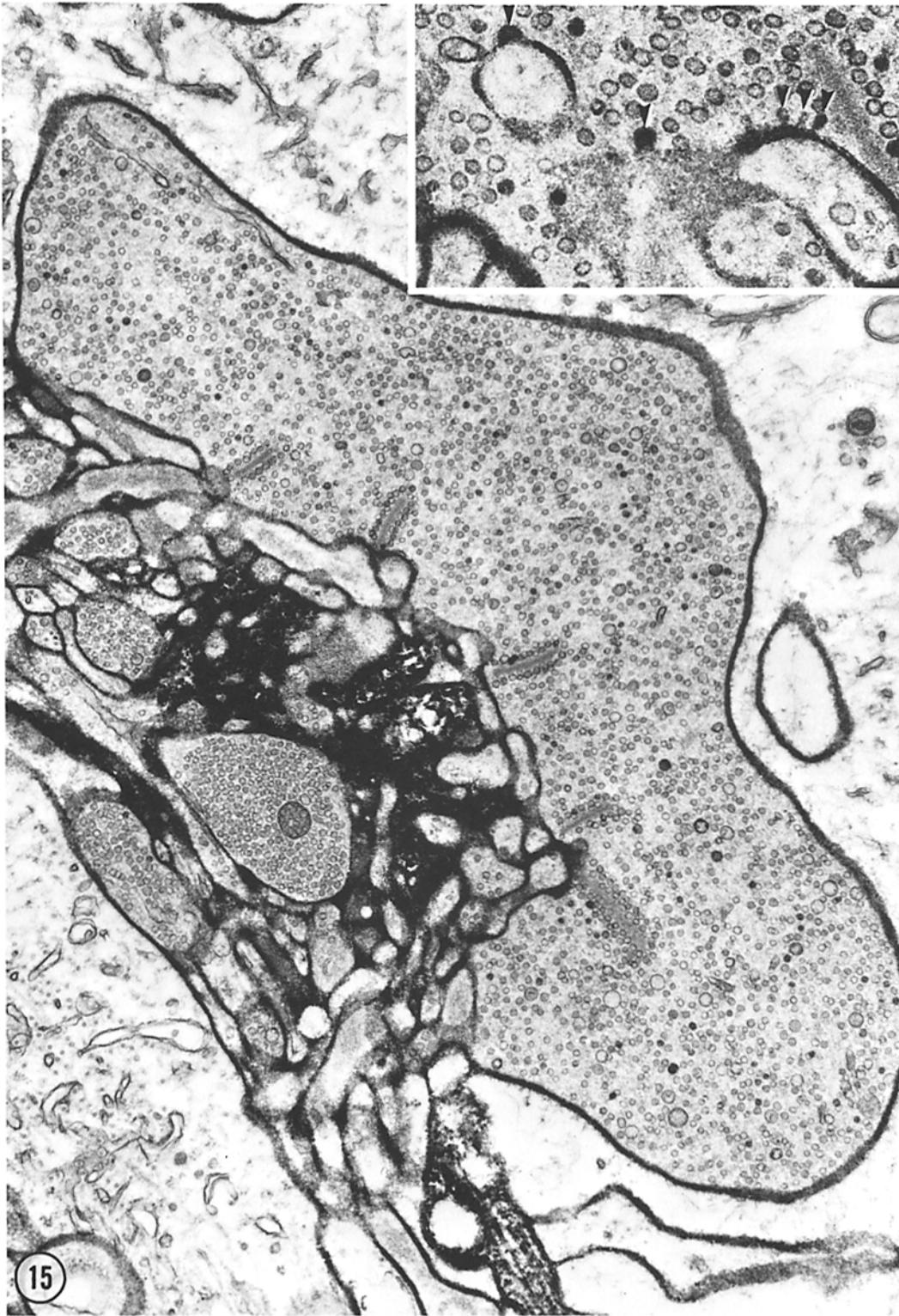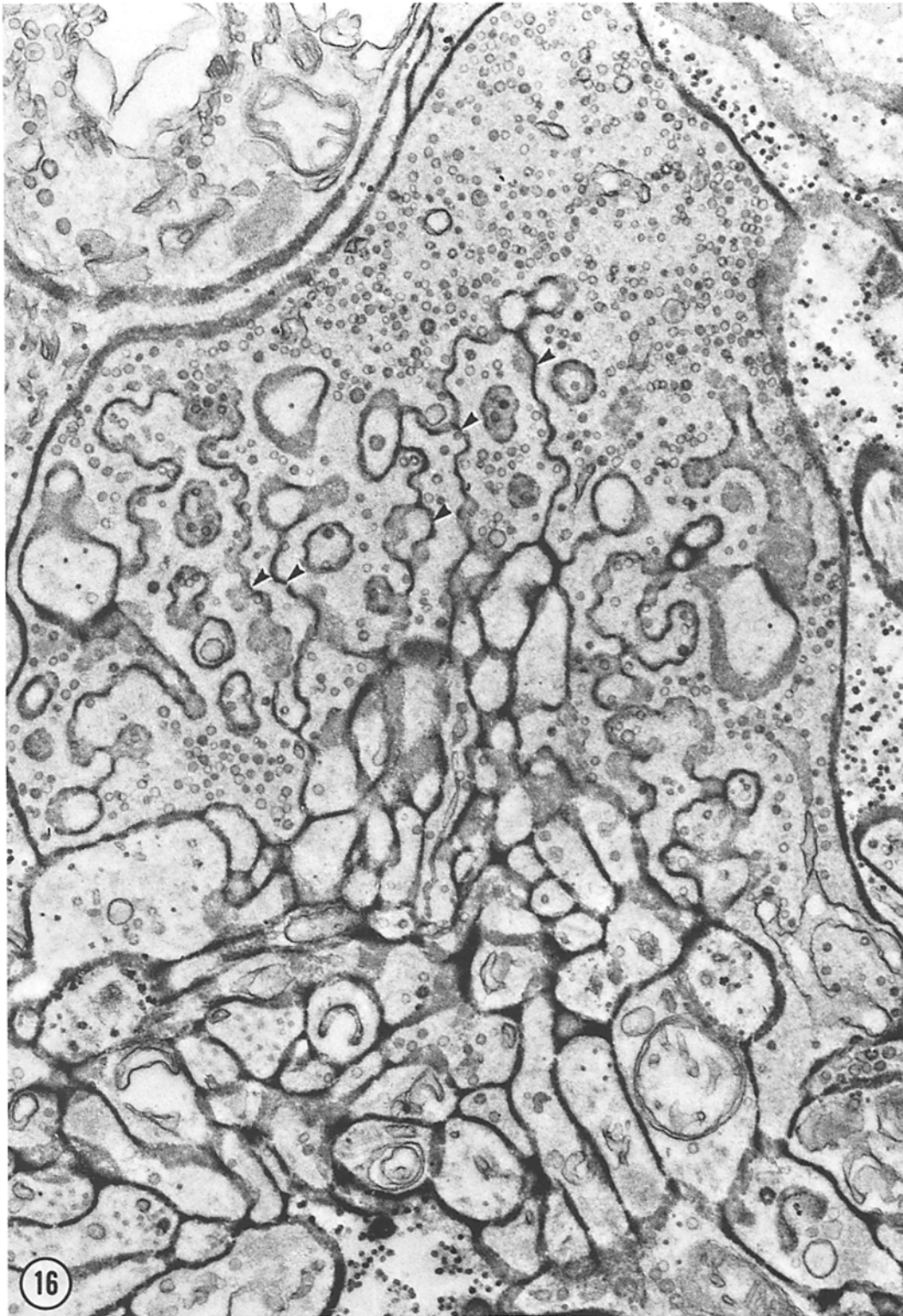
FIGURE 15 1 h light 22°C, horseradish peroxidase. After illumination, only a few synaptic vesicles are labeled and these appear randomly distributed throughout the cytoplasm. Most of the vacuoles and cisterns are unlabeled. *Inset:* Coated invaginations of the plasmalemma (arrowheads) are filled with dense reaction product, × 37,800; *inset* × 61,200.

FIGURE 16   12 h dark 4°C, horseradish peroxidase. The invaginating processes are so deeply inserted into the ending that the pedicle cytoplasm is permeated by meandering mesodyads and mesotriads (arrowheads). In the pedicle cytoplasm, synaptic vesicles are few and only exceptionally labeled. × 37,100.
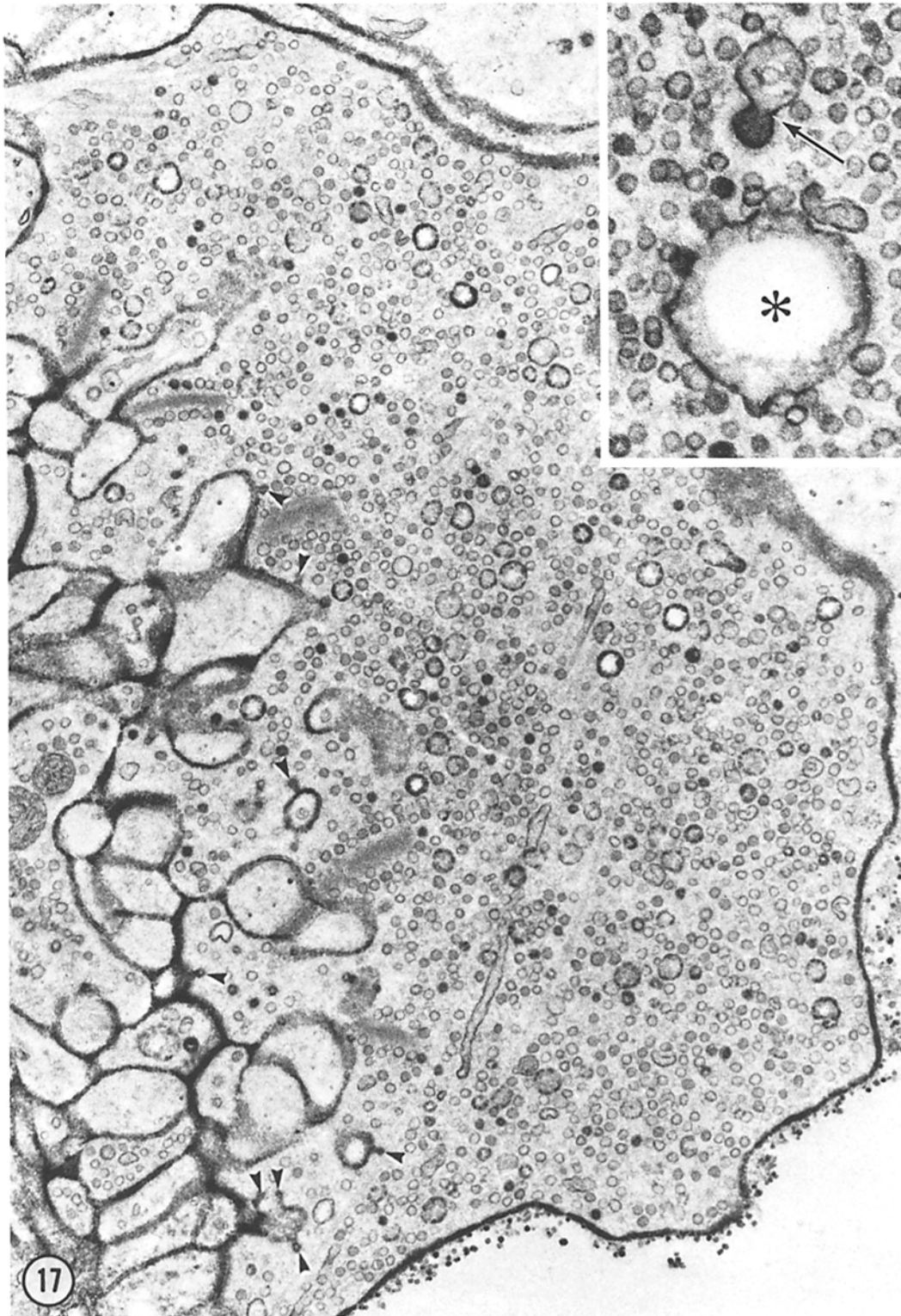
FIGURE 17 1 h dark 4°C, followed by 45 min light 22°C, horseradish peroxidase. Most vacuoles and cisterns contain horseradish peroxidase, whereas few synaptic vesicles are labeled. Forming coated vesicles filled with reaction product (arrowheads) are present at the base of the ending. *Inset:* A tracer-filled coated vesicle is fusing with or budding from a vacuole which is either empty or lightly labeled (arrow). Note that the circular profile at the center of the vacuole is continuous with the limiting membrane and therefore represents a surface evagination which is included in the thickness of the section. Below, a labeled vacuole (asterisk) is provided with numerous hemispherical surface protrusions. × 41,400; *inset* × 85,400.
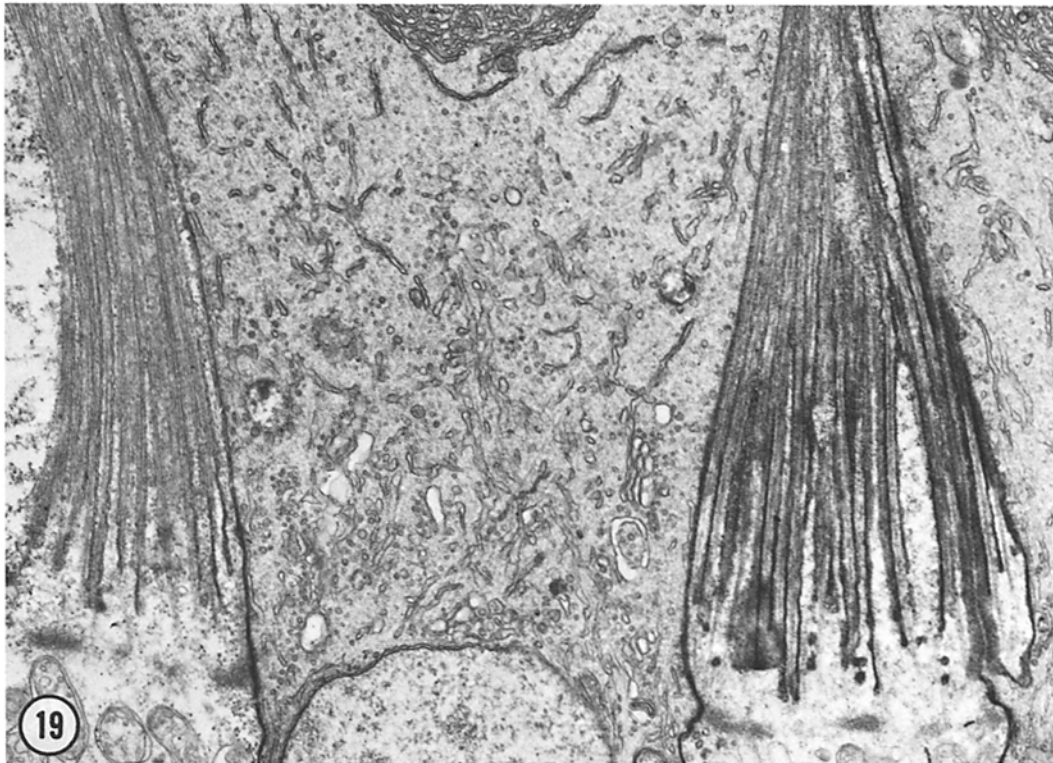
FIGURE 18 4 h dark 4°C with horseradish peroxidase, 1 h light 22°C with horseradish peroxidase, 1 h dark 22°C without horseradish peroxidase. Vacuoles and cisterns have disappeared from the cone cell endings, and a proportion of the synaptic vesicles is labeled. In spite of the 1 h chase, the intercellular spaces still contain horseradish peroxidase. × 13,300.
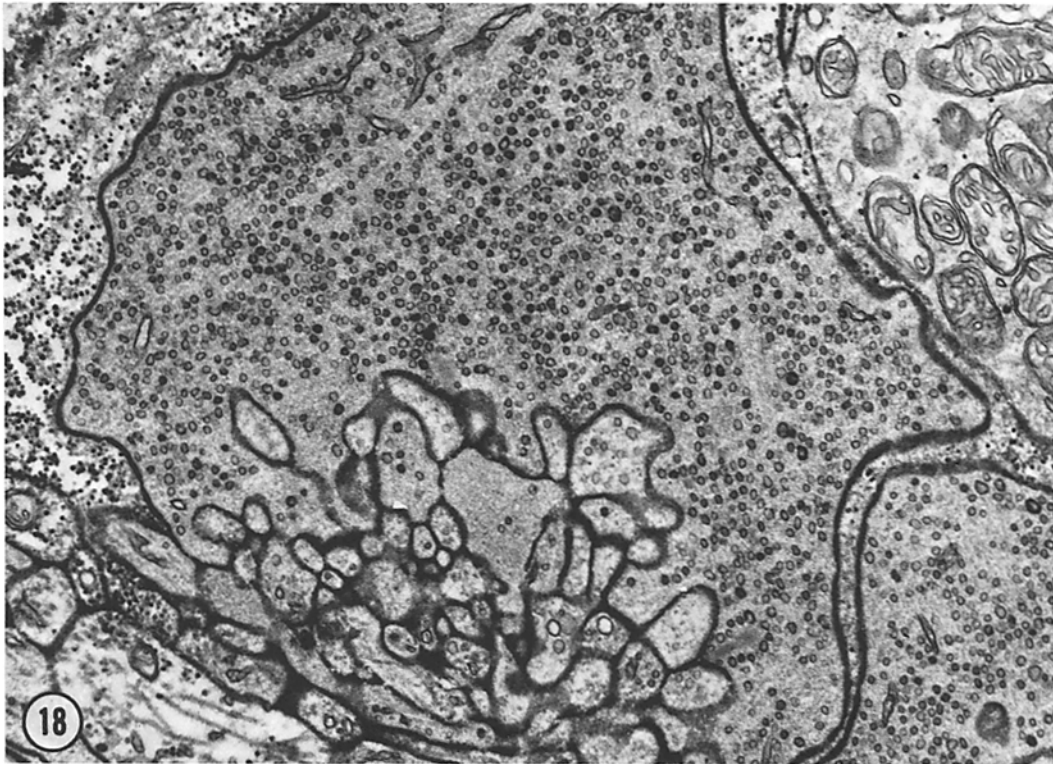
FIGURE 19 4 h dark 4°C with horseradish peroxidase, 1 h light 22°C with horseradish peroxidase, 1 h dark 22°C without horseradish peroxidase. At the time in which labeled vacuoles and cisterns have disappeared from the cone cell endings, few tracer-containing bodies are present in the myoid. × 23,200.

endings were stable—they did not change when the length of the period of dark or light exposure was further increased—the rate of membrane retrieval must equal the rate of membrane addition in both the dark- and light-adapted states. However, more synaptic surface was present in the dark: thus, membrane addition must increase upon turning the light off and abate upon illumination without a parallel adjustment of the retrieval process.

The observation that the synaptic surface of the endings progressively increased with longer periods of exposure to 4°C indicates that cold blocks or greatly depresses a mechanism of membrane interiorization which continuously operates at 22°C. In keeping with this hypothesis are the following facts: (a) the excess surface membrane is interiorized again when the retina returns to room temperature; (b) illumination causes a decrease in synaptic surface at 22°C, but it has no influence on the surface of the endings at 4°C; (c) in the dark, fewer vesicles are labeled with horseradish peroxidase at 4°C than at 22°C. Probably, membrane retrieval is mediated by a process of endocytosis, because in Amoeba the uptake of polystyrene beads is inhibited by temperatures of less than 10°C (69).

The source of the membrane that is continuously added to the plasmalemma in the dark, light, and cold is most likely represented by synaptic vesicle membrane that becomes incorporated into the cell surface upon exocytosis. It has been repeatedly shown that upon electrical or chemical stimulation of the axonal endings of the neuromuscular junction (12, 13, 15, 29), superior cervical ganglion (51, 52), and Torpedo electric organ (71), synaptic vesicles decrease in number and the surface of the presynaptic membrane increases. In this respect, presynaptic endings just conform to the general rule that the surface of secretory cells increases upon fusion of the limiting membrane of secretory granules with the plasmalemma (1, 26, 46, 47). Indirect evidence that in the dark at 22°C more vesicles fuse with the plasmalemma than in the light emerges from the tracer experiments (see below). Indirect evidence that synaptic vesicle membrane is added to the plasmalemma in the cold emerges from the observation that the endings become depleted of synaptic vesicles with prolonged periods of exposure to 4°C. Finally, direct evidence that synaptic vesicles do indeed fuse with the cell membrane in all experimental conditions tested was obtained with

the freeze-fracturing technique, which demonstrated sites of vesicle exocytosis on the slopes of the ridges in the light, dark, and cold. It must be noted that one cannot imply from the freeze-fracture findings that the rate of vesicle fusion was the same in all three experimental conditions. Quantitative estimates, however, were made difficult by the fact that few synaptic ridges were exposed in each replica. Furthermore, the number of vesicle sites per ridge varied greatly and ridges were always found that were devoid of vesicle sites; these variations inevitably arise when a short-lived process such as vesicle exocytosis is investigated with the slow-acting chemical fixation. Thus, the freeze-fracture findings are not necessarily in contradiction with the results of the tracer experiments (see below). It is interesting to note that vesicle exocytosis also occurs at low temperature; this is consistent with the observation that in the frog neuromuscular junction, miniature end-plate potentials continue to be generated in the cold, although at a low frequency (34, 38).

In conclusion, the increase in the synaptic surface of the cone cell endings in the dark and in the cold strongly supports the hypothesis, based on physiological experiments, that transmitter is released in the dark (65). Furthermore, the observation that low temperature reversibly blocks the process of retrieval of synaptic vesicle membrane from the plasmalemma provides a useful model for investigating the mechanism of membrane interiorization in the photoreceptor endings.

Membrane Recycling in the Photoreceptor Endings

In addition to their complement of ribbons and synaptic vesicles, the photoreceptor endings contained a variable but generally small population of vacuoles and coated vesicles; furthermore, coated vesicles were seen budding from the plasmalemma on either side of the synaptic ridges (25, 53) or along the unspecialized regions of the pedicle base. The observation that upon illumination the number of cytoplasmic vacuoles slightly increased in parallel with a decrease in the amount of the synaptic surface of the pedicles suggests that vacuoles may be involved in the process of membrane retrieval. Crucial evidence, however, emerged only from an analysis of the cytological events which occur during recovery from cold exposure. When the retina was warmed up and illuminated, the amount of synaptic surface in the endings de-

creased and a large number of vacuoles, curved cisterns, and coated invaginations of the plasmalemma transiently appeared; they disappeared again after the endings had returned to their room temperature configuration. These findings clearly show that the decrease in synaptic surface of the endings is due to a process of internalization of the plasmalemma mediated by vacuoles, cisterns, and coated vesicles. A similar phenomenon of membrane retrieval after cold exposure has been recently reported by Model et al. (45) in a synapse of the central nervous system of the hatchetfish; furthermore, participation of vacuoles, cisterns, and coated vesicles in retrieval of surface membrane has been repeatedly described in both neural and nonneural elements (1, 8, 10, 11, 29, 31-33, 47, 55, 59, 63, 64, 67, 70).

The vacuoles and curved cisterns of the photoreceptor endings are clearly equivalent to the membranous profiles described by Heuser and Reese in the frog neuromuscular junction after repetitive electrical stimulation (29); in fact, when the retina was exposed to osmium tetroxide as a primary fixative, all of these organelles acquired the appearance of irregular membrane-bounded compartments with a dilated lumen. The precise interrelationships between round vacuoles and curved cisterns are unclear; transitional forms, however, are often present, and the morphological heterogeneity of these membranous profiles may well represent a fixation artifact. It was suggested that cup-shaped cisterns represent precursors of multivesicular bodies (10, 11, 31, 33, 58, 59, 70), and an increase in labeling of these lysosomal equivalents was reported in photoreceptor endings exposed to horseradish peroxidase in the dark (58, 59). The present study does not support this hypothesis, for multivesicular bodies were exceedingly rare in cone cells which had recovered from cold exposure.

In all experimental conditions, the peripheral cytoplasm of the photoreceptor endings contained profiles of the agranular reticulum which were typically associated with bundles of microtubules. These cisterns were easily distinguished from the vacuoles and curved cisterns because of their branching and anastomosing pattern and their specific location in the endings. Furthermore, their number did not vary in the course of the experiments performed in this study. After primary osmium fixation, however, they lost their distinctive appearance and could not be differentiated from the other membranous profiles of the endings.

The aim of the ultrastructural tracer analysis was to label selectively with horseradish peroxidase the structures responsible for internalization of the plasmalemma. Because this enzyme does not cross intact membranes (24), its presence in a membrane-walled compartment indicates that this compartment either transiently opened at the cell surface or fused with another organelle that picked up tracer from the extracellular space.

In cone pedicles, when the retina was kept at room temperature, a much larger proportion of synaptic vesicles was labeled in the dark than after light exposure. Similar results were obtained in the rod cell endings of the skate (55) and frog (59). In the cone pedicles of the frog (59), the difference between light and dark was less striking, except when high intensity illumination was used for a long period of time. The fact that more synaptic vesicles are labeled in the dark than in the light shows that the rate of tracer exchange between the extracellular and the vesicular compartment is higher in the dark than in the light, and thus provides indirect evidence that in the photoreceptor endings: (a) more vesicles fuse with the plasmalemma in the dark, and (b) vesicular membrane is reutilized after exocytosis. Thus, this finding further supports the hypothesis that the photoreceptor endings release more transmitter in the dark than upon illumination (65).

In the dark, labeled synaptic vesicles do not seem to be preferentially associated with the synaptic ribbons. This is in agreement with the observation of both Ripps et al. (55) and Schacher et al. (59), and suggests that the ribbon randomly selects its complement of vesicles from the pool available in the synaptic ending.

When the retina was exposed to dark at low temperature, labeled synaptic vesicles were considerably fewer than at room temperature. Because the synaptic surface of the endings increased in the cold, the result of this tracer experiment strongly supports the hypothesis that low temperature blocks the process of retrieval of synaptic vesicle membrane from the plasmalemma. When the retina was warmed up and illuminated for short time intervals after cold exposure, few synaptic vesicles were labeled, whereas tracer appeared in vacuoles, cisterns, and coated vesicles. These findings provide decisive evidence that vacuoles, cisterns, and coated vesicles are responsible for internalization of the surface membrane.

2 h After the retina was returned to room temperature, vacuoles, cisterns, and coated vesicles had disappeared, and synaptic vesicles were

labeled. A likely interpretation for this finding is that vacuoles, cisterns, and coated vesicles ultimately give rise to synaptic vesicles. Conclusive evidence in favor of this hypothesis could not be obtained because it was impossible to rinse peroxidase from the extracellular space before completion of the experiment.

However, additional observations document the origin of synaptic vesicles from vacuoles and cisterns: (a) vacuoles are often provided with uncoated hemispherical or flask-shaped protrusions that may represent nascent synaptic vesicles; (b) both vacuoles and cisterns are often connected to the ribbons and thus possess binding properties similar to those of synaptic vesicles; (c) finally, after recovery from cold exposure, vacuoles and cisterns disappear from the photoreceptor cell without giving rise to lysosomal equivalents.

Thus, the results of the experiments performed on turtle cone pedicles seem to substantiate the hypothesis of synaptic vesicle membrane recycling originally proposed by Heuser and Reese for the frog neuromuscular junction (29): during transmitter release, synaptic vesicle membrane is reversibly incorporated into the plasmalemma and is returned to the vesicular compartment through coated vesicles and vacuoles or cisterns. The various steps of this membrane cycle are spatially segregated and well-ordered: synaptic vesicles discharge transmitter and fuse with the plasmalemma on the slopes of the synaptic ridges (53). Subsequently, constituents of their membrane move laterally in the plane of the pedicle plasmalemma and are retrieved by coated vesicles which pinch off on either side of the synaptic ridges or from the base of the endings. Thus, the invaginating synapse is a sensitive morphological indicator of the rate of transmitter release, for deep penetration of dyads and triads into the pedicle cytoplasm reflects transitory accumulation of synaptic vesicle membrane at the cell surface.

The present study does not rule out the possibility that vesicles may also reform directly from the plasmalemma (12, 13): in fact, no increase in the number of vacuoles, cisterns, and coated vesicles was recently observed in the photoreceptor endings of the skate during recovery from exposure to a high potassium medium (56). Perhaps, cooperation of two different mechanisms is required to ensure a precise adjustment of the cell surface. There is no doubt, however, that vacuoles, cisterns, and coated vesicles become the primary mechanism of membrane retrieval when the surface of the ending has greatly increased

upon cold exposure.

Several questions remain to be elucidated in the process of synaptic vesicle membrane recycling in turtle cone cell endings. Because horseradish peroxidase diffuses quite slowly through the intercellular spaces of the retina, it was impossible to dissect out the various phases of the recycling process. Thus, the problem of the precise interrelationships between coated vesicles, vacuoles, and cisterns was not solved. The presence of tracer in the interior of vacuoles and cisterns indicates that these structures form either as invaginations of the plasmalemma or by coalescence of coated vesicles. However, only coated vesicles appeared to arise from the plasmalemma, and on occasions, coated vesicles were seen in continuity with vacuoles. When tracer was added to the incubation medium, the coated evaginations stained more intensely than the lumen of the vacuoles: this suggests that coated vesicles fuse with the vacuoles, although the possibility cannot be ruled out that the difference in staining intensity may be due to geometrical reasons or the fact that vacuoles arise during fixation from swelling of labeled cisterns. Thus, it is unclear whether vacuoles and cisterns arise exclusively from fusion of coated vesicles (29), or rapidly pinch off from the plasmalemma (27) and subsequently fuse with or give rise to coated vesicles.

The role of the profiles of agranular reticulum at the periphery of the endings is unknown. As previously noted (11, 63), these membrane-walled compartments were never penetrated by tracer and therefore cannot be involved in local recycling. Perhaps they originate in the cell body, move down the fiber in close association with microtubules, and ultimately divide to form synaptic vesicles. A continuous inflow of new vesicles would explain why it has never been possible to label the whole population of synaptic vesicles with extracellular tracers.

Finally, part of the vesicle population may have a finite life-span and be eventually turned over. In other systems, it has been shown that labeled coated vesicles and multivesicular bodies are transported towards the perikaryon (40, 42, 43), and it has been proposed that multivesicular bodies contain the products of synaptic vesicle degradation (31, 33, 43, 59, 63). In the present study, however, multivesicular bodies were rarely seen in the pedicles. Furthermore, because lysosomes were consistently absent from the fiber and cell body of the photoreceptor cells, it seems unlikely that the membrane retrieved from the surface of

the endings could be degraded in the inner segment. Probably, the tracer-containing organelles which are occasionally found in the myoid region are involved in local endocytotic events.

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