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Acidification and endosome-like compartments in the presynaptic terminals of frog retinal photoreceptors

D. SULZER* and E. HOLTZMAN‡

Department of Biological Sciences, Columbia University, New York 10027, USA

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Summary

By using the 'acidotropic' vital dye, Acridine Orange, we have found that the presynaptic terminals of rod and cone photoreceptors in retinas of *Rana pipiens* maintain a low pH relative to the surrounding medium through an energy dependent mechanism. When this pH is raised, by exposing the retinas to weak bases like ammonium chloride, the terminals exhibit large, membrane-delimited compartments, many of which accumulate endocytic tracers. This effect is partly reversed when the weak bases are removed. We infer that among the acidified structures within the terminals are endocytic compartments with at least some of the characteristics of the endosomes that participate in receptor-mediated endocytosis in other cell types. One role of these structures in the terminals may be in the recycling of synaptic vesicles.

Introduction

It has been known for some time that nerve terminals can exhibit considerable endocytosis-like activity. The most prominent of such activities is the retrieval of membrane added to the cell surface by the fusion of synaptic vesicles during transmitter release; the retrieved membrane eventually regenerates synaptic vesicles (Holtzman *et al.*, 1971; Heuser & Reese, 1973; Ceccarelli *et al.*, 1973; for review see Holtzman & Mercurio, 1980; Kelly, 1988; Rash, 1988). Some nerve terminals also exhibit endocytic uptake of biologically active growth factors, toxins or other molecules (reviewed, e.g. in Hawrot, 1984) by processes that may not be tied to the cycling of membranes during neurotransmission. The tips of growing nerve processes at stages before synapses form can also show considerable endocytosis (reviewed, e.g. in Jacobson, 1978).

There are many important unresolved issues about the mechanisms of endocytosis and membrane cycling at nerve terminals. For example, the extent to which clathrin-coated structures are involved remains in dispute (cf. Holtzman, 1977; Torri-Tarelli *et al.*, 1987; Miller & Heuser, 1984; Kelly, 1988). This involvement is of interest given the central participation of coated vesicles of non-neuronal cells in receptor-mediated endocytosis and other processes dependent on coupled endocytosis and exocytosis (reviewed in Tartakoff, 1987; Holtzman, 1989).

In receptor-mediated endocytosis 'endosomes' serve critical roles as the structures to which endocytosed materials are delivered and from which the materials are sorted to their subsequent fates. These compartments are characterized by pH values considerably below that of the surrounding cytoplasm (cf. Maxfield, 1985; Mellman *et al.*, 1986). Acidification of the endosomes and of related compartments is central to determining whether membranes and ligands eventually become distributed to lysosomes, move on to the Golgi region, or are delivered back to the cell surface (reviewed in Anderson & Orci, 1988; Tartakoff, 1987; Holtzman, 1989).

It is not known at present whether endosomes or comparable acidified compartments occur in neurons. However, relatively large endocytic compartments, that could be endosome-related, are easily observed in nerve terminals and elsewhere, both normally and under various experimental conditions. For instance, during retrieval of synaptic vesicle membrane, especially when transmitter release is very intense (Bennett *et al.*, 1976), when the temperature is low (Heuser & Reese, 1973; Schaeffer & Raviola, 1978) or under some ionic conditions (Liscum *et al.*, 1982), a considerable

* Present address: Box 62, New York State Psychiatric Institute, Columbia University, New York 10032, USA.
‡ To whom correspondence should be addressed.

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proportion of the retrieved membrane can accumulate in intermediate compartments. These are distinguishable from typical synaptic vesicles but are fated eventually to give rise to such vesicles (e.g. Heuser & Reese, 1973; Bennett *et al.*, 1976; Miller & Heuser, 1984). Depending on system and circumstance the compartments vary in size and other properties. Some are a few tens of nanometres in diameter and thus are not much larger than typical synaptic vesicles. Others are sacs or vacuoles with lengths or diameters of hundreds of nanometres; the term 'cisternae' has sometimes been used to designate such large intermediates in synaptic vesicle recycling. If cisternae do turn out to be endosome-like, an interesting point deserving attention will be that the cycling membrane appears only transiently at the cell surface; in the 'resting' state, the membrane is held in intracellular compartments (synaptic vesicles). In contrast, the membrane cycling in receptor-mediated endocytosis is generally regarded either as in continuous movement between the cell surface and interior compartments, or as membrane that 'resides' at the cell surface but can transiently be internalized (reviewed in Holtzman, 1989).

Retinal photoreceptors are among the neurons in which extensive endocytic activity, especially that linked to synaptic activity, has been demonstrated (Schacher *et al.*, 1974, 1976; Ripps *et al.*, 1976). Under normal circumstances endocytic tracers are incorporated chiefly into synaptic vesicles, but some tracer also passes into larger sacs and tubules and vacuole-like 'cisternae' of endocytic origin can become quite prominent under several experimental conditions (Schaeffer & Raviola, 1978; Liscum *et al.*, 1982; Cooper & McLaughlin, 1983). In the present study we establish that frog photoreceptor terminals contain structures whose pH is below that of the extracellular milieu and we demonstrate that the terminals contain compartments which respond to treatments that raise intracompartmental pH, in a manner similar to the responses of acidified endosomes in other cell types. Our methodology derives from the numerous observations on endosomes and lysosomes indicating that weak bases accumulate selectively in compartments with interior pH values lower than their surroundings (reviewed in Anderson & Orci, 1988; Holtzman, 1989). Weak base vital dyes ('acidotropic dyes'), including aminoacridine derivatives like Acridine Orange, have been used extensively to stain acidified compartments. Other bases, especially ammonium chloride, have been widely employed to raise intracompartmental pH values, thereby perturbing a variety of phenomena. (For reports of various effects of ammonium ions on nervous tissue see Baguena-Cerevellera *et al.*, 1987; Erecinska *et al.*, 1987; Van der Kloot, 1987; Fitzpatrick *et al.*, 1988). Abstracts reporting our findings have appeared (Sulzer *et al.*, 1987b; Sulzer & Holtzman, 1988; Holtzman *et al.*, 1988).

Materials and methods

Rana pipiens fed with meal worms were kept at room temperature on a 12 h light/12 h dark cycle. Retinas for our experiments were typically taken at 2 h before light onset. The neural retinas were dissected from the pigment epithelium by our usual procedures (Schacher *et al.*, 1976). Incubations were in amphibian Ringer's solution (111 mM NaCl, 2 mM KCl, 2.3 mM $NaHCO_3$, 0.45 mM $CaCl_2$, 5.0 mM glucose, pH 7.5) in the dark, at room temperature; the medium was kept oxygenated throughout the incubation with 100% O_2. When compounds were added to the Ringer's solution at concentrations of more than 5 mM, NaCl concentrations were reduced accordingly to maintain osmolarity.

The high-potassium Ringer's solution was that used in our previous work (Evans *et al.*, 1981; 113 mM K propionate, 1.1 mM Ca propionate, 2.3 mM $NaHCO_3$, 5.0 mM glucose, pH 7.2).

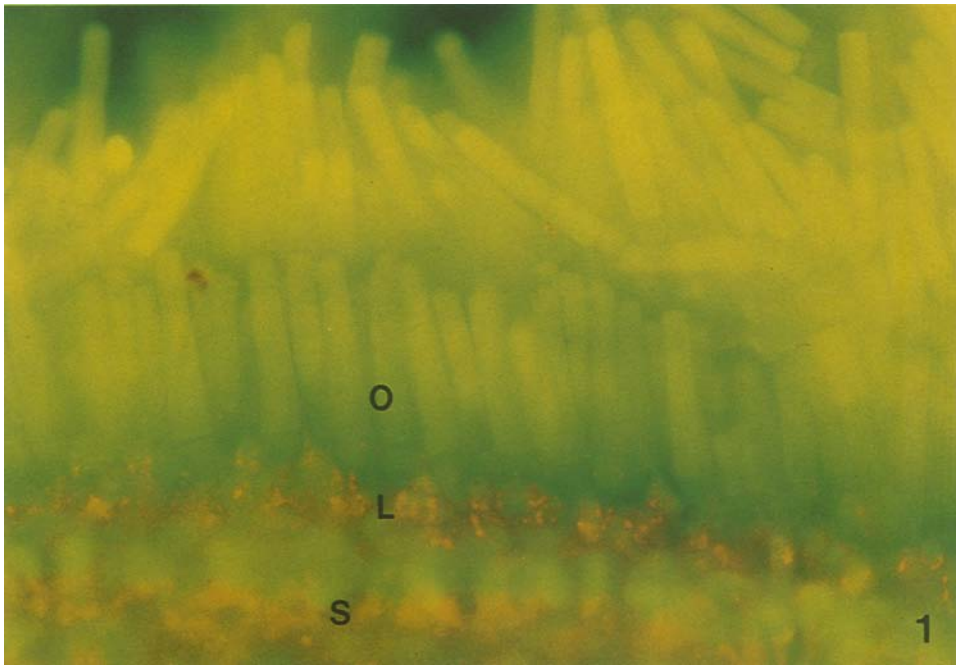
Acridine orange (AO) experiments

Retinas were incubated in Ringer's solution with 10–270 μM AO for between 30 min and 1 h. Our experiments were routinely done with 20–40 μM AO; the higher concentrations were used for photography and for experiments on vacuole formation (see results). For examination and photography, the retinas were then rinsed briefly in three changes of Ringer's solution and sliced with a sharp razor blade into sections oriented parallel to the long axis of the outer segments. Fluorescence observations were made with a Nikon Optiphot epifluorescence system using a Nikon B-2 filter.

Experiments with inhibitors of energy metabolism (protocols based on Matsumoto & Besharse, 1985): each inhibitor was studied in at least three experiments and in each case companion retinas were incubated for similar times in AO alone. Experimental retinas were exposed to 10 μM iodoacetic acid plus AO for 1 h; or to 1 μM dinitrophenol (DNP) plus AO for 10 min; or to AO for 1 h followed by 10 min in 1 μM DNP plus AO; to 5 mM potassium cyanide plus AO for 1 h; or to AO for between 20 min and 1 h followed by 10–40 min in AO plus 5–10 mM potassium cyanide. Note that as well as interfering with energy production, DNP can itself directly influence proton distributions across membranes.

Experiments with weak bases: retinas were exposed for between 30 min and 1 h to 10–30 mM ammonium chloride (NH_4Cl), 10 mM tributylamine or 5 mM benzylamine with AO. In the recovery experiments, following 30–60 min in AO

Fig. 1. Fluorescence micrograph of the photoreceptor layer of a free hand section of an Acridine Orange-stained living retina. The stain is present in lysosomes in the cell bodies (retinal layer indicated by L) and within the presynaptic terminals of the photoreceptors (retinal layer indicated by S). O indicates outer segments. × 600.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with NH_4Cl or benzylamine, the retinas were placed for 30 min in AO alone.

Horseradish peroxidase (HRP) experiments

Retinas were incubated at room temperature in Ringer's solution with 0.5% HRP (Sigma Biochemicals, St Louis, Missouri, USA; Type II) for between 15 min and 1 h. All key experiments were replicated at least three times. When weak bases were used along with HRP they were added at the same concentrations as were used for the AO experiments. Unless otherwise indicated the base-containing medium was added after 5 min incubation in HRP-Ringer's solution without the bases. In 'recovery' experiments retinas that had been incubated for 15 min with NH_4Cl and HRP were rinsed three times in Ringer's solution and then incubated in Ringer's solution which lacked both HRP and NH_4Cl for an additional 1 h. The 'recovery' retinas were compared with companion preparations which had been fixed after the initial period in NH_4Cl and HRP. In each of these recovery experiments we also examined a retina which was exposed to NH_4Cl–HRP, rinsed and then incubated for 1 h in NH_4Cl without HRP.

In a few experiments 9 kD dextran (Sigma, St Louis, Missouri, USA) was used as an endocytotic tracer in place of HRP (cf. Liscum *et al.*, 1982).

After incubation, HRP-exposed retinas were fixed for 1.5 h at room temperature in 2.5% glutaraldehyde in cacodylate buffer, pH 7.4, chopped on a Smith–Farquhar tissue chopper, incubated to demonstrate peroxidase activity by our usual methods (Graham & Karnovsky, 1966; Schacher *et al.*, 1976) postfixed in 1% osmium tetroxide at pH 7.4 and embedded in epoxy resin (Ladd, LX-112, Burlington, Vermont, USA).

Surface areas of the membranes of compartments were estimated from intersections with a morphometric grid (Weibel, 1981). The data in Fig. 8 were collected from three independent recovery experiments (see above for procedures); a fourth experiment was discarded because the preparations contained many dead or moribund photoreceptors. Each individual experiment showed the same pattern indicated by the grouped data. For each experiment, grids bearing thin sections from three different portions of the retina were scored; randomness in choice of cells was achieved by selecting, in each grid, three different zones of the thin sections and, in each zone, scoring the first five profiles of different rod cells and the first five profiles of different cone cells whose terminals could be clearly identified.

Results

Acridine orange (AO) uptake

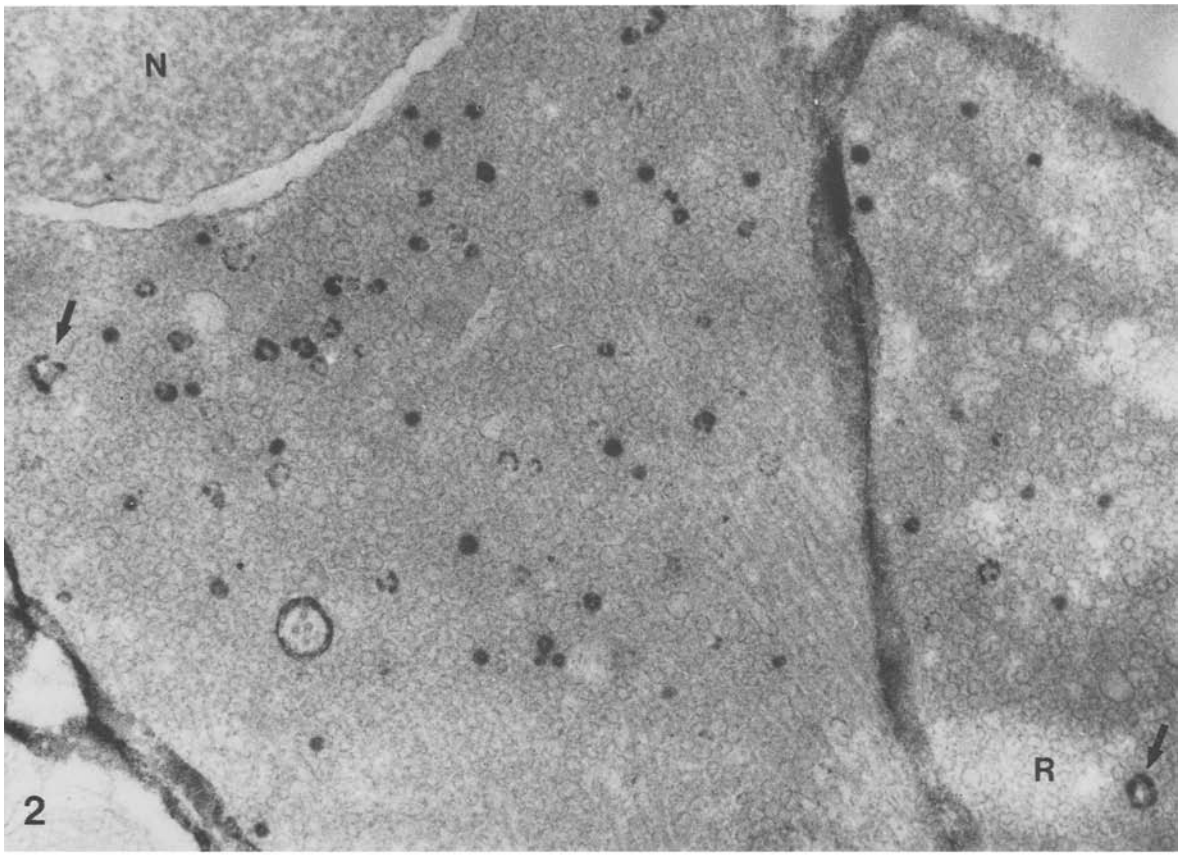
At all concentrations used the principal sites of AO accumulation in the retinas were in two zones of the photoreceptors: the myoid region of the cell bodies and the outer plexiform layer, composed largely of the photoreceptor's presynaptic terminals (Fig. 1). Some stain was also seen in the inner plexiform layer, where retinal interneurons make synapses, and surrounding the nuclei of the interneurons in the inner nuclear layer. In the photoreceptor cell bodies, staining was distinctly particulate (Fig. 1) with the bodies that stained having the size and distribution expected for lysosomes (cf. Schmied & Holtzman, 1987). Staining in the outer plexiform layer was confined to the presynaptic terminals and seemed to be associated with structures ranging in size from near the limit of resolution to large bodies; the details of staining within the terminal were difficult to make out because the preparations are relatively thick and the terminals are closely grouped in an extended layer.

Incubation with the inhibitors of energy metabolism prevented the AO staining, as did incubation with weak bases: 10 mM NH_4Cl reduced the stain markedly, but 20–30 mM was needed to inhibit all staining reliably; 5 mM benzylamine or 10 mM tributylamine prevented all staining. Retinas exposed to NH_4Cl or benzylamine and then placed in Ringer's solution which was free of the base regained their ability to accumulate AO in the photoreceptor cell bodies and terminals. Retinas incubated in the high potassium medium had the same distribution of stain as those incubated in ordinary Ringer's solution, though we often had the impression that the staining was more intense with the high potassium medium.

HRP uptake

The photoreceptors in control material – preparations incubated without weak bases – resembled those in our previous studies (Schacher *et al.*, 1974, 1976; Evans *et al.*, 1978; Liscum *et al.*, 1982). Peroxidase was demonstrable (Fig. 2) principally in a proportion of the structures with the size, appearance and distribution of synaptic vesicles. A few structures with diameters larger than the 40–50 nm typical of the synaptic vesicles also showed an HRP label. Most of these structures were vesicles, small sacs, and tubules with largest dimensions of 60–120 nm; the smaller amongst such structures cannot reliably be distinguished from large synaptic vesicle profiles. As in our earlier work most terminals in preparations incubated in standard Ringer's solution showed labelling in 5–15% or more of their synaptic vesicles while preparations incubated in high-potassium Ringer's solution showed labelling in two to three times as many vesicles (cf. Evans *et al.*, 1981; Liscum *et al.*, 1982). This latter effect reflects the depolarization of the photoreceptors by the medium, with consequent intensification of transmitter release.

The most obvious change resulting from incubation in NH_4Cl was the presence of vacuoles, most with diameters of 100–500 nm, but some ranging up to 1 μm or more (Figs 3–8). In the NH_4Cl-exposed preparations such structures accounted for 5–20% of the intracellular membrane surface area in the photoreceptor terminals as against 1–3% in the retinas not exposed to the base. There were no obvious changes in the agranular reticulum-related compartments that



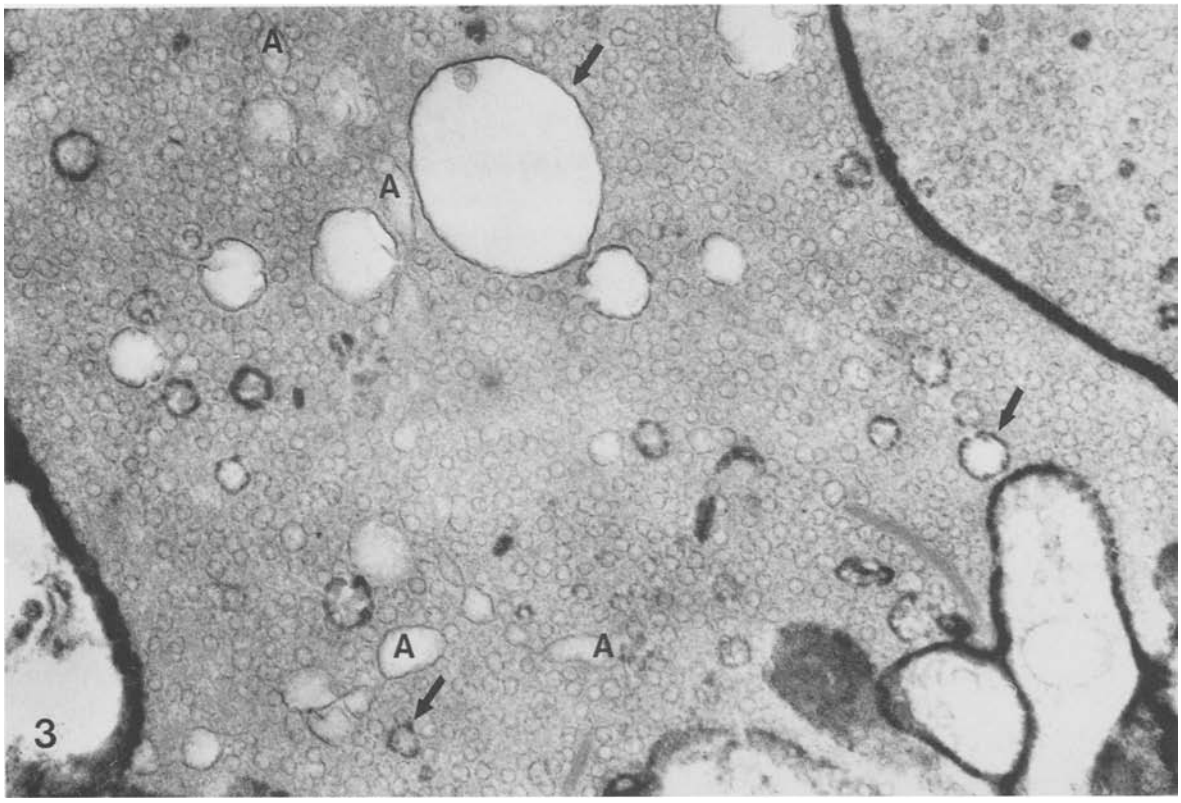


Fig. 2. HRP uptake in the presynaptic terminals of a cone cell (identifiable by the nucleus at N) and of an adjacent rod (R) in a retina incubated for 30 min in HRP, under control conditions. Most of the labelled structures are of the size and appearance of typical synaptic vesicles but occasional, somewhat larger, structures also show reaction product (arrows indicate two of the smaller of these). × 48 000.

Fig. 3. Presynaptic terminal of a rod cell from a retina incubated for 30 min with 30 mM ammonium chloride and HRP. Most of the HRP labelled structures are larger than typical synaptic vesicles. The arrows indicate examples spanning much of the range of sizes of such structures. A indicates agranular reticulum which lacks HRP under all conditions we have studied thus far (cf. Fig. 6 and Mercurio & Holtzman, 1982). × 48 000.

are present in the terminals (Fig. 6; cf. Mercurio & Holtzman, 1982). The vacuoles were surrounded by a normal-looking population of synaptic vesicles. Vacuoles were more consistently obvious in rod terminals than in cones (see Fig. 8 and Liscum *et al.*, 1982). The vacuoles were present whether or not the preparations had been exposed to HRP (Angenbraun & Holteman, unpublished observations). In retinas exposed to NH_4Cl and HRP by our routine protocol the majority of the vacuoles were HRP labelled, but unlabelled vacuoles were often present as well. Unlabelled vacuoles were particularly prominent if we omitted the preincubation in HRP we routinely included to permit the tracer to percolate into the regions of the synapses (cf. Schacher *et al.*, 1976), and especially if we exposed the terminals to NH_4Cl without HRP before exposing them to NH_4Cl with HRP (Fig. 5). The percentage of labelled synaptic vesicles in NH_4Cl–HRP exposed retinas varied from experiment to experiment. Sometimes it was markedly less than in the controls but sometimes it approached control levels.

The 'vacuologenic' effect of NH_4Cl was partly reversible: when retinas that had taken up HRP in the presence of NH_4Cl were incubated in Ringer's solution lacking both HRP and NH_4Cl the abundance of the large vacuoles decreased markedly while the abundance of HRP-labelled synaptic vesicles increased (Fig. 8). Terminals of retinas transferred from NH_4Cl–HRP medium to NH_4Cl without HRP continued to show numerous labelled vacuoles (Fig. 6).

Retinas incubated in high-potassium Ringer's solution with HRP (Fig. 9) showed extensive vacuolation of the terminals as well as folding and inward bulging of the terminal's plasma membrane reminiscent of that seen in other situations of intense stimulation and altered recycling (e.g. Schaeffer & Raviola, 1978; Cooper & McLaughlin, 1983; for potassium effects see Florey & Kriebel, 1988).

Retinas incubated in tributylamine or benzylamine with HRP (Fig. 10) showed numerous modest sized HRP-labelled vacuoles (60–100 nm in diameter) instead of the large ones seen in NH_4Cl. Retinas incubated in HRP with AO at concentrations above 30 μM showed numerous labelled vacuoles 100–500 nm in diameter (Fig. 11).

The vacuoles and vesicles in NH_4Cl treated retinas accumulated dextran particles when this tracer was used in place of HRP.

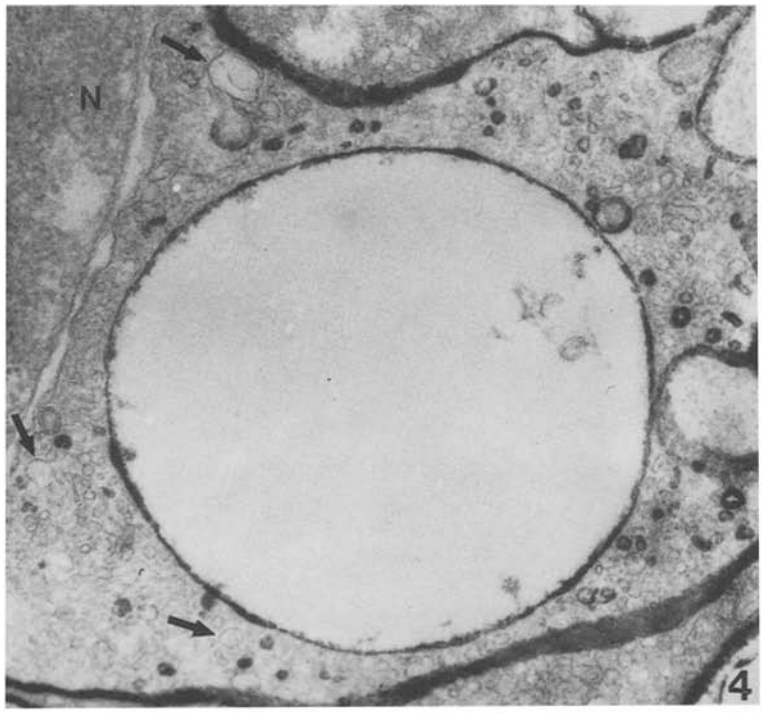
Discussion

Our findings with AO and with ammonium chloride indicate that compartments within the nerve terminals of photoreceptor cells *in situ* maintain a pH lower than their surroundings, through the operation of an energy-dependent mechanism. To our knowledge this is the first such demonstration of acidification for nerve terminals *in situ* though, as mentioned below, proton pumps have been detected in isolated synaptic vesicles. The present findings explain our previous observations of staining of retinal terminals with another acidotropic weak-base vital dye, Neutral Red (Sulzer & Holtzman, 1986).

By themselves our observations with vital dyes do not establish which structures within the terminals are staining, though the fact that AO engenders accumulation of vacuoles suggests that these are among the structures it stains (see below). We have tried to use an electron microscopic method, based on the accumulation of immunocytochemically-demonstrable DAMP (Anderson & Orci, 1988), but have found that the label is too diffusely distributed to be cleanly interpreted even in the vicinity of the mitochondria packed in the ellipsoid region or of known acidified structures such as lysosomes in the cell body. Our control preparations and our studies on other systems suggest that we face technical problems quite difficult to deal with. Not only are there problems in penetration of the detecting antibodies to the photoreceptor interiors, but we suspect also that we cannot fix DAMP molecules rapidly enough to keep them in place because the retinas are too thick, and the synaptic structures lack a dense enough internal matrix of macromolecules with available amino groups to which DAMP can be attached by the aldehyde fixative.

Our conclusion that endocytic vacuoles in the terminal are among the acidified structures rests on the close similarity of the effects of weak bases on the vacuoles to the effects of the bases on endosomes and other known acidified compartments. In the presence of ammonium chloride or of AO, relatively large vacuoles accumulate. This vacuologenic effect of weak bases like ammonium chloride, at concentrations on the same order as those we use, has long been recognized in various endocytosing, non-neuronal cells (Okhuma & Poole 1981; Cain & Murphy, 1986; Cain *et al.*, 1989). It is attributed to the accumulation of protonated forms of the bases within acidified compartments. The effect is held to be a result of low membrane permeability to the protonated forms of the bases, which are generated within the compartments because of the low pH: the vacuolation reflects principally the consequent osmotic entry of water (DeDuve, *et al.*, 1974; Okhuma & Poole, 1981), probably coupled with alterations in the patterns and relative rates of membrane cycling into and out of the compartments (cf. Holtzman, 1989; see also Heuser, 1989).

That the vacuolation is likely to involve osmotic effects rather than being due directly to changes in pH *per se* or to related alterations in H^+ gradients, is inferred from experiments on actively endocytosing cells, showing that weak bases which are membrane-permeant even when protonated can markedly reduce acidification without engendering vacuolation of the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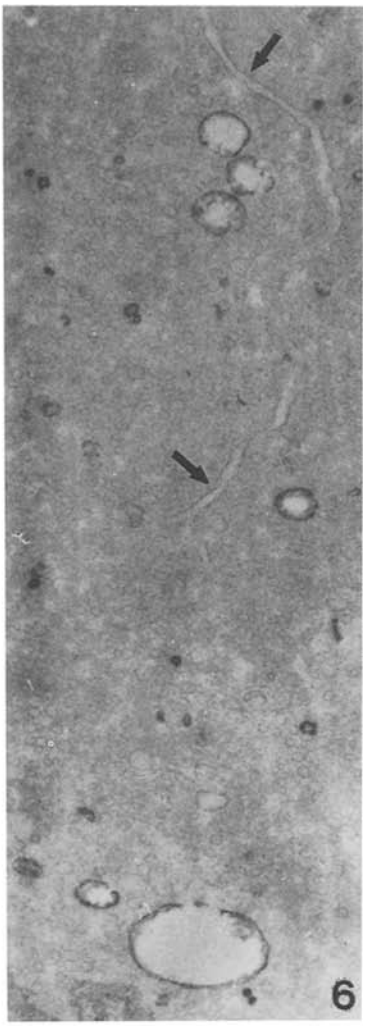
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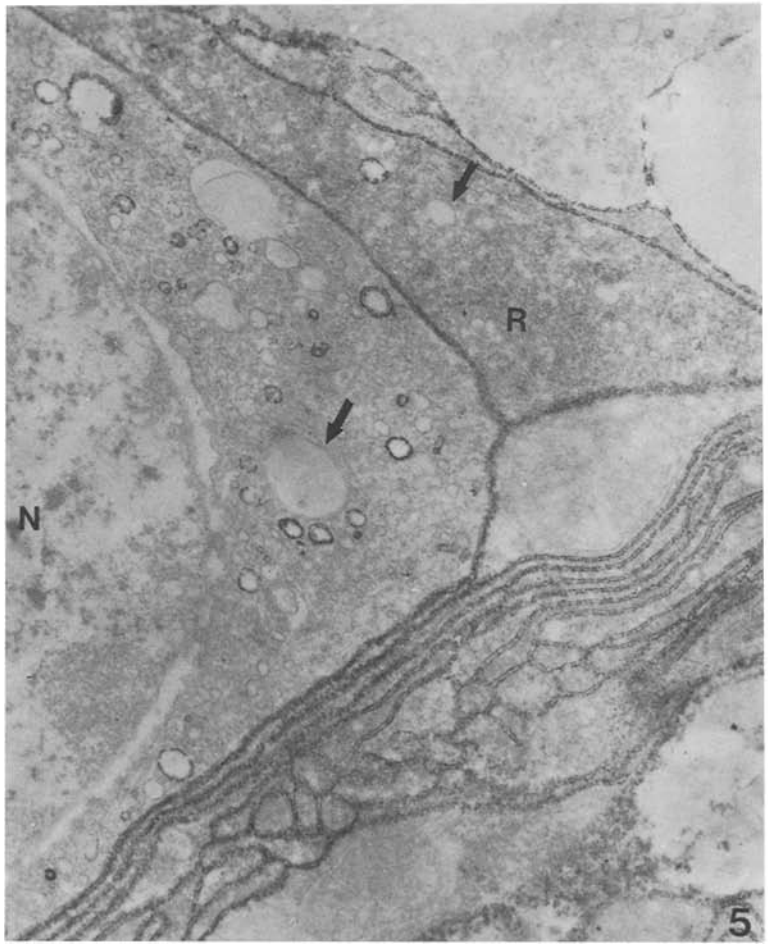
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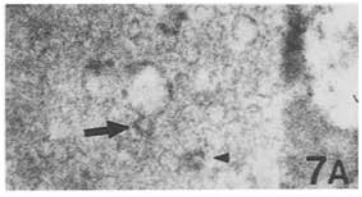
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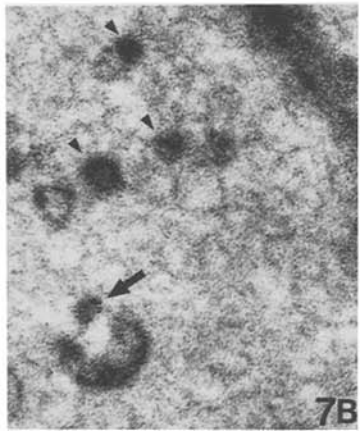
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endocytic system (Okhuma & Poole, 1981; Cain & Murphy, 1986). This was true in our system with the two such bases we used, benzylamine and tributylamine, even though these bases did have the expected effect on the pH in the terminals, as judged by AO staining. On the other hand, we previously found (Matheke *et al.*, 1983) that the Na^+/H^+ ionophore monensin, which has a number of effects including vacuolation on acidified compartments in other cell types (reviewed in Tartakoff, 1987; Holtzman, 1989), produces vacuoles in the photoreceptor terminals like the vacuoles seen in the present study.

The presence of unlabelled vacuoles along with the HRP-labelled ones could signify functional heterogeneity among the vacuoles or imply that at least some of the vacuoles arise from intracellular fusions, including ones involving structures that have not recently participated in endocytosis. Changes in cystolic pH could have some influence on such fusions (cf. Heuser, 1989) or even on retrieval of membrane from the cell surface (see Fig. 9). Alternatively, our observations may well mean that vacuoles eventually lose their capacity to acquire endocytosed tracers so that at least some of the vacuoles formed before HRP was present did not become labelled. This would parallel the indications in other cell types that endosomes, shortly after they form, cease fusing with newer endocytic structures, probably because the endosomes undergo changes in intrinsic fusion capacities and also move away from the zones of endocytosis (Gruenberg & Howell, 1987; Diaz *et al.*, 1988; reviewed in Holtzman, 1989).

The vacuoles we see are strongly reminiscent of the 'cisternae' thought to pertain to the recycling of synaptic vesicles (Schaeffer & Raviola, 1978; Liscum *et al.*, 1982) – the major endocytic process evident in photoreceptor terminals. In this connection it is interesting that when the ammonium chloride-HRP medium is replaced by one lacking ammonium chloride and HRP, labelled vesicles with the size and distribution of synaptic vesicles increase in abundance as the vacuoles disappear. This behaviour has previously been reported for cisternae in several systems under other experimental circumstances: the cisternae regenerate synaptic vesicles when conditions are returned to normal (Heuser & Reese, 1973; Miller & Heuser, 1984). For retina this was first shown by Schaeffer and Raviola (1978). In these reports, and in our work on retina (present study; Liscum *et al.*, 1982; Mercurio & Holtzman, 1982) the amounts of membrane that accumulate as cisternae represent a surface area sufficient to account for the subsequent regeneration of vesicles. Moreover in the present work (Fig. 7) and in several of the other studies (for retina see Schaeffer & Raviola, 1978; Liscum *et al.*, 1982; see also Miller & Heuser, 1984) images are encountered which seem to represent synaptic vesicles continuous with the cisternae as if trapped or fixed in the act of budding (or fusing). We see no sign of an increase in the multivesicular bodies and other degradative structures we have previously investigated in the terminals and cell bodies of photoreceptors (Holtzman & Mercurio, 1980; Schmied & Holtzman, 1987): degradation of the vesicles and other membranes of the terminals seems to be a slow, steady-state process that does not respond markedly to even substantial changes in rates of transmission and associated membrane cycling (cf. Holtzman & Mercurio, 1980).

Still, our findings with the weak bases indicate that the conditions under which vacuoles appear need not block the passage of endocytic label into synaptic vesicles (see e.g. Fig. 4). For neuromuscular junctions it has been posited that two or more routes by which synaptic vesicle membrane is retrieved can operate simultaneously. One such route may be relatively direct, involving the pinching off of small vesicles from the plasma membrane (cf. also Ceccarelli *et al.*, 1973 and Torri-Tarelli *et al.*, 1987) whereas other routes, more variable, seem to involve intermediate, cisternal-like compartments (Miller & Heuser, 1984). This would fit well with our present observations. However, with the protocols we have used thus far we

Fig. 4. Presynaptic terminal of a cone cell (nucleus at N) incubated in 30 mM ammonium chloride for 30 min and then in ammonium chloride plus HRP for 30 min. The centre of the field is occupied by a very large HRP-containing vacuole within the terminal. Note also the unlabelled vacuoles at the arrows. × 35 000.

Fig. 5. Extensively vacuolated regions of the presynaptic terminals of a cone cell (nucleus at N) and an adjacent rod (R) from a retina incubated in 30 mM ammonium chloride for 30 min and then in ammonium chloride plus HRP for 30 min. Unlabelled vacuoles are readily evident (examples at the arrows) along with the HRP-labelled ones. × 34 000.

Fig. 6. Portion of a rod terminal from a retina incubated for 15 min in 30 mM ammonium chloride with HRP and then for 1 h in ammonium chloride without HRP. The HRP labelled structures include large vacuoles. Several sacs of agranular reticulum are evident in the field (arrows); they are of normal appearance and lack HRP. × 34 000.

Fig. 7. Portions of presynaptic terminals of rod cells showing configurations (arrows) suggestive of synaptic vesicles attached to larger vacuoles. HRP-labelled synaptic vesicles are seen at the arrowheads. (A) From a retina exposed to 30 mM ammonium chloride with HRP for 15 min and then for 30 min to ammonium chloride lacking HRP. × 57 000. (B) From a retina exposed to ammonium chloride plus HRP for 30 min. × 100 000.

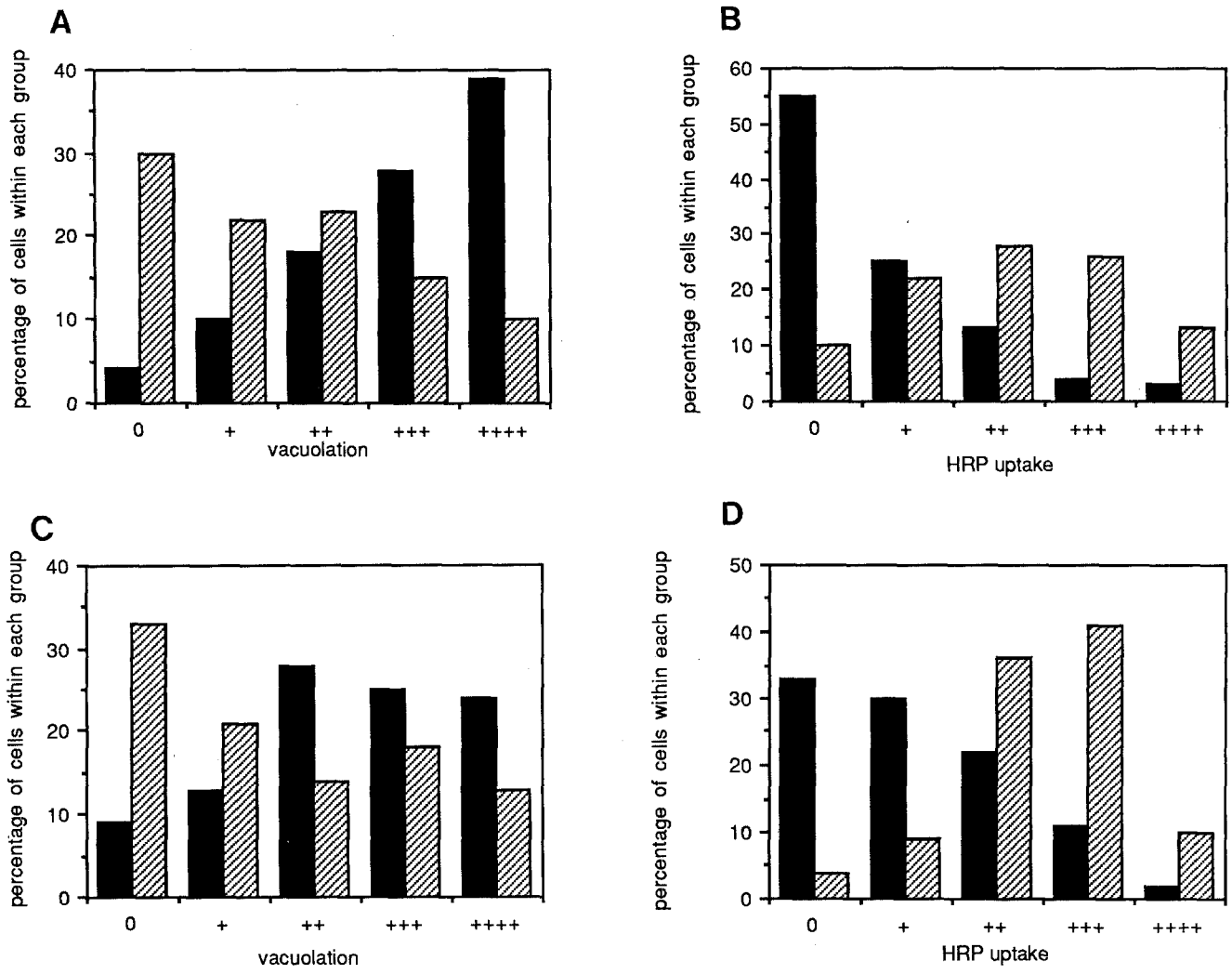
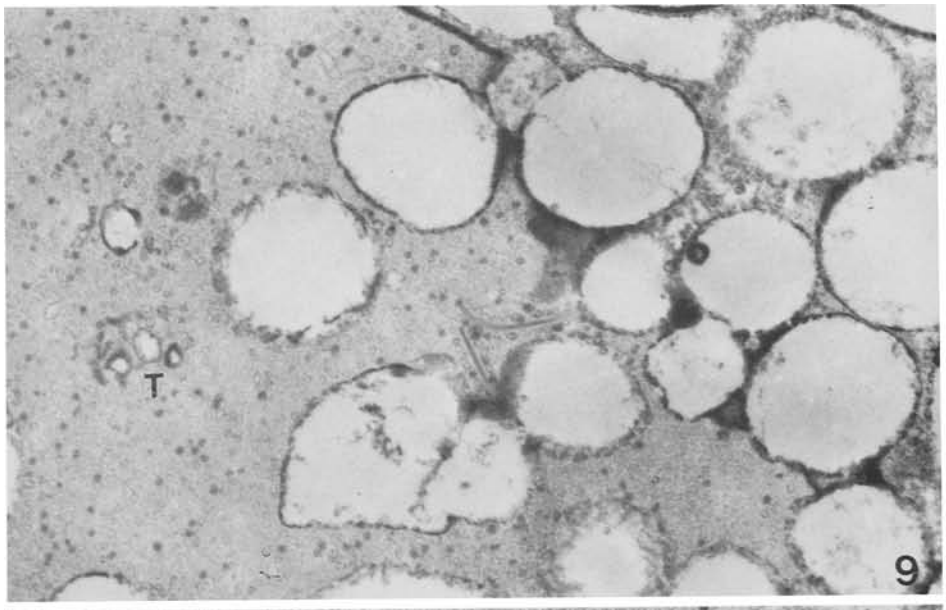


Fig. 8. Morphometry of structure changes in rod and cone terminals during and after exposure to ammonium chloride. (A) and (C) Illustration of the degree of vacuolation observed in rods and cones respectively before and after recovery. Full shaded bars denote weak base exposure, half-shaded bars recovery. Terminals of rod and cone photoreceptors prepared and selected as described in the Materials and Methods section were scored for the abundance of HRP-labelled vacuoles with diameters of 150 nm or greater. Profiles of terminals that had no such vacuoles were scored 0; + indicates the presence of one or two such vacuoles; ++ the presence of three or four such vacuoles; +++ the presence of more than four such vacuoles; ++++ the presence of very large vacuoles (1 μm or more in diameter, as in Fig. 4) or of vacuoles whose cross sectional area totalled one-third or more of the cross sectional area of the terminal. (B) and (D) Illustration of the relative degree of synaptic vesicle labelling with HRP in rods and cones respectively before and after recovery. Full shaded bars denote weak base exposure, half-shaded bars recovery. Profiles of terminals were scored 0 if they had only one or two labelled synaptic vesicles; + indicates terminals in which several vesicles were labelled but labelled vesicles amounted to less than 1% of the total synaptic vesicle population; ++ indicates terminals with 1–5% of the vesicles labelled; +++ terminals with 5–15% of the vesicles labelled; ++++ terminals with greater than 15% of the vesicles labelled.

Fig. 9. Presynaptic terminal of a rod cell from a retina incubated for 15 min in high-potassium Ringer's solution with 30 mM ammonium chloride and HRP. The terminal shows numerous labelled synaptic vesicles and large, vacuole-like structures, some of which are probably inward bulges of the plasma membrane. The structures clustered near T are particularly reminiscent of the morphology of 'early' endosomes in other cell types (cf. Holtzman 1989) – the profiles are of tubules and of vacuoles, some of which have short, tubular extensions (see also Liscum *et al.*, 1982 for similar structures). × 23 000.

Fig. 10. Portions of presynaptic terminals of a rod cell (lower two-thirds of field) and a cone (nucleus at N) from a retina incubated for 30 min in 5 mM benzylamine and HRP. Label is seen in synaptic vesicles and in somewhat larger structures (examples at the arrows). × 36 000.

Fig. 11. Portion of a presynaptic terminal of a rod cell from a retina incubated for 30 min in 100 μM Acridine Orange and HRP. Labelled vacuoles are present along with some labelled synaptic vesicles. (The section selected for this micrograph was somewhat thicker than the ones we routinely use, to aid in illustrating the distribution and abundance of the labelled vacuoles.) × 23 000.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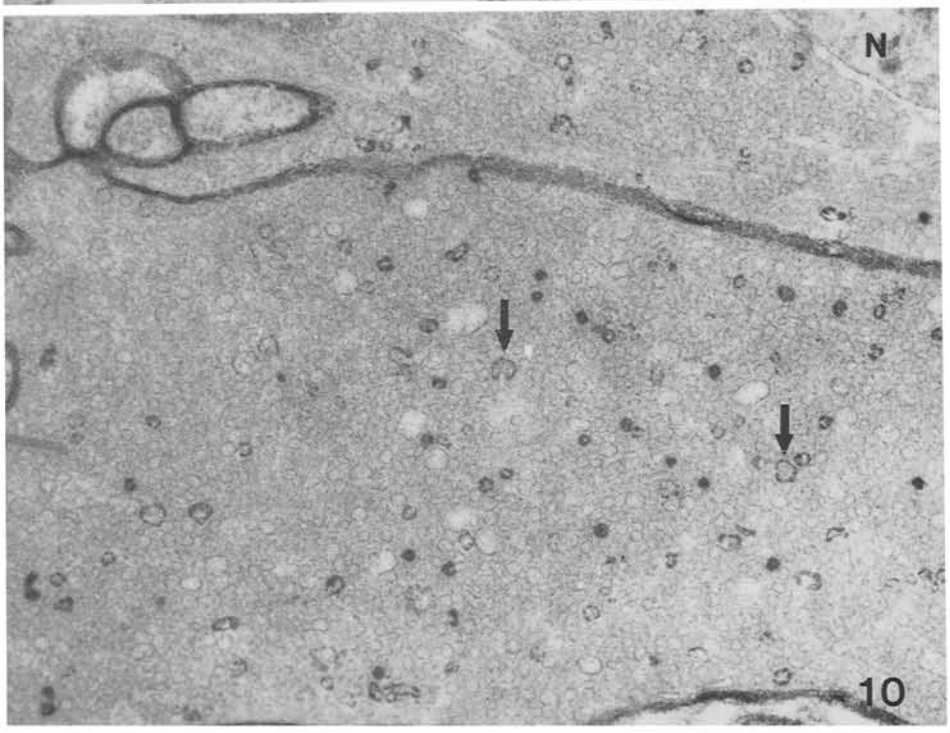
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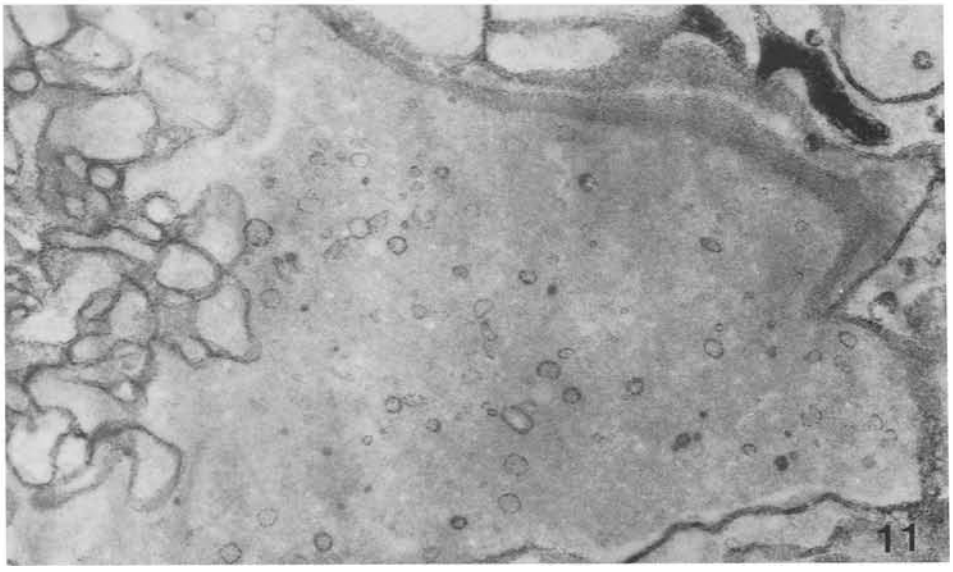
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cannot definitively evaluate this model. For example, we cannot adequately evaluate the possible toxic effects of our procedures, or the extent to which traces of HRP might persist in extracellular spaces after the rinses used in our recovery experiments. Thus, we think it premature, for example, to argue against the possibility that the vacuoles we observe are involved in yet-to-be discovered endocytic processes that coexist with the recycling of synaptic vesicles in the photoreceptors.

As summarized in the Introduction, cisternae do become prominent in various types of neurons under several conditions of physiological interest. But this by no means rules out the possibility that there is something special about the photoreceptors' vacuoles, related perhaps to the cells' use (Massey & Redburn, 1986) of acidic amino acids as transmitters. Movements of anions into acidifying compartments can be important in controlling the degree of acidification (reviewed in Mellman *et al.*, 1986; Moriyama & Nelson, 1987; Barasch *et al.*, 1988; Holtzman, 1989; see also Stanley *et al.*, 1988). Do our experimental conditions exaggerate normal pathways (e.g. enlarging compartments that ordinarily are much smaller), or do they divert endocytosis or recycling into an atypical mode? At present this is an unanswerable question, tied closely to the unresolved general disputes about synaptic vesicle recycling mentioned already. It is, however, easy to find modest-sized endocytic structures that could well be recycling intermediates in photoreceptors and other neurons releasing transmitters under normal stimulation conditions. Cell fractionation studies also suggest that there may be intermediate stages in the regeneration of synaptic vesicles from recycled membrane (Whittaker, 1987).

Another question of some interest is why the synaptic vesicles in the terminals fail to swell notably in the presence of ammonium ions, given that synaptic vesicles of several classes of neurons have been found to possess proton pumps (Johnson, 1987). The synaptic vesicles of photoreceptors have not been isolated but the transmitters used probably include glutamate (Massey & Redburn, 1986). Vesicles from other types of glutamatergic neurons seem to use proton pumps to help store transmitters (Naito & Ueda, 1983; Maycox *et al.*, 1988). Perhaps in the terminals we study the vesicles use their proton pumping machinery largely to generate a membrane potential rather than a pH gradient, as is the case under some circumstances with secretory bodies in gland cells, or with isolated synaptic vesicles (Johnson, 1987; Barasch *et al.*, 1988; Maycox *et al.*, 1988). Or it could be that the effects of the small radius of curvature of the vesicles or the formation of multimolecular complexes within them (cf. Sulzer *et al.*, 1987a) counteract expected osmotic effects; these types of explanations have been used to account for previous observations that synaptic vesicles and other small lipoprotein vesicles fail to swell or shrink markedly in response to large osmotic changes (e.g. Holz & Stratford, 1979; Van der Kloot & Spielholz, 1987).

Most discussion of the importance of the low pH in endosomes focuses on the impact on associations between cellular receptors and exogenous ligands. However, acidification could also have an impact on the interactions of the constituent molecules of membranes with one another or on the state of aggregation of compartmental contents, and these effects could influence, for example, the recycling and sorting of the membrane molecules (cf. Kelly, 1985; Caplan *et al.*, 1987; Sulzer *et al.*, 1987a; Tooze *et al.*, 1987; Turkewitz *et al.*, 1988; Chung *et al.*, 1989). Or pH might be important for the transmembrane movements of materials – solutes or water – into or out of the vacuoles; key roles have been assigned to osmotic effects and to movements of ions additional to H^+ in controlling the membrane reorganizations involved in exocytosis and in endosome functions (e.g. Harding *et al.*, 1985; Finkelstein *et al.*, 1986), although the proposals remain controversial (Holz, 1986).

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