Effect of Barium and Tetraethylammonium on Membrane Circulation in Frog Retinal Photoreceptors

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ABSTRACT We studied the influence of altered ionic conditions on the recycling of synaptic vesicle membrane in frog retinal photoreceptors using horseradish peroxidase to monitor synaptic activity and trace the fate of internalized membrane. The addition of 1.2 mM barium or 20 mM tetraethylammonium to isolated retinas maintained in Ringer's solution, changes the usual balance of membrane circulation in the rod cells; the cone cells are much less affected. Retrieval of synaptic vesicle membrane in the rods, which normally regenerates small vesicles, becomes mediated predominantly by large sacs and vacuoles ("cisternae"). Because these cisternae can be labeled with peroxidase, they appear to arise from endocytized membrane. Morphometric analysis suggests strongly that the cisternae are formed of circulating synaptic vesicle membrane. The effects of barium and tetraethylammonium can be inhibited by high extracellular potassium, by high intensity light, and by 5 mM cobalt. They seem likely to depend on potassium channels, though additional more complex mediation may also be involved. The alterations in membrane retrieval that we find are of interest in terms of the multiple pathways of membrane cycling now being uncovered. They open potential experimental approaches to the controls of this circulation. In addition, the findings extend our previous ones demonstrating that rod cells and cone cells differ in their responses to divalent cations in ways that seem likely to be of physiological importance.

Photoreceptors are specialized nerve cells that transduce light energy into membrane potential changes which in turn modulate the release of neurotransmitter. In the dark, the photoreceptors are depolarized and synaptically active (55, 59–61). Moderate levels of light hyperpolarize rod cells and reduce their synaptic activity, while higher light intensities are required to affect cone cells (67).

As with conventional neurons, neurotransmitter release in photoreceptors is accompanied by a stimulation-responsive cycle of exocytosis and endocytosis; we showed this originally for frog retinas (59, 60) and confirmatory findings have been published for skate and turtle preparations (55, 61). The accumulation of exogenous macromolecular tracers had provided direct evidence of such a cycle in invertebrate neuromuscular junctions (35) and vertebrate neuromuscular junctions (7, 30), among other preparations (reviewed in references 33, 76).

The conditions that control exocytosis have been extensively studied (reviewed in reference 43), and it is generally accepted that calcium plays an integral role in triggering synaptic transmission. Much less is known about the controls of endocytosis in general (63) and of membrane retrieval in particular. For instance, membrane retrieved after exocytosis in nerve terminals can take the form either of small vesicles or of larger sacs and vacuoles called "cisternae" (30). But there is virtually no information about what governs retrieval of synaptic vesicle membrane beyond the facts that calcium may be an important factor (6; see also reference 44), and that the relative prominence of large and small retrieval structures can vary with rates of transmission (19, 30, 47, 51, 54) and temperature (30, 61).

Issues of the form of retrieval and its controls are potentially of broad significance, in view, for example, of the long standing literature indicating the existence of intermediate prelysosomal endocytic compartments (32) and of current theories of adsorptive endocytosis and endocytic membrane cycling which place an emphasis on supposed large intermediate structures (53). In general, the behavior of internalized membrane seems more complex than was hitherto thought to be the case (18, 34).

In this paper, we describe the effects of divalent and monovalent cations, and of agents that affect channels for these ions, on membrane recycling in frog photoreceptors. Synaptic vesicle

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membrane retrieval in rod cells, in which small vesicles normally predominate, is altered by barium, so that retrieval becomes mediated predominantly by larger cisternae. This change may be due in part to barium's interaction with potassium channels since, for example, the effects of barium are paralleled by those of tetraethylammonium (TEA). As with other effects of divalent cations on photoreceptors that we have observed (15), the cone cells are much less affected by barium than are the rods.

Preliminary reports of this work have been published (45, 46).

MATERIALS AND METHODS

Animals

Frogs, *Rana pipiens*, ~3 in in body length, were obtained from J. M. Hazen Frog Farm (Alburg, VT). In the laboratory, frogs were kept at room temperature in plastic bins containing tap water that was changed daily. Most of the frogs were maintained on a 12-h light/12-h dark cycle, and were fed meal worms on a regular basis.

Horseradish Peroxidase Experiments

Before each experiment, frogs were kept in total darkness for 12 h. The retinas were isolated under a dim red light as described by Schacher et al. (60). Control Ringer's solution (normal Ringer's) contained 111 mM NaCl, 2.3 mM $NaHCO_3$, 2 mM KCl, 1.1 mM $CaCl_2$, and 5 mM D-glucose, pH 7.2. High potassium Ringer's (20) had 113 mM potassium propionate, 2.3 mM $NaHCO_3$, 1.1 mM calcium propionate, and 5 mM D-glucose, pH 7.2. When Ringer's solution contained 5 mM or more of other salts, the NaCl concentration was adjusted to maintain osmotic constancy. A concentration of 0.5% horseradish peroxidase (HRP) (Sigma Chemical Co., St. Louis, MO; Type II) was included in the Ringer's solutions. Retinas were exposed to HRP-Ringer's for 15 or 30 min in the dark at room temperature, during which time the retinas were kept on a moistened Telfa pad in a physiological chamber with oxygen bubbling into the Ringer's surrounding the pad. We find periods of this length suitable to permit both recovery of the retinal physiological activities from the effects of manipulation and adequate penetration and uptake of peroxidase. After HRP exposure, the retinas were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, sectioned on a Smith-Farquhar tissue chopper at a thickness setting of 40 μm, incubated to demonstrate peroxidase activity, and prepared for electron microscopy by the procedures used routinely in our laboratory (60). Thin sections were stained with lead citrate and examined in a Philips 201 electron microscope.

Dextran Experiments

10% dextran T-10 or T-40 (Pharmacia Fine Chemicals, Uppsala, Sweden) was dissolved in reduced sodium Ringer's, sonicated for 20 min, then filtered through a 0.22-μm Millipore filter to remove aggregates. Retinas were isolated and incubated as described for HRP experiments, and then fixed for 2 h in fixative made by mixing 3 parts 3.5% formaldehyde and 5% glutaraldehyde with 2 parts 2% osmium and 1 part saturated lead citrate, in 0.07 M cacodylate buffer (V. Herzog, personal communication). The fixative was changed once after 1 h. Retinas were then rinsed overnight in buffer, cut with a razor blade into 1-mm^2 pieces, and dehydrated and embedded as described for HRP preparations. Thin sections were stained with 0.04% bismuth subnitrate and 0.08% sodium tartrate in 0.04 N sodium hydroxide according to the method of Ainsworth and Karnovsky (1).

Electrophysiology

Mass retinal potentials were recorded using the extracellular recording system and the optical system described in Hood and Hock (38). The intensities of "white" light used for the tracer experiments, expressed as the log of the fraction of full intensity (130 ft-candles) were: moderate intensity, −3.2; and high intensity, −2.1 (see reference 60).

Analysis of Tracer Uptake

After the retinas were thin sectioned, the grids were relabeled so that the microscopist was unaware of the tissue's history. To obtain numerical data, two to three tissue blocks were usually examined from each retina for three separate preparations. Exceptions are noted in the figure legends. We typically examined 30 rod and 30 cone cells per block.

The "synaptic activity" of a given terminal was quantified directly under the electron microscope by calculating the percentage of HRP-labeled synaptic vesicles in the vesicle population (see reference 60 for more details). Vesicle density (number of vesicles/unit area) was calculated from electron micrographs by randomly selecting three regions of 0.5 μm^2/terminal and counting the vesicles.

Morphometry

Morphometric analysis was done using a computer program and tracing system designed by Lee D. Peachey (University of Pennsylvania). This system employs a bitpad and microprocessor to measure perimeters and areas. For each tissue block analyzed, we used one thin section per grid and photographed the first 20 cones encountered whose profile included a nucleus and synaptic ribbon, and the first 20 rods whose profile included the base of the axon and a synaptic ribbon. This procedure ensured that our sample was unbiased, yet included complete terminal regions. The micrographs were printed at a final magnification of 21,000 or 25,000.

From area and vesicle density data, we calculated the average number of synaptic vesicles per thin section of a terminal. The amount of synaptic vesicle membrane per thin section of a terminal was determined using the formula $\pi \times$ mean vesicle diameter $\times$ number of vesicles. For example, control terminals have 1,546 (SD = 194) vesicles with a mean diameter of 47.4 nm, which gives 230 (SD = 24) μm of membrane. This figure must be adjusted to correct for errors due to finite section thickness, the "Holmes effect" (31). We calculated a correction factor of 0.33 for synaptic vesicles using an equation for the sphere model (70, 71), and estimating a section thickness of 80 nm from interference color. With this correction, we calculate that control terminals have 76 (SD = 10) μm of synaptic vesicle membrane. Note that the linear measurements so obtained are a morphometric estimate of relative membrane area (22, 70, 73).

RESULTS

Use of HRP as a Tracer of Membrane Retrieval

CONTROLS: After a 30-min incubation in normal Ringer's with HRP in the dark, HRP is observed within synaptic vesicles and blunt-ended tubules in the presynaptic terminals (Fig. 1). In many terminals, a few HRP-labeled cisternae are also present. Included in the "cisternae" category are bodies with three or more times the area of synaptic vesicles, appearing as either rounded or flattened vacuoles or sacs. The cisternae in photoreceptors resemble structures which participate in membrane retrieval in a variety of other nerve terminals.

In the myoid region, HRP is found within multivesicular bodies (MVBs) and lysosomal structures, but not within the rough endoplasmic reticulum, the smooth endoplasmic reticulum (SER) (49), or Golgi-associated sacs.

In preparations fixed and stained by our routine methods, only a few of the vesicles in terminals are "coated;" most often these are still attached to cell surface structures, as if caught in the aid of budding (see Fig. 8). Coating is detectable with moderate frequency on vesicles within the terminal when tannic acid procedures are used (41).

MULTIVESICULAR BODY FORMATION: It is important to determine if our tracer, HRP, has marked effects on the structures and processes studied. In previous work, we failed to find appreciable physiological or morphological effects (60). However, dramatic HRP effects on nerve terminals have been recently claimed to occur (8, 57). In our studies here, retinas incubated in Ringer's with HRP show no differences in the numbers or types of membranous structures present from those incubated without HRP. For example, neither our incubation conditions nor the presence of HRP cause increases in the frequency of degradative structures such as MVBs (Table I; for more details see reference 49) (Subtle effects cannot be ruled out—our concern is with marked changes in the frequency of vesicles, lysosomes, cisternae, and so forth that could mislead us.) MVBs are encountered regularly in frog photoreceptor

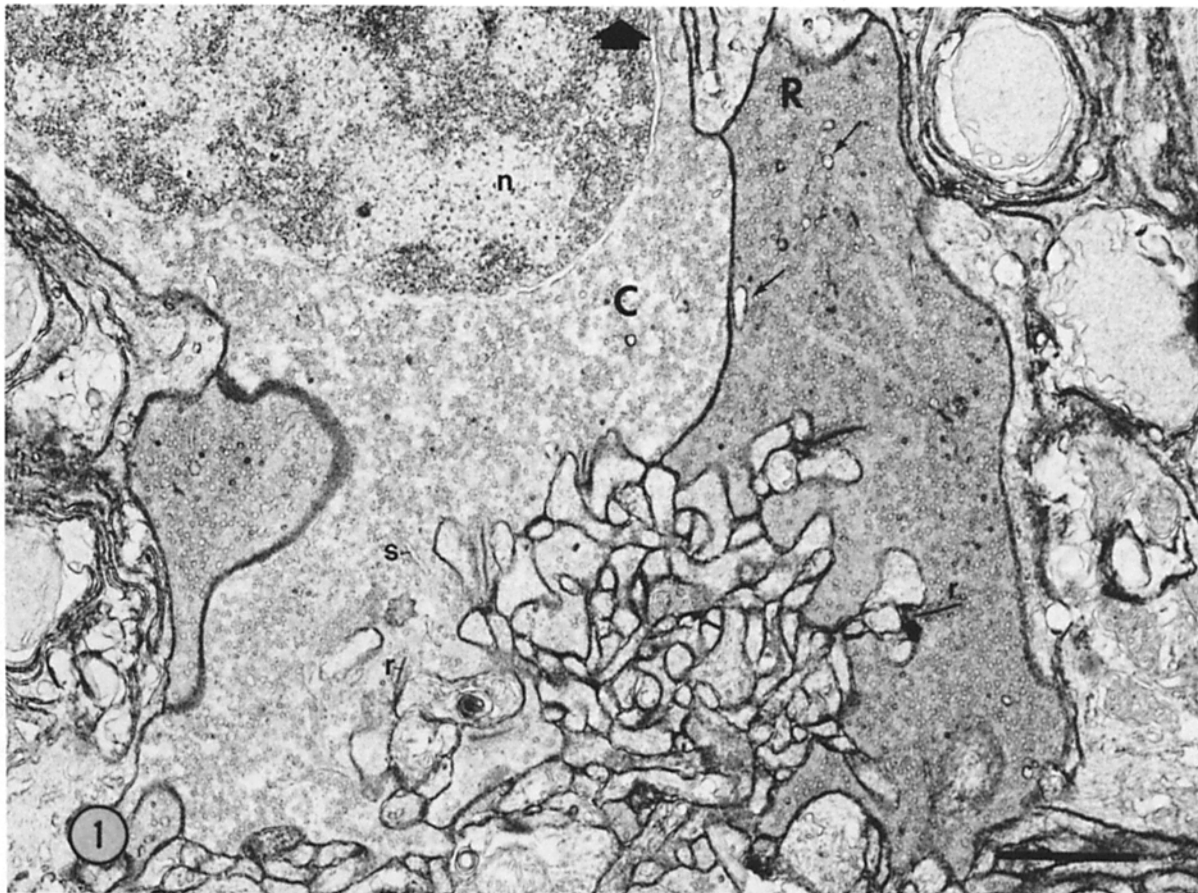


FIGURE 1 Presynaptic terminals of a rod (*R*) and a cone (*C*) from a retina incubated (30 min) in normal Ringer's with HRP in the dark. Both terminals show HRP-labeled vesicles. In the rod, a few tubules and cisternae (small arrows) are also labeled; the SER (*s*) in the cone is not. The cone nucleus is seen at *n* and synaptic ribbons at *r*. The large arrow in this and the following figures points in the direction of the outer segment region of the receptors. Bar, 1 μm. × 18,000.

terminals, in contrast to reports in other systems (61); their numbers are relatively constant within a batch of frogs obtained at a given time and in isolated retinas incubated under varying conditions (49).

COMPARISON OF TRACERS: To further evaluate possible tracer effects on the routes of endocytosis in the terminals, dextrans were employed as extracellular tracers in four experiments (27, 28). Dextran is readily endocytized by the photoreceptors and is seen in the same types of intracellular compartments in terminals as HRP (Fig. 2). After 90 min of incubation, dextran is found in 24.8% (SD = 8) of the synaptic vesicles. Control retinas incubated in normal Ringer's without dextran have dextran-like particles in 3.1% (SD = 2.2) of the synaptic vesicles. However, dextran is an unsuitable tracer for general use with isolated frog retinas under the incubation conditions we employ, as it causes swelling of the rod outer segments and autophagy in the inner segments in most preparations. Attempts to reduce rod damage by decreasing the dextran concentration or dialyzing the dextran solution to remove low molecular weight impurities resulted in little to no uptake.

Effects of Barium

Incubation of the retina in 1.2 mM barium-Ringer's results in the accumulation of cisternae in the rod terminals (Fig. 3). The morphology of the cone terminals is much less altered. Incubation in higher concentrations of barium, up to 5 mM, results in an even more dramatic accumulation of cisternae in the rods (Fig. 4), and the cones begin to exhibit an effect as well. The cisternae in barium preparations resemble those seen in controls, suggesting that we are exaggerating a cycle rather than creating a new one. In fact, cells are occasionally observed in control preparations with "cisternal" numbers approximating those seen with barium.[1]

Table II shows that the number of cisternae in a thin section of a rod terminal increases ~15-fold on exposure to 1.2 mM barium, while the increase in the cones is not significant. The frequency distribution of cisternae for barium-treated rods is quite broad, with most containing 30 to 50 cisternae, while the majority of barium-treated cones, like control cones, have fewer than 10 cisternae (Fig. 5).

Cisternae are seen in rods incubated in barium-Ringer's both with and without HRP. When retinas are incubated with HRP,

[1] In 2 out of 19 barium experiments, the control retinas included many rods with appreciable numbers of vacuoles in their terminals. We excluded these experiments from our analysis (although their inclusion would not have significant effect on our conclusions) since we are still not sure of the nature of these vacuoles. This occasional occurrence of extensively vacuolated control retinas has been noted in other experimental series in our laboratory. As yet, we are unable to ascribe this phenomenon to the batch of frogs, seasonal changes, or contamination of the incubation media. We are interested in the phenomenon since it may represent an effect on membrane circulation similar to the ones being analyzed in the present paper.

Table I
Frequency of Multivesicular Bodies in Terminals

	Overall	Rods	Cones
No incubation*	10.3 (1.5)	14.3 (2.2)	5.4 (0.8)
90 min, dark	8.4 (0.6)	13.7 (2.5)	1.8 (0.6)
90 min, light	7.5 (1.5)	13.4 (3.6)	3.7 (0.8)
90-min incubation, dark‡			
No HRP	12.8 (1.5)	18.0 (3.2)	7.0 (0.7)
HRP present	10.8 (1.2)	15.6 (3.0)	4.9 (3.0)
Light/dark cycle§			
Condition at time of fixation			
Light	6.2 (0.6)	7.1 (1.3)	4.7 (0.6)
Dark	6.5 (0.9)	7.9 (1.8)	4.7 (0.4)
No cycle	6.7 (1.4)	7.0 (1.6)	6.0 (2.0)

Multivesicular body frequencies were determined for the presynaptic terminals of the photoreceptors for two to three experiments. The numbers represent the mean (mean deviation) of the percentage of terminals in a thin section that show a MVB. For each data point, 150–200 teminals per frog were examined.

* Comparison between retinas fixed directly after isolation and retinas incubated for 90 min in the dark or under moderate intensity light.

‡ Comparison between retinas fixed after a 90-min incubation in normal Ringer's with or without HRP present.

§ Comparison between frogs on a 12-h light/12-h dark cycle, and frogs kept in constant darkness for 3 d, then fixed. For the frogs that were cycled, retinas were fixed at the end of a light period and at the end of a dark period.

75%–90% of the cisternae are labeled. Levels of HRP uptake into synaptic vesicles in barium-treated retinas are comparable to controls (Table III). Therefore, the total number of endocytically derived compartments labeled with HRP, synaptic vesicles plus cisternae, is much greater in barium-exposed rods than in control rods.

The cisternae are distinguishable from the SER present in the rod and cone terminals and the rod axons (Fig. 6 and reference 49) which does not acquire HRP under any of our incubation conditions.

Morphometric analysis, illustrated in Tables IV and V, provides evidence that the cisternae are formed of synaptic vesicle membrane. Barium-exposed rods have 52% fewer synaptic vesicles than control rods and the amount of vesicle membrane lost approximates the gain in cisternal and plasma membrane. The increase in plasma membrane length is visible in some preparations as an increase in the finger like invaginations of postsynaptic processes, and as infoldings of plasma membrane.

As is true in neuromuscular junctions (30), cisternae are seen with vesicles attached as if caught in the act of budding or fusing (Fig. 4). Cisternae also are seen associated with synaptic ribbons (Fig. 7).

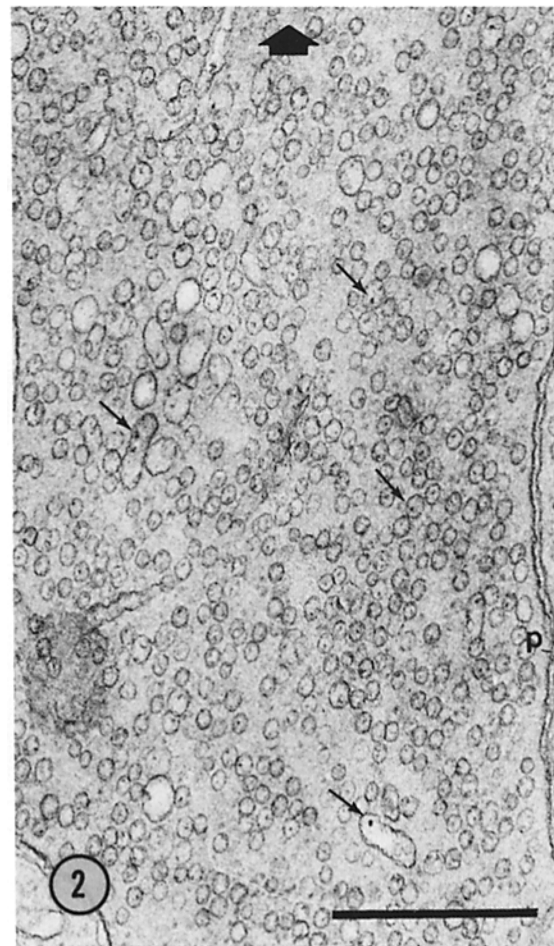


Figure 2 Portion of a rod terminal from a retina incubated (90 min) in normal Ringer's with 10% dextran (10,000 mol wt). Small arrows point to dextran particles within synaptic vesicles and cisternae. The plasma membrane is indicated by (p). Bar, 0.5 μm. × 53,000.

What Is Barium Doing?

Barium is reported to have several effects on ion balances and membrane activity of neurons and other cells. We selected for study a number of possible actions of barium. These are: depolarization, inhibition of the Na/K ATPase, calcium like or calcium-competitive action, and blockade of potassium channels.

Depolarization? We attempted to increase synaptic activity and induce the appearance of cisternae by incubating the retinas in elevated potassium (39, 55) 30 min of incubation in high potassium Ringer's does result in substantially increased uptake of HRP into synaptic vesicles in both rod and cone terminals (Fig. 8, Table III); there was, however, no unusual accumulation of cisternae or depletion of synaptic vesicles. Networks of HRP-labeled tubules distinct from the SER as seen in Fig. 8 are normal components of the terminals (16).

Inhibition of Na-K ATPase? Drugs such as ouabain that inhibit the Na/K ATPase also increase trasmitter release (69). Since barium can inhibit the ATPase, the effects of barium were compared to the effects of incubation in ouabain to determine if ATPase inhibition might contribute to the accumulation of cisternae. Incubation in 100 μM ouabain results in substantially increased HRP uptake into synaptic vesicles of both rods and cones (Table III), but no accumulation of cisternae.

Calcium-like or -competitive actions? Barium could be interfering with the available concentration or action of ions, such as calcium, that might serve as endocytic control factors. We find that raising the calcium concentration in normal Ringer's to 8.4 or 10 mM has no consistent effect on cisternae formation. Neither does raising calcium concentrations in the presence of high potassium, which should open more "calcium gates" and allow more calcium to enter. In two out of nine retinas, 50%–60% of the rod cells in preparations incubated with elevated calcium did show higher-than-control numbers of cisternae but, interestingly, up to 75% of these cisternae showed no HRP. The remaining retinas incubated in

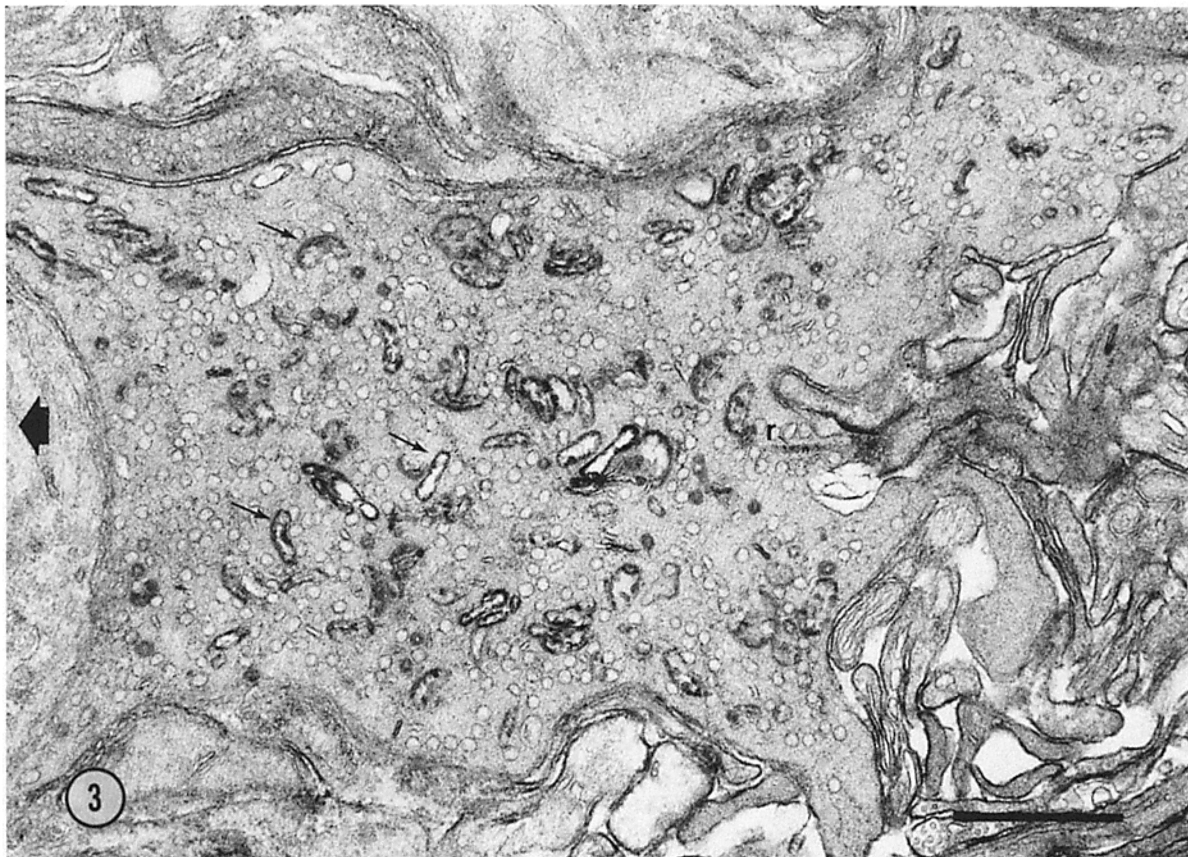


FIGURE 3 Rod terminal from a retina incubated (30 min) in 1.2 mM barium-Ringer's with HRP in the dark. Numerous HRP-labeled cisternae are seen (small arrows). A synaptic ribbon is present at *r*. Bar, 0.5 μm. × 43,500.

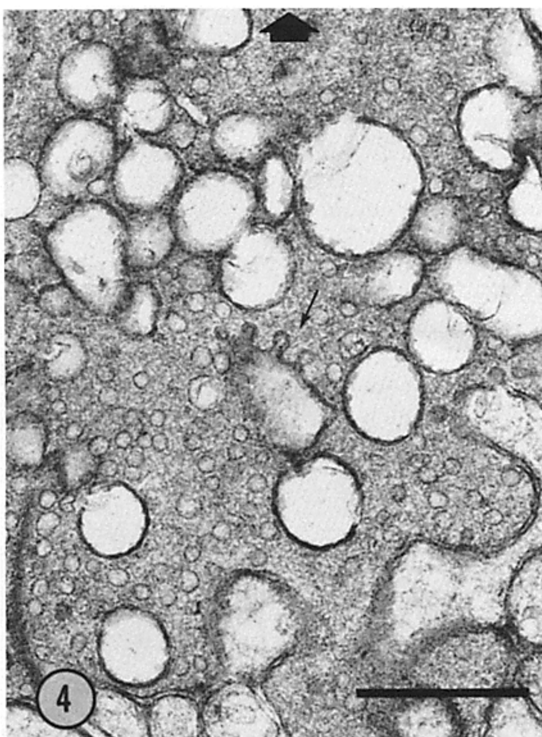


FIGURE 4 Portion of a rod terminal from a retina incubated (15 min) in 5 mM barium-Ringer's with HRP in the dark. One of the numerous HRP-labeled cisternae (small arrow) has vesicles attached. Bar, 0.5 μm. × 43,400.

TABLE II

Numbers of Cisternae/μm²

	Rods	Cones
Normal	0.2 (0.2)	0.2 (0.2)
Barium	2.9 (1.8)	0.4 (0.3)*
Normal	0.7 (0.5)	0.5 (0.4)
TEA	2.8 (1.1)	0.7 (0.5)*

Retinas incubated in normal Ringer's were compared with ones incubated in 1.2 mM barium or 20 mM TEA. Method of selecting cells is given in Materials and Methods. All sacs larger than three times the area of a synaptic vesicle were counted. The mean (SD) number of cisternae in each population of cells was divided by the mean terminal area to obtain a cisternae number/μm². Numbers represent the mean (SD) for two to three experiments, where the *n* per point per experiment was 40–60 terminals. * Indicates that the increase is not statistically significant for $P = 0.05$ using Student's *t* test.

Ringer's with elevated calcium were indistinguishable from control retinas.

Incubation in calcium-free Ringer's with 2 mM EGTA reduces HRP uptake into vesicles of both rods and cones from 4–6% in controls to <1%. No cisternae are induced. However, 1.2 mM barium added to calcium-free Ringer's engenders the formation of cisternae.

Trifluoperazine (Stelazine), a calmodulin antagonist does not consistently engender cisternae when retinas are exposed to concentrations of 10 or 25 μM in normal Ringers.

BLOCKADE OF POTASSIUM CHANNELS? Barium has been shown to block the potassium channels in numerous systems, including the toad rod (17). When retinas are incubated in 20 mM TEA, a pharmacological agent that blocks the potassium channels, the effects of barium are mimicked: cister-

nae accumulate in the rod terminals (Fig. 9), while the cone cells are much less affected (Fig. 10).

The cisternae that form with TEA treatment are very similar in appearance to those that form with barium, and ~80% are labeled with HRP. Table II shows that the number of cisternae in rods increases four- to fivefold with TEA treatment. The frequency distribution in Fig. 10 indicates that the change in the number of cisternae with TEA is similar to, but less pronounced than that with barium. Morphometric analysis, illustrated in Tables IV and V, was qualitatively similar to the barium analysis, and the depletion of synaptic vesicles in the rods is balanced by the gain in cisternal and plasma membrane. As with barium, HRP uptake into synaptic vesicles is not significantly different from controls (Table III), but the total amount of endocytized membrane is much greater. Incubation in 5 mM 4-aminopyridine, another potassium channel blocker, does not consistently engender formation of cisternae.

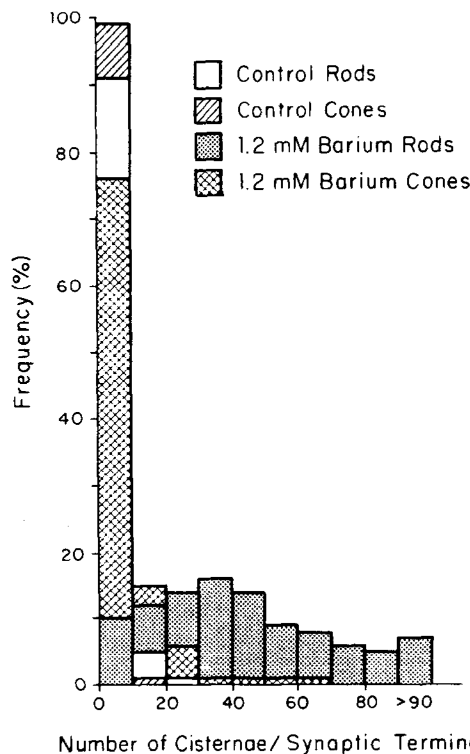


FIGURE 5 Frequency distribution illustrating the numbers of cisternae per synaptic terminal in rod and cone terminals incubated (15′ and 30′) in normal Ringer's (control) or in 1.2 mM barium-Ringer's. Incubation for 15 or 30 min resulted in similar numbers of cisternae; therefore, data for both times were combined in this figure. Each bar represents the relative frequency of terminals found with the indicated numbers of cisternae. The bar heights correspond to the average frequency of each category (percent of total population of terminals) observed in 7-10 experiments; *n* per experiment was 60-90 terminals.

BARIUM, TEA, AND POTASSIUM: If barium and TEA are interacting with the potassium channels perhaps they could be cleared by raising the extracellular potassium concentration. We find that when retinas are incubated in 1.2 mM barium or 20 mM TEA in a high potassium Ringer's, HRP uptake is increased in both rods and cones (Table III), but the numbers of cisternae are reduced to control levels. Figs. 11 and 12 show the effect of high potassium on the frequency distribution of cisternae in barium-exposed and TEA-exposed retinas. Numbers of cisternae are also reduced, though not quite to control levels, in retinas incubated in higher concentrations (5 mM) barium added to high potassium Ringer's (Fig. 13).

BARIUM, TEA, AND LIGHT: Do cisternae form when the cells are not synaptically active and hence are not circulating membrane between their synaptic vesicle population and the plasma membrane? High intensity light hyperpolarizes the rod cells and markedly depresses HRP uptake into rod synaptic vesicles. Uptake into cones is less affected (60). We find that high intensity light suppresses the accumulation of cisternae in most rod cells incubated in barium and TEA media (Figs. 14 and 15). The rod cells resemble those incubated in light with normal Ringer's. The cone cells resemble controls in morphology and HRP uptake (Table III). Moderate intensity light does not suppress the formation of cisternae in barium-treated rod cells (Fig. 16).

BARIUM, TEA, AND COBALT: Cobalt can block the calcium channels and, presumably as a result of this, it can depress membrane cycling and the uptake of HRP. We previously found (15) that 5 mM cobalt blocks uptake in rods and cones,

TABLE III
HRP Uptake into Synaptic Vesicles of Rod and Cone Terminals

	Rods		Cones		
Condition	Experimental	Control	Experimental	Control	n
Potassium	22.2 (5.2)	5.8 (2.4)	21.8 (5.6)	4.2 (1.8)	9
Barium	5.0 (2.2)*	5.3 (2.5)	4.3 (1.9)*	3.7 (1.8)	6
TEA	6.7 (2.1)*	4.6 (1.6)	6.2 (2.6)*	3.4 (1.1)	2
Ouabain	14.1 (4.6)	2.4 (1.0)	13.7 (3.9)	2 (0.9)	2
	Experimental	Barium	Experimental	Barium	
Ba + potassium	17.3 (4.2)	5.1 (2.6)	16.7 (4.7)	5.0 (2.0)	2
Ba + moderate light	3.0 (1.7)*	5.4 (2.2)	3.5 (1.7)*	4.4 (1.3)	2
Ba + high light	0.5 (0.5)	3.3 (1.1)	3.2 (1.1)*	3.6 (1.2)	3
Ba + 5 mM Co	1.7 (1.3)	4.5 (1.5)	3.3 (1.3)*	3.6 (1.4)	2
	Experimental	TEA	Experimental	TEA	
TEA + potassium	18.9 (4.2)	6.7 (2.1)	17.2 (4.5)	6.2 (2.6)	2
TEA + high light	0.6 (0.7)	4.3 (1.6)	1.3 (0.8)*	2.4 (1.1)	2
TEA + 5 mM Co	0.3 (0.5)	4.2 (1.5)	1.4 (0.9)*	2.6 (1.2)	3

Retinas incubated under the experimental conditions indicated were compared to retinas incubated in normal Ringers (control), 1.2 mM barium, or 20 mM TEA. Numbers represent the mean (SD) of the percentage of vesicles showing HRP. *n* is the number of experiments (the *n* per data point per experiment was 20-90 terminals). * Indicates that the difference is not statistically significant for $P = 0.05$ using Student's *t* test.

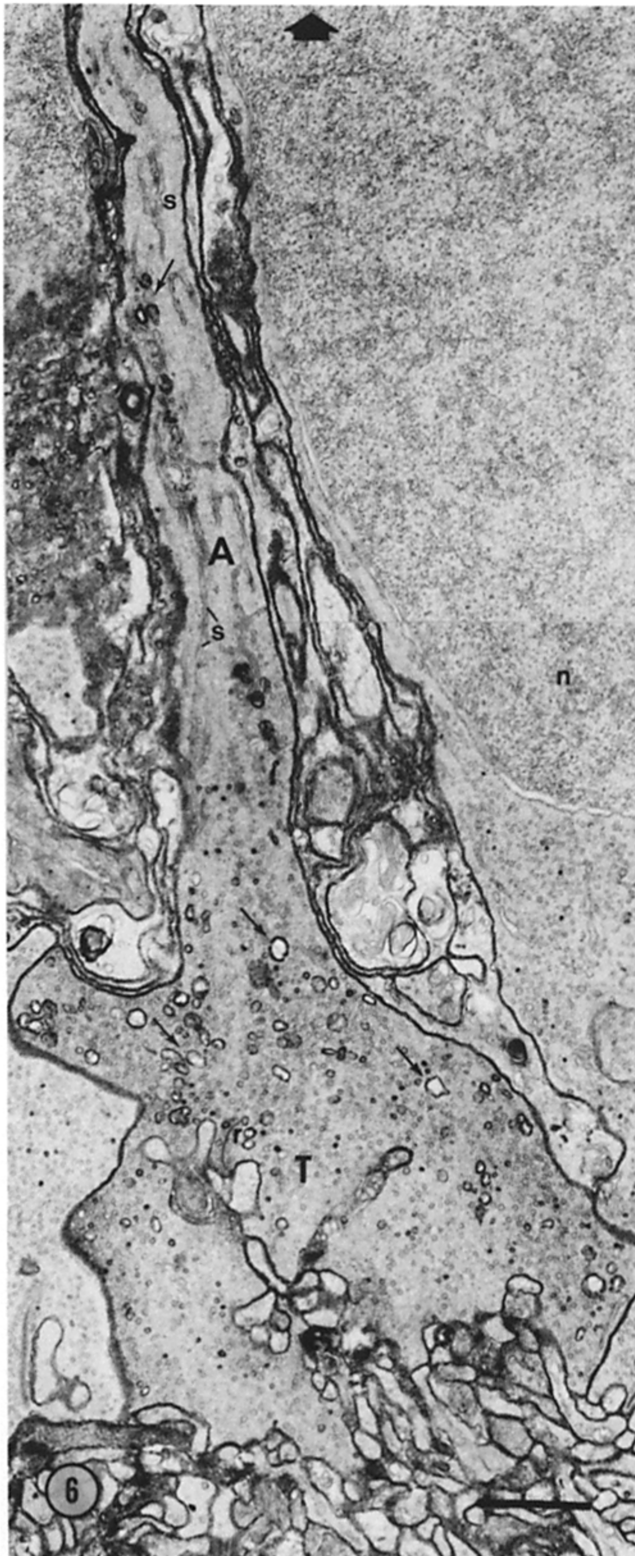


FIGURE 6 Rod axon (*A*) and terminal (*T*) from a retina incubated (30 min) in 1.2 mM barium-Ringer's with HRP in the dark. Cisternae (small arrows) are labeled with HRP, while the SER (*s*) is not. A synaptic ribbon is seen at *r*, and the nucleus of an adjacent cone at *n*. Bar, 1 μm. × 15,400.

while lower levels, like moderate intensities of light, differentially block rods. Addition of 5 mM cobalt to barium-Ringers or TEA-Ringers reduces HRP uptake and the number of cisternae in rod cells (Table III, Figs. 17 and 18).

TABLE IV
Vesicle Density

	Rods	Cones
	No. of vesicles/μm²	
Normal	117.1 (14.7)	131.1 (18.1)
Barium	56.4 (16.9)	121.0 (15.8)
Decrease	51.8%	7.7%*
Normal	123.4 (14.1)	130.9 (14.7)
TEA	91.8 (15.0)	114.4 (15.2)
Decrease	25.6%	12.6%*

Retinas incubated in normal Ringer's were compared with ones incubated in 1.2 mM barium or 20 mM TEA. For each cell, three regions of 0.5 μm^2 were randomly selected, and the vesicles counted. Numbers represent the mean (SD) for two to three experiments, where the *n* per point per experiment was 40-60 terminals. * Indicates that the decrease is not statistically significant for $P = 0.05$ using Student's *t* test.

2 mM cobalt added to 1.2 mM barium-Ringer's is not effective in reducing the formation of cisternae (data not shown) suggesting that barium and cobalt effects compete to some extent.

Electrophysiology

The electroretinogram from a 20 mM aspartate-isolated retina, termed the receptor potential, is a measure of the summed electrical activity of the rods and cones (38). The effects of barium on the rod and cone receptor potentials were tested by measuring the spectral sensitivity of the retina. The relative intensity, measured as the log of relative number of quanta, required to elicit a 20 μV criterion response at various wavelengths was determined, before and after application of 1.2 mM barium in Ringer's. We find that applying 1.2 mM barium Ringer's has a relatively small effect (cf. reference 38); it causes a loss of ~1 log unit of sensitivity in both the rod and the cone receptor potentials. This decrement is only slightly larger than that produced by applying control Ringer's which engenders a loss of ~0.5 log units of sensitivity.

DISCUSSION

Membrane Retrieval

We previously showed that dark-adapted frog photoreceptors take up extracellular tracers into synaptic vesicles, endocytic vacuoles, tubules and sacs ("cisternae"), and lysosome-related compartments and that this uptake is reduced in response to illumination (59, 60). Here, we report that the addition of modest concentrations of barium or TEA to normal Ringer's changes the usual balance of membrane circulation in the rod cells, causing synaptic vesicle membrane to be retrieved largely in the form of cisternae.

These results are of interest from two viewpoints. First, we have altered the pathway of membrane retrieval by agents expected to affect the ionic balances of the cell. The alterations in membrane retrieval that we find bear on the multiple pathways of membrane cycling (18, 34). They may open useful experimental approaches for analysis of the retrieval process. Second, these findings extend our earlier ones demonstrating significant differences between rod and cone terminals in their responses to divalent cations (15, 45, 46). While the functional differences of rods and cones have been intensively analyzed and the differences at the outer segment studied, much remains unclear regarding the differences between these major photoreceptor types.

TABLE V
Morphometric Analysis of Control and Experimental Terminals

	Rods			Cones		
	Control	Barium	(Ba-Control)	Control	Barium	(Ba-Control)
Area (μm^2)						
Terminals	13.2 (4.5)	13.2 (4.3)	0.0*	11.0 (4.3)	10.0 (4.6)	−1.0*
Membrane length (μm)						
Plasma membrane	28.4 (7.2)	35.4 (13.0)	+7.0*	24.9 (13.2)	23.3 (8.7)	−1.6*
Cisternae	1.0 (1.4)	20.0 (12.4)	+19.0	0.8 (0.9)	1.8 (1.8)	+1.0*
Synaptic vesicles	76.8 (10.0)	37.0 (11.0)	−39.8	71.6 (10.0)	61.2 (9.0)	−10.4
	Control	TEA	(TEA-Control)	Control	TEA	(TEA-Control)
Area (μm^2)						
Terminals	12.0 (4.1)	13.1 (4.5)	+1.1*	11.3 (4.8)	13.0 (4.0)	+1.7*
Membrane length (μm)						
Plasma membrane	26.9 (7.3)	27.4 (8.8)	+0.5*	23.7 (8.7)	26.0 (8.4)	+2.3*
Cisternae	3.3 (2.4)	13.8 (5.7)	+10.5	1.9 (1.4)	3.5 (2.2)	+1.6*
Synaptic vesicles	73.5 (8.3)	59.7 (9.7)	−13.8	73.5 (8.4)	73.9 (9.8)	+0.4*

Retinas incubated in control Ringers were compared with ones incubated in 1.2 mM barium or 20 mM TEA. Methods of selecting cells, making measurements, and calculating area and membrane length are given in Materials and Methods. Numbers represent the mean (SD) for two to three experiments, where the n per point per experiment was 40–60 terminals. * Indicates that the difference is not statistically significant for $P = 0.05$ using Student's t test.

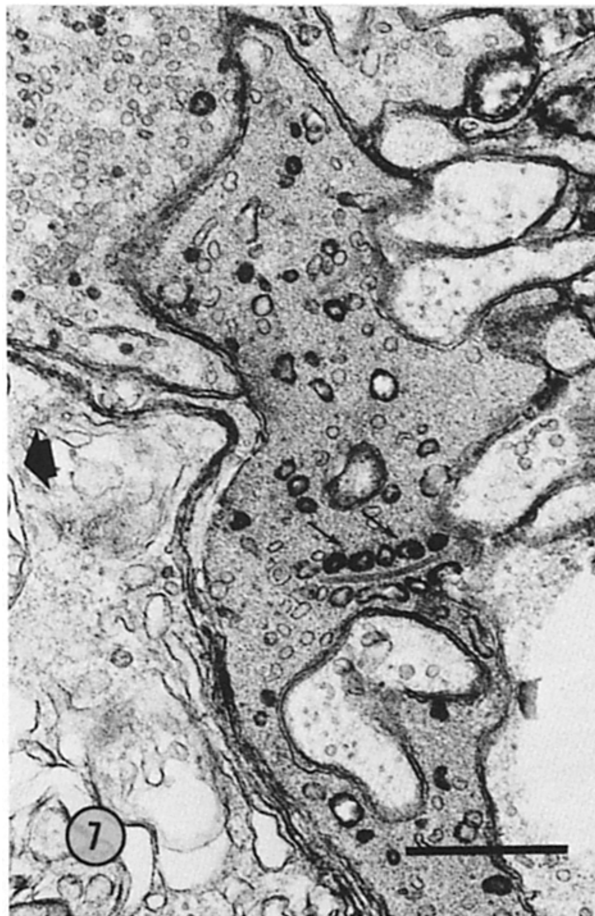


FIGURE 7 Portion of a rod terminal from a retina incubated (30 min) in 5 mM barium-high potassium Ringer's with HRP in the dark. Arrows point to small cisternae aligned along the synaptic ribbon. Bar, 0.5 μm. × 41,300.

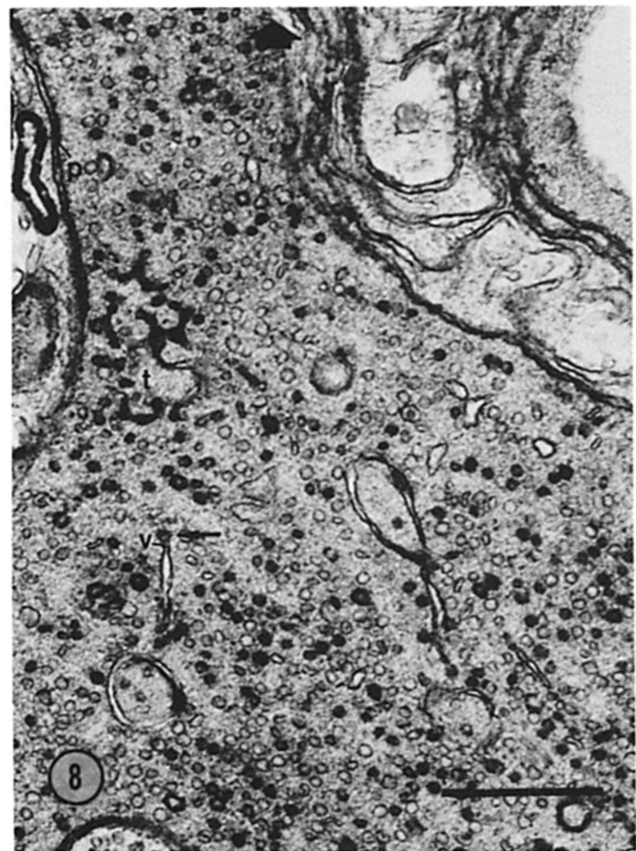


FIGURE 8 Portion of a rod terminal from a retina incubated (30 min) in high potassium Ringer's with HRP in the dark. Numerous synaptic vesicles are labeled with HRP. A network of HRP-labeled tubules is indicated by *t*. The plasma membrane is indicated by *p*. Note the vesicle—a coated vesicle (arrow)—apparently budding from a flat cisterna at *v*. Bar, 0.5 μm. × 44,100.

Formation of Cisternae

Cisternae were initially detected in terminals studied at low temperatures or with very high transmission rates (30, 47, 51, 61). Our observations and those by others in synaptosomes (19), chick photoreceptors (9), hair cell terminals (24), and neurosecretory cells (4, 52, 66) strongly suggest that at least some synaptic vesicle retrieval under normal conditions is also mediated by large sacs, tubules, and vacuoles (see Fig. 8). Many of these larger structures probably form directly from

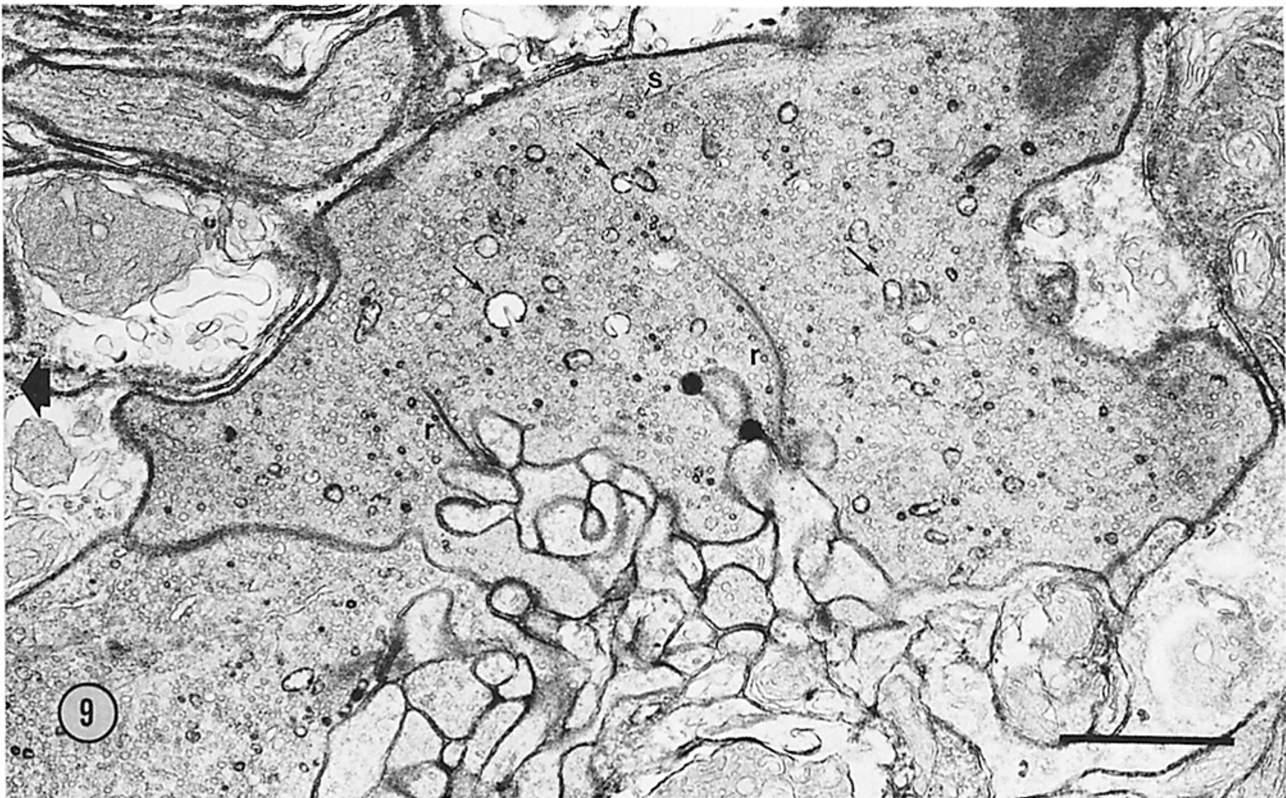


FIGURE 9 Rod terminal from a retina incubated (30 min) in 20 mM TEA-Ringer's with HRP in the dark. Small arrows point to HRP-labeled cisternae; SER (*s*) is unlabeled. Synaptic ribbons are seen at *r*. Bar, 0.5 μm. × 25,200.

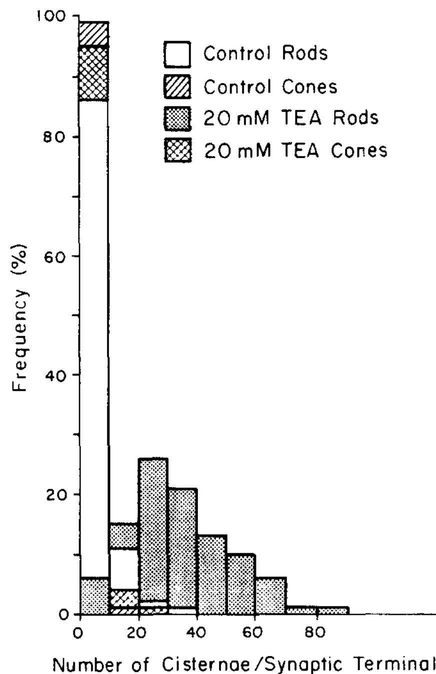


FIGURE 10 Frequency distribution illustrating the numbers of cisternae per synaptic terminal in rod and cone terminals incubated (15 and 30 min) in normal Ringer's (control) or in 20 mM TEA-Ringer's. As in Fig. 5, the bar heights represent averages (in this case, from three to five experiments; the *n* per experiment was 60 terminals).

the cell surface and some may retain long-term continuity with the surface (24, 50, 58).

Since 75%–90% of the cisternae in our preparations are labeled with HRP, we believe they originate from endocytized membrane, though conceivably they receive additional contributions from synaptic vesicles fusing directly with them. Most are no longer continuous with the cell surface. Morphometric analysis indicates that the amount of cisternal membrane that accumulates can be accounted for by the amount of synaptic vesicle membrane that disappears. A reasonable interpretation of these results is that after barium treatment, synaptic vesicle membrane that has fused exocytotically with the plasma membrane is endocytized in the form of cisternae, rather than as smaller vesicles. It is an open question as to what proportion of retrieval in photoreceptors is normally mediated in this way. For one thing, the cisternae normally may rapidly generate new vesicles as they can in photoreceptors (61) and other neurons (30). For another, in a number of cases (19, 50) the retrieval structures larger than synaptic vesicles are only a few times the volume of the vesicle and are apt to have been overlooked in pertinent studies. Therefore, while the most likely explanation of our findings is that barium is interfering with the processing of retrieved membrane into synaptic vesicles we cannot determine whether the events affected by barium normally occur at the time of membrane internalization or subsequent to internalization.

Fate of Cisternae

Just as the origin of different retrieval structures may be varied, so may the fate of such structures. It is not clear that the membrane retrieved in cisternae inevitably gives rise to new vesicles, as some may be slated for degradation via retrograde transport and inclusion in lysosomes (see references 33, 34, 36). The ultimate fate of cisternae in our barium preparations is unknown. We are currently trying to reverse the

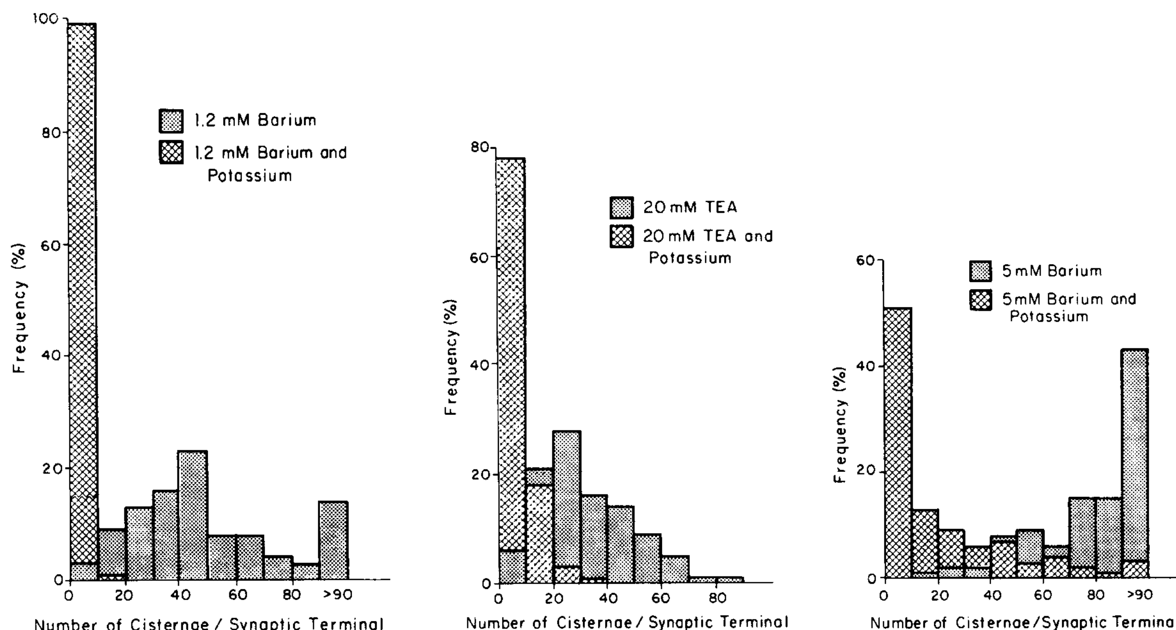


FIGURE 11-13 Frequency distributions illustrating the numbers of cisternae per synaptic terminal in rod terminals incubated (30 min) in 1.2 mM barium-Ringer's or 1.2 mM barium-high potassium Ringer's (Fig. 11), (15 and 30 min) in 20 mM TEA-Ringer's or in 20 mM TEA-high potassium Ringer's (Fig. 12), and (15 and 30 min) in 5 mM barium-Ringer's or 5 mM barium-high potassium Ringer's (Fig. 13). As in Figure 5, the bar heights in each figure represent averages (in these cases, from 2-3 experiments; the *n* per experiment was 60-90 terminals).

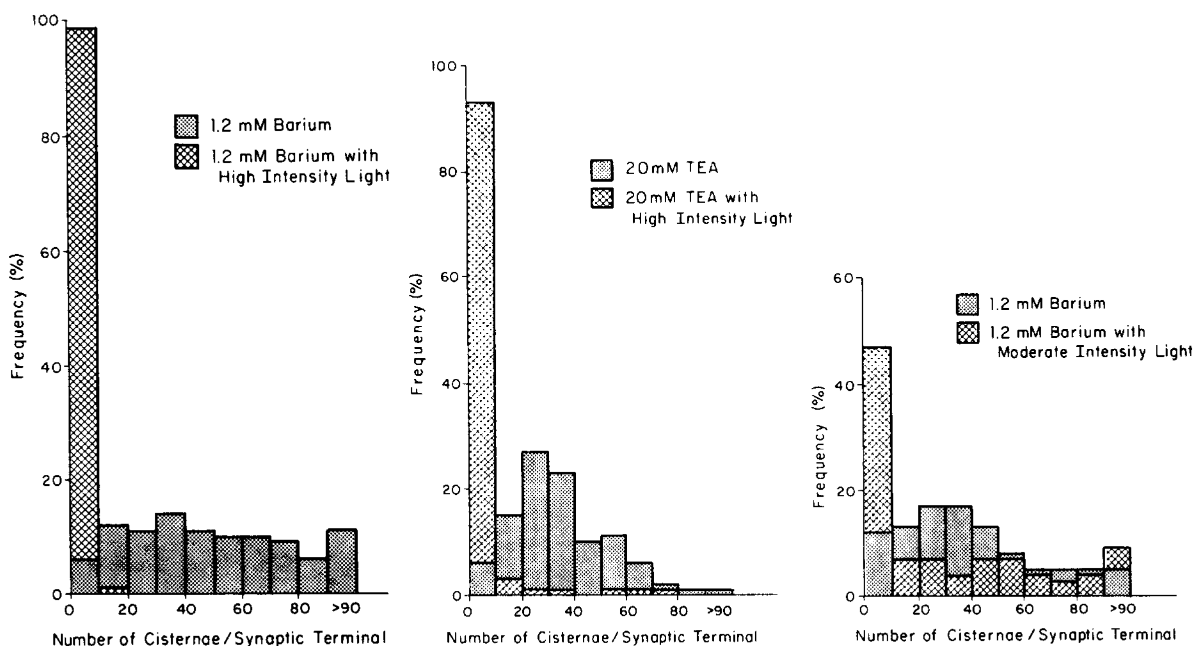
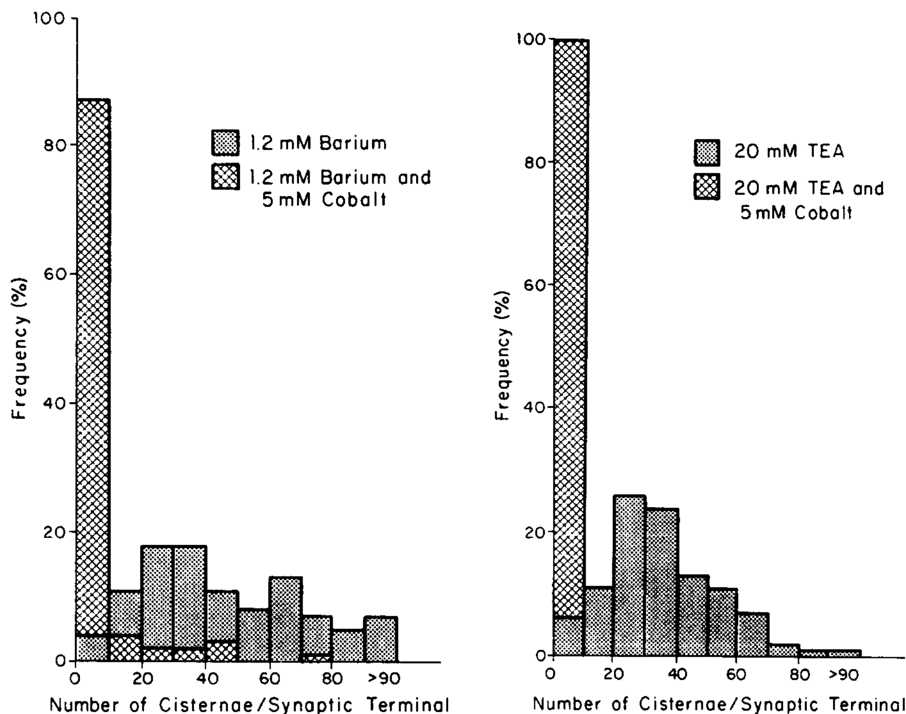


FIGURE 14-16 Frequency distributions illustrating the numbers of cisternae per synaptic terminal in rod terminals incubated (15 and 30 min) in 1.2 mM barium-Ringer's (Fig. 14) or (15 and 30 min) in 20 mM TEA-Ringer's (Fig. 15) in the dark or in high intensity light, and (15 and 30 min) in 1.2 mM barium-Ringer's (Fig. 16) in the dark or in moderate intensity light. As in Fig. 5, the bar heights in each figure represent averages (in these cases, from two to three experiments; the *n* per experiment was 60 terminals).

formation of cisternae in our barium preparations by washing out the ions. Unfortunately, the retina does not withstand the manipulations involved very well and a more elaborate perfusion-based experimental system may be required. As outlined below, our observations do suggest that some cisternae are transported up the axon, like similar structures in other neurons (33, 36).

That direct rerelease of large vesicles or cisternae could occur under some circumstances is suggested by the observations of Heuser (28) who noted large vesicles in frog neuromuscular junctions coincident with the occurence of "giant" miniature end-plate potentials. These cisternal-like structures might be postulated to fuse with the plasma membrane and release neurotransmitter, producing the giant potentials. In rod terminals, cisternae are found associated with synaptic ribbons (Fig. 7) where vesicles "awaiting" release normally accumulate (60). This seemingly indicates that cisternal membrane is recognized by the ribbon as vesicle membrane and could conceivably point towards exocytotic release of cisternal contents.

One important point to recall is that the fate of retrieval



FIGURES 17 and 18 Frequency distributions illustrating the numbers of cisternae per synaptic terminal in rod terminals incubated (30 min) in 1.2 mM barium-Ringers (Fig. 17) or (15 min) in 20 mM TEA-Ringer's (Fig. 18), alone or with 5 mM cobalt added. As in Fig. 5, the bar heights in each figure represents averages (in these cases, from two to three experiments; the *n* per experiment was 60 terminals).

structures is likely to differ substantially in different nerve terminals. Neurosecretory cells perhaps represent one extreme since direct local reuse of membrane for hormone storage is unlikely (4, 36, 52, 66). Neuromuscular junctions, with their very high rates of transmission which requires rapid reformation and reuse of membrane, may fall at the opposite extreme (7, 29, 30, 47).

Membrane Circulation

One can think of a number of ways in which cisternal retrieval might be important in the cells we study and in others. For example, it might permit selective intracellular sorting out of the components destined for different fates—reuse or degradation. This would be in accord with speculation about lysosomal activities (18, 34) and with schemes for adsorptive endocytosis put forth to explain recycling of cell surface receptors (53). In the case of nerve terminals, large intermediate structures could also serve to reaccumulate some of the components lost during exocytosis, perhaps not the transmitters themselves but other ions and macromolecular components. Molecules or structures coming down the axon might interact with the cisternae and replenish such components, and correspondingly the cisternae might interact in a variety of ways with the surrounding axoplasm. We are, for example, intrigued by the fact (68) that in photoreceptors, structures resembling cisternae are among the chief sites where we find active accumulation of calcium in terminals and that calcium binding is evident in the synaptic vesicles.

Like other endocytic structures, cisternae are seen in rod axons (Fig. 6), maintaining their distinctness from the ER (see references 36, 47, 49 for discussion). Since rod axons show little evidence of engaging appreciably in endocytosis, we believe that the cisternae in axons are being transported in the retrograde direction—up the rod axons from the synaptic terminals (60).

In our study as in past ones (36), we looked for involvement of the SER in the recycling mechanism by searching for tracer in the SER under various conditions. No such tracer was detected, even when the cells were depolarized with potassium producing increasing HRP uptake or when the frequency of tubules and cisternae was increased as with barium or TEA treatment. In line with findings in other neurons (40), tracers that bind firmly to the plasma membrane, such as cholera toxin, do enter certain compartments in photoreceptors that are not markedly labeled by HRP, notably Golgi complex-associated sacs (48), as well as entering compartments accessible to HRP. Even such tracers are not seen in the SER. Nonetheless, if the SER does contribute to axonal transport of synaptic vesicle components, as seems very likely, (reviewed in references 36 and 49), the vesicles that bud from the SER (49) could conceivably feed components into the cycling synaptic vesicle population by fusing with cisternal like intermediates in a manner reminescent of Golgi apparatus-secretory vacuole relations (18).

Barium's Mechanism of Action

What is the basis of our ability to produce alterations in retrieval by changes in ionic balance? The effects of barium might simply reflect interaction of this ion with sites normally influenced by calcium that participate in membrane fissions or related processes of vesicle formation. As already mentioned, calcium does seem to be required for membrane retrieval (6, 44). There also is ample precedent from studies of exocytosis for divalent cations affecting membrane fission/fusion events (reviewed in reference 43). On the other hand, experiments with monensin offer possible precedent for effects of *monovalent* ions on relevant membrane behavior. Monensin, a monovalent ion ionophore, causes rapid dilation of the Golgi apparatus (65). It is postulated that monensin-induced high sodium, low potassium conditions (or perhaps, changes in H^+ balances) arrest membrane fission while allowing continued fusion of ER-derived membrane with the Golgi apparatus. (What may be a comparable ion-sensitive step in transport has been localized to the Golgi apparatus in frog spinal neurons [25]. In this case, the transfer of glycoproteins from the Golgi system to the fast axonal transport was found to be a calcium-dependent,

cobalt-sensitive step.) Could there be points of similarity between the impact of changes in monovalent ion balances on processes in nerve terminals that normally lead to synaptic vesicle formation from recycled membrane, and the impact on Golgi apparatus-associated packaging of secretions in membrane delineated bodies (which can also involve recycled membrane [18])? Are there ion pumps or carriers in the membranes whose functioning generates conditions needed for membrane reorganizations like fission of vesicles? This speculation is triggered by the analogies between packaging of secretions and production of synaptic vesicles and by the fact that synaptic vesicles do seem to maintain ionic gradients such as pH differentials (see reference 36 for references), as well as by notions that osmotic effects may help account for features of exocytic membrane fusions (75).

Detailed interpretation of our data in terms of ionic mechanisms is rendered difficult by the fact that the agents we use have multiple effects, and the cells themselves engage in numerous ion-sensitive processes. High potassium for instance does not simply depolarize the plasma membrane. Via the depolarization it also alters calcium distributions in the cell. Commercially available TEA has very recently been reported possibly to be contaminated to a small extent with triethylamine which can perturb pH and calcium buffering (77; even if so, at the TEA concentrations we use this seems likely to be of at most quite minor import). For such reasons, in this section we can only sketch outlines; details will require much more elaborate studies than those we have done so far.

Our results with barium, TEA, and high potassium, alone and in combination, strongly suggest that the effects we see on membrane circulation result from blockade of potassium channels. Barium has been shown to interfere with potassium conductance in muscle fibers (23, 64, 72) and pace-maker neurones (26), among other cell types. In squid giant axon, internally or externally applied barium blocks the potassium channels and stabilizes their closed conformation (3, 14).

The ability of barium or TEA to affect retrieval is inhibited by high extracellular potassium. Among other conclusions, this result suggests that barium is not simply engendering some general "toxic" effects. One explanation for the countervailing influence of high potassium is that potassium relieves a blockade by competing with barium or TEA for the potassium channel. However, it is still unclear if barium or TEA are actually occupying a site in the potassium channel or if their effect on potassium conductance is indirect. For example, these ions may either be acting extracellularly, or they may be endocytized and exert an effect from within circulating membranous compartments.

The formation of cisternae is also blocked by concurrent exposure of the retina to high intensity light or 5 mM cobalt. These results could signify that barium must gain entry into the cells through calcium channels to exert its effects. Cobalt, or hyperpolarization due to light, would prevent entry by closing the calcium channel, thus prevent barium from taking action. Alternatively, the fact that cisternae do not form could simply be due to the blocking of membrane cycling by cobalt (15) or light (59, 60), so that there is no retrieval going on for barium to affect. This last possibility could most readily account for the ability of light or cobalt to prevent TEA from engendering cisternae. TEA is too large to cross the plasma membrane and probably exerts its effects extracellularly, as it does at the frog node of Ranvier (2).

Moderate intensity light does not suppress the formation of cisternae, although it has been shown to reduce synaptic activity in rod cells from retinas incubated in normal Ringers (60). Perhaps the hyperpolarization caused by moderate intensity light is partially overcome by the depolarization caused by barium (5). Similarly, 2 mM cobalt does not prevent formation of cisternae. This may reflect competition between cobalt and barium for specific channels, perhaps the calcium channels.

In theory, barium or TEA could have an impact on the modulation of intracellular calcium concentration. This might, for instance, be a secondary result of potassium channel blockade. In photoreceptor cells of toad (17), giant barnacle (56), and scallop (10), barium and TEA are reported to produce calcium-dependent regenerative potentials. In addition, barium is found to have electrophysiological effects similar to calcium in several systems (12, 13, 42), although in toad rods barium's effects are opposite to that of calcium (5). Some of our retinas incubated in elevated concentrations of calcium show increased numbers of cisternae in the rod terminals. The majority of cisternae are, however, not labeled with HRP suggesting that the cisternae in calcium-treated rod cells may form inside the terminal through the direct fusion of synaptic vesicles with one another, (11, 74) and not by endocytosis as with barium or TEA. Although the manipulation of external calcium concentration does not mimic the effects of barium, the phenomena of interest to us could be due partially to the calcium that circulates between the cytosol and intracellular stores (68).

Our observations with ouabain indicate that if barium is inhibiting the photoreceptor's Na/K ATPase, this is not responsible for the formation of cisternae. Incubation in ouabain results in enhanced HRP uptake in both rods and cones, which could reflect inhibition of ATPase activity. ATPase inhibition could lead to a rise in intracellular sodium and a loss of potassium (69) which would, in turn, produce depolarization and increased synaptic activity.

HRP Effects

We believe that the alteration in form of retrieval with barium or TEA treatment is not due to the presence of HRP. The suggestion has been made that HRP induces membrane damage and causes membrane to become incorporated into large vesicular structures (57). Our control preparations do not show such effects, nor are vacuoles engendered when HRP uptake is dramatically enhanced, as with high potassium. On the other hand, vacuolelike cisternae are produced by barium treatment in the absence of HRP.

Rods vs. Cones

Two systems have evolved in the frog retina to cover a wide range of light intensities. One system, driven by the rod receptors, is more sensitive at lower light levels, and the other system, driven by the cone receptors, becomes the more sensitive one at higher light levels (reviewed in reference 21).

Part of the differences between these systems is due to the differences in light absorption by the rods and cones (37, 38). The results of this and our other studies seem to point to interesting differences in the response of these receptors to ions. Cobalt (15) and nickel (45, 46) at appropriate concentrations block transmission and membrane cycling in rods selectively. We have found a similar selective rod response to cobalt in the goldfish retina. (Liscum, L., D. C. Hood, and E. Holtzman, unpublished observations). Barium and TEA at the concentrations used in this study also affect primarily the rod cells,

although the manifestations of the effects are quite different from those with cobalt and nickel. The element tying these observations together could well be the calcium channel. Cobalt and nickel block the calcium channels, and Fain et al. (17) postulate that barium and TEA cause increased calcium entry when they block the potassium channels in toad rods. Our results with calcium-free Ringer's indicate that calcium is required for transmission in both rods and cones. This suggests that rods and cones may differ in the permeability properties, abundance, or control of their calcium channels. Such differences might underlie some of the physiological differences between rod and cone synapses such as those recently reported for turtle retina (62).

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Note Added in Proof: A recent report by Baenziger and Fiete (*J. Biol. Chem.* 1982. 257:6007–6009) indicates that changes in monovalent ion concentrations, in this case sodium, may alter the recycling of the asialoglycoprotein receptor.

REFERENCES

1. Ainsworth, S. K., and M. J. Karnovsky. 1972. An ultrastructural staining method for enhancing the size and electron opacity of ferritin in thin sections. *J. Histochem. Cytochem.* 20:225–229.
2. Armstrong, C. M., and B. Hille. 1972. The inner quaternary ammonium ion receptor in potassium channels of node of Ranvier. *J. Gen. Physiol.* 59:388–400.
3. Armstrong, C. M., and S. R. Taylor. 1980. Interaction of barium ions with potassium channels in squid giant axons. *Biophys. J.* 30:473–488.
4. Baker, P. F., M. Ravazzola, F. Malaisse-Lague, and L. Orci. 1974. Secretion-dependent uptake of extracellular fluid by the rat neurohypophysis. *Nature (Lond.)* 250:155–157.
5. Brown, K., and D. Flaming. 1978. Opposing effects of calcium and barium in vertebrate rod photoreceptors. *Proc. Natl. Acad. Sci. U. S. A.* 75:1587–1590.
6. Ceccarelli, B., and W. P. Hurlbut. 1980. Calcium-dependent recycling of synaptic vesicles at the frog neuromuscular junction. *J. Cell Biol.* 87:297–303.
7. Ceccarelli, B., W. P. Hurlbut, and A. Mauro. 1973. Turnover of transmitter and synaptic vesicles at frog neuromuscular junctions. *J. Cell Biol.* 57:499–524.
8. Chan, K. Y., and R. H. Haschke. 1981. A re-evaluation of the retrograde axonal transport of horseradish peroxidase isoenzymes by semi-quantitative cytochemical analysis. *Neuroscience* 6:1145–1156.
9. Cooper, N., and B. McLaughlin. 1977. Peroxidase uptake with time by chick photoreceptor terminals. *J. Cell Biol.* 75:(2, Pt. 2): 111*a* (Abstr.)
10. Cornwall, M. C., and A. L. F. Gorman. 1979. Contribution of calcium and potassium permeability changes to the off response of scallop hyperpolarizing photoreceptors. *J. Physiol. (Lond.).* 291:207–232.
11. Dahl, G., M. Gratzl, and R. Ekerdt. 1976. In vitro fusion of secretory vesicles isolated from pancreatic cells and from the adrenal medulla. *J. Cell Biol.* 70(2, Pt. 2):180*a* (Abstr.).
12. Douglas, W. W., and D. W. Lywood, and R. W. Straub. 1961. The stimulant effect of barium on the release of acetylcholine from the superior cervical ganglion. *J. Physiol. (Lond.).* 156:515–522.
13. Douglas, W. W., and R. P. Rubin. 1964. The effects of alkaline earth and other divalent cations on adrenal medullar secretion. *J. Physiol. (Lond.).* 175:231–241.
14. Eaton, D. C., and M. S. Brodwick. 1980. Effects of barium on the potassium conductance of squid axons. *J. Gen. Physiol.* 75:727–750.
15. Evans, J. A., D. C. Hood, and E. Holtzman. 1978. Differential effects of cobalt ions on rod and cone synaptic activity in the isolated frog retina. *Vision Res.* 18:5–151.
16. Evans, J. E., L. Liscum, D. C. Hood, and E. Holtzman. 1981. Uptake of horseradish peroxidase by presynaptic terminals of bipolar cells and photoreceptors of the frog retina. *J. Histochem. Cytochem.* 29:511–518.
17. Fain, G. L., H. M. Gerchenfeld, and F. N. Quandt. 1980. Calcium spikes in toad rods. *J. Physiol. (Lond.).* 303:495–513.
18. Farquhar, M. G., and G. E. Palade. 1981. The Golgi apparatus (complex)—(1954–1981)—from artifact to center stage. *J. Cell Biol.* 9(3, Pt. 2):88S-103S.
19. Fried, R. C., and M. P. Blaustein. 1978. Retrieval and recycling of synaptic vesicle membrane in pinched-off nerve terminals (synaptosomes). *J. Cell Biol.* 78:685–700.
20. Gennaro, J., W. Nastuk, and D. Rutherford. 1978. Reversible depletion of synaptic vesicles induced by application of high external potassium to frog neuromuscular junctions. *J. Physiol. (Lond.).* 280:237–247.
21. Gordon, J., and D. C. Hood. 1976. Physiology of the frog retina. *In* The Amphibian Visual System: A Multidisciplinary Approach. K. V. Fite, editor. Academic Press, Inc., New York. 1–68.
22. Gronowicz, G., S. K. Masur, and E. Holtzman. 1980. Quantitative analysis of exocytosis and endocytosis in the hydroosmotic response of toad bladder. *J. Membr. Biol.* 52:221–235.
23. Hagiwara, S., J. Fukada, and D. C. Eaton. 1974. Membrane currents carried by Ca, Sr, and Ba in barnacle muscle fiber during voltage clamp. *J. Gen. Physiol.* 63:564–578.
24. Hama, K., and K. Saito. 1977. Fine structure of the hair cells in the saccular macula of the goldfish, with special reference to the anastomosing tubules. *J. Neurocytol.* 6:361–373.
25. Hammerschlag, R., and P.-A. Lavoie. 1979. Initiation of fast axonal transport: involvement of calcium during transfer of proteins from Golgi apparatus to the transport system. *Neuroscience.* 4:1195–1201.
26. Herman, A., and A. L. F. Gorman. 1979. Blockade of voltage-dependent and Ca^{2+} dependent K^+ current components by internal Ba^{+2} in molluscan pacemaker neurons. *Experientia (Basel).* 35:229–231.
27. Herzog, V., and M. Farquhar. 1978. Luminal membrane retrieved after exocytosis reaches most Golgi cisternae in secretory cells. *Proc. Natl. Acad. Sci. U. S. A.* 74:5073–5077.
28. Heuser, J. E. 1974. A possible origin of the "giant" spontaneous potentials that occur after prolonged transmitter release at frog neuromuscular junctions. *J. Physiol. (Lond.).* 239:106P–8P.
29. Heuser, J. E., and R. Miledi. 1971. Effect of lanthanum ions on function and structure of frog neuromuscular junctions. *Proc. R. Soc. Lond. B Biol. Sci.* 179:247–260.
30. Heuser, J. E., and T. S. Reese. 1973. Evidence for recycling of synaptic vesicle membrane during transmitter release at the frog neuromuscular junction. *J. Cell Biol.* 57:315–344.
31. Holmes, A. 1927. Petrographic Methods and Calculations. Murby, London.
32. Holtzman, E. 1976. Lysosomes, A Survey. Cell Biology monograph series, vol. 3. Springer, Vienna.
33. Holtzman, E. 1977. The origin and fate of secretory packages, especially synaptic vesicles. *Neuroscience.* 2:327–355.
34. Holtzman, E. 1981. Membrane circulation: an overview. *Methods Cell Biol.* 23:379–397.
35. Holtzman, E., A. Freeman, and L. Kashner. 1971. Stimulation-dependent alterations in peroxidase uptake at lobster neuromuscular junctions. *Science (Wash. D. C.)* 173:733–736.
36. Holtzman, E., and A. M. Mercurio. 1980. Membrane circulation in neurons and photoreceptors: some unresolved issues. *Int. Rev. Cytol.* 67:1–67.
37. Hood, D. C. 1978. Psychophysical and electrophysiological test of physiological proposals of light adaptation. *In* Visual Psychophysics: its physiological basis. J. Armington, J. Krauskopf, and B. Wooten, editors. Academic Press, Inc., New York. 141–155.
38. Hood, D. C., and P. A. Hock. 1975. Light adaptation of the receptors: increment threshold functions for the frog's rods and cones. *Vision Res.* 15:545–553.
39. Hubbard, J. I., and S. Kwanbunbumpen. 1968. Evidence for the vesicle hypothesis. *J. Physiol. (Lond.).* 194:407–420.
40. Joseph, K. C., A. Stieber, and N. K. Gonatas. 1979. Endocytosis of cholera toxin in GERL-like structures of murine neuroblastoma cells pretreated with GM_1 ganglioside. *J. Cell Biol.* 81:543–554.
41. Kalina, M., and D. C. Pease. 1977. The preservation of ultrastructure in saturated phosphatidyl cholines by tannic acid in model systems and type II pneumocytes. *J. Cell Biol.* 74:726–741.
42. Katz, B., and R. Miledi. 1969. Tetrodotoxin-resistant electric activity in presynaptic terminals. *J. Physiol. (Lond.)* 203:459–487.
43. Kelly, R. B., J. W. Deutsch, S. S. Carlson, and J. A. Wagner. 1979. Biochemistry of neurotransmitter release. *Annu. Rev. Neurosci.* 2:399–446.
44. Koike, M., and J. Meldolesi. 1981. Post-stimulation retrieval of luminal surface membrane in parotid acinar cells is calcium dependent. *Exp. Cell. Res.* 134:377–388.
45. Liscum, L., E. Holtzman, and D. C. Hood. 1980. Effects of divalent cations on photoreceptor synapses. *Invest. Ophthal. Visual Sci.* 80 (Supplement):131 (Abstr.).
46. Liscum, L., D. C. Hood, and E. Holtzman. 1981. Effects of divalent cations on membrane cycling in photoreceptor presynaptic terminals. *J. Cell Biol.* 91(2, Pt. 2):88*a* (Abstr.).
47. Lynch, K. 1982. The effects of chronic stimulation on the morphology of the frog neuromuscular junction. *J. Neurocytol.* 11:81–107.
48. Matheke, M. L., and E. Holtzman. 1981. Golgi apparatus and coated vesicles in membrane circulation in *Rana pipiens* retinas: studies with cholera toxin and monensin. *Soc. Neurosci. Abstr.* 7:306.
49. Mercurio, A. M., and E. Holtzman. 1982. Smooth endoplasmic reticulum and other agranular reticulum in frog retinal photoreceptors. *J. Neurocytol.* 11:263–293.
50. Miller, T., and J. Heuser. 1979. Membrane retrieval following transmitter release at the neuromuscular junction. *J. Cell Biol.* 83(2, Pt. 2):292*a* (Abstr.).
51. Model, P. G., S. M. Highstein, and M. V. L. Bennett. 1975. Depletion of vesicles and fatigue of transmission at a vertebrate central synapse. *Brain Res.* 98:209–228.
52. Morris, J. F., J. J. Nordmann, and R. E. G. Dyhall. 1978. Structure function correlation in mammalian neurosecretion. *Int. J. Exp. Pathol.* 18:1–28.
53. Pastan, I. H., and M. C. Willingham. 1981. Journey to the center of the cell: role of the receptosome. *Science (Wash. D. C.).* 214:504–509.
54. Reinecke, M., and C. Walther. 1981. Ultrastructural changes with high activity and subsequent recovery at locust motor nerve terminals. A sterological analysis. *Neuroscience.* 6:489–503.
55. Ripps, H., M. Shakib, and E. D. MacDonald. 1977. On the fate of synaptic vesicle membrane in photoreceptor terminals of skate retina. *Biol. Bull.* 153:443.
56. Ross, W. N., and A. E. Stuart. 1978. Voltage sensitive calcium channels in the presynaptic terminals of a decrementally conducting photoreceptor. *J. Physiol. (Lond.).* 274:173–191.
57. Rutherford, D. T., and J. F. Gennaro. 1979. A mechanism for horseradish peroxidase induced membrane damage in recycling membrane systems. *J. Cell Biol.* 83(2, Pt. 2):438*a* (Abstr.).
58. Rutherford, D. T., W. L. Nastuk, and J. F. Gennaro. 1976. Reversible depletion of synaptic vesicles in K^+ depolarized junctions. *J. Cell Biol.* 70(2, Pt. 2) 70:375*a* (Abstr.).
59. Schacher, S., E. Holtzman, and D. C. Hood. 1974. Uptake of HRP by frog photoreceptors in the dark and in the light. *Nature (Lond.).* 249:261–263.
60. Schacher, S., E. Holtzman, and D. C. Hood. 1976. Synaptic activity of frog retinal photoreceptors: peroxidase uptake study. *J. Cell Biol.* 70:178–192.
61. Schaeffer, S., and E. Raviola. 1978. Membrane recycling in the cone cell endings of the turtle retina. *J. Cell Biol.* 79:802–825.
62. Schnapf, J. L., and D. R. Copenhagen. 1982. Differences in the kinetics of rod and cone synaptic transmission. *Nature (Lond.)* 296:862–864.
63. Silverstein, S., R. Steinman, and Z. Cohn. 1977. Endocytosis. *Ann. Rev. Biochem.* 46:669–722.
64. Standen, N. B., and P. R. Stanfield. 1978. A potential and time-dependent blockade of inward rectification in frog skeletal muscle fibers by barium and strontium ions. *J. Physiol. (Lond.)* 280:169–191.
65. Tartakoff, A., and P. Vassalli. 1978. Comparative studies of intracellular transport of secretory proteins. *J. Cell Biol.* 79:694–707.
66. Theodosis, D. T., J. J. Dreifuss, M. C. Harris, and L. Orci. 76. Secretion-related uptake of horseradish peroxidase in neurohypophysial axons. *J. Cell Biol.* 70:294–303.

67. Tomita, T. 1970. Electrical activity of vertebrate photoreceptors. *Q. Rev. Biophys.* 3:179–222.
68. Ungar, F., I. Piscopo, and E. Holtzman. 1981. Calcium accumulation in intracellular compartments of frog retinal photoreceptors. *Brain Res.* 205:200–206.
69. Vizi, E. S. 1978. Na^{+}-K^{+}-activated adenosinetriphosphatase as a trigger in transmitter release. *Neuroscience.* 3:367–384.
70. Weibel, E. R., and R. P. Bolender. 1973. Stereological techniques for electron microscopic morphometry. *In* Principles and Techniques of Electron Microscopy. M. A. Hayat, editor. Van Nostrand Rheinhold, New York. 3:237–246.
71. Weibel, E. R., and D. Paumgartner. 1978. Integrated stereological and biochemical studies on hepatocytic membranes. II. Correction of section thickness effect on volume and surface density estimates. *J. Cell Biol.* 77:584–597.
72. Werman, R., and H. Grundfest. 1961. Graded and all-or-none electrogenesis in arthropod muscle. II. The effects of alkali-earth and onium ions on lobster muscle fibers. *J. Gen. Physiol.* 44:997–1027.
73. Williams, M. A. 1977. Quantitative methods in biology. *In* Practical Methods in Electron Microscopy. A. M. Glauert, editor. North-Holland, New York. Vol. 6.
74. Wilschut, J., and D. Papahadjopoulus. 1979. Ca^{2+}-induced fusion of phospholipid vesicles monitored by mixing of aqueous contents. 1979. *Nature (Lond.).* 281:690–692.
75. Zimmerberg, J., F. S. Cohen, and A. Finkelstein. 1980. Fusion of phospholipid vesicles with planar phospholipid bilayers. *J. Gen. Physiol.* 75:241–250.
76. Zimmerman, H. 1979. Vesicle recycling and transmitter release. *Neuroscience.* 4:1773–1803.
77. Zucker, R. S. 1981. Tetraethylammonium contains an impurity which alkalizes cytoplasm and reduces calcium buffering in neurons. *Brain Res.* 208:473–478.