Rod Photoreceptors Dissociated from the Adult Rabbit Retina

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Rod photoreceptors have been isolated from the adult rabbit retina using enzymatic and mechanical dissociation procedures; their fine structure, synaptic activity, and long-term viability were examined using conventional electron-microscopic, quick-freezing, and cell culture techniques. Freshly dissociated photoreceptors were well-preserved compared to their counterparts in the intact retina. About half of the cells, however, exhibited broad continuity between inner and outer segments. Quick-frozen, freeze-substituted rods differed from chemically fixed cells in 3 respects: (1) there was an increased amount of granular matrix in the cytoplasm, mitochondria, and rough endoplasmic reticulum; (2) branching and anastomosing profiles of smooth endoplasmic reticulum had disappeared from the inner segment; and (3) the number of synaptic vesicles within the spherule was highly variable, in some cases leaving synaptic ribbons completely denuded of their halo of vesicles. Light-adapted, solitary rod cells continued to be synaptically active: their endings were capable of endocytosis when placed in the dark in the presence of extracellular ferritin and tracer was incorporated into vesicles and vacuoles; this uptake was much reduced when the cells were incubated with the tracer in the light. Thus, synaptic vesicle regeneration was stimulated in the dark, suggesting that vesicles underwent exocytosis in the dark. Isolated rod cells adhered poorly to most standard substrates; without proper adhesion, cells deteriorated in 2–4 hr. However, photoreceptors did adhere to glutaraldehyde-fixed Vitrogen gels and could be maintained for over 48 hr on this substrate if kept in a complete medium at 22°C. In contrast, Müller cells adhered quickly to a laminin substrate with their endfoot processes. The differential adhesion properties of Müller and photoreceptor cells may be useful in obtaining pure populations of glial cells or neurons from the adult mammalian retina.

The study of the cell biology of retinal photoreceptors has been greatly facilitated by the development of techniques for their isolation from the adult retina (Lam, 1972). Solitary rod cells from the salamander retina (1) have normal light responses (Bader et al., 1979), (2) appear structurally intact when examined with the electron microscope (Townes-Anderson et al., 1985),

(3) are capable of synaptic activity (Townes-Anderson et al., 1985), and (4) can be maintained in culture for several weeks (MacLeish et al., 1983). Preliminary observations in mammals indicate that a full complement of cell types can be obtained using enzymatic and mechanical dissociation procedures. The isolated cells retain their shape (Sarthý and Lam, 1979; Vaughan and Fisher, 1983) and are capable of protein phosphorylation (Lolley et al., 1986).

In this study, solitary rods were obtained from the rabbit retina. Their structure was examined using both conventional electron-microscopic techniques and quick-freezing. Membrane recycling in their synaptic endings was investigated by exposing the cell suspensions to light and dark in the presence of native ferritin as an ultrastructural tracer. Finally, the potential for long-term survival of isolated rod cells was explored in a variety of culture conditions.

A preliminary report of this work was presented elsewhere in abstract form (Townes-Anderson et al., 1984).

Materials and Methods

Cell dissociation. These procedures closely follow those developed for tiger salamander retinal dissociations (Bader et al., 1979; Townes-Anderson et al., 1985). Light-adapted New Zealand albino rabbits, weighing 1.5–4.5 kg, were anesthetized by intraperitoneal injection of urethane (1 gm/kg). The lids were infiltrated with lidocaine hydrochloride (2%) and the eyes enucleated. Globes were hemisected at the ora serrata, the vitreous was removed, and the posterior optic cup was cut into 4 pieces. These were immersed in a Ringer's solution at pH 7.4 containing (in mM) 120 NaCl, 3 KCl, 25 NaHCO₃, 0.1 NaH₂PO₄, 0.8 Na₂HPO₄, 1 MgSO₄, 0.2 CaCl₂, 40 glucose, 0.06 ascorbic acid, and saturated with 5% CO₂/95% O₂. The neural retina was peeled away from pigmented epithelium and transferred to Ringer's solution with added cysteine (2.7 mM) and papain (14 U/ml; Cooper Biomedical, Malvern, PA), but no MgSO₄. Retinas were incubated with the enzyme for 30 min at 30°C with mild agitation, rinsed in Ringer's containing 2% NuSerum (Collaborative Research, Lexington, MA), and triturated with a wide-bore glass pipette. Resulting cell suspensions were placed into either flat-bottomed test tubes for tracer experiments or modified petri dishes for cell culture.

Electron microscopy. For examination of the intact rabbit retina, posterior eyecups were processed as previously described (Dacheux and Raviola, 1982). Dissociated cells were fixed in 2.5% glutaraldehyde, 2.5% formaldehyde, and 0.05% picric acid in 0.1 M cacodylate buffer, pH 7.4 (Ito and Karnovsky, 1968), and then pelleted. Pellets were post-fixed in a solution of 1% OsO₄, 1.5% potassium ferrocyanide (Karnovsky, 1971). Those cells that had not been treated with the tracer ferritin were also stained *en bloc* with 1% uranyl acetate in maleate buffer. After dehydration and embedding, thin sections were stained with lead citrate, either alone or after uranyl acetate staining, and examined in a Jeol 100-B or 100-CX2 electron microscope.

Quick-freezing and freeze substitution. A drop of the cell suspension was placed on a mica coverslip and maintained at room temperature in an oxygenated atmosphere for 1–4 hr. For freezing, mica coverslips, with attached cells, were cut into small squares. Individual squares were mounted on a transparent slab of 3% agarose and slammed against a

Received Apr. 13, 1987; accepted June 17, 1987.

We gratefully acknowledge Dr. Michael Rutten's advice and help in mammalian cell culture techniques. This work was supported by National Institutes of Health Grants EY 06135, EY 03011, and EY 01344.

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copper block cooled to liquid helium temperature (Heuser et al., 1979). Frozen specimens were transferred to liquid nitrogen for storage. For freeze substitution, specimens were placed in scintillation vials containing the primary fixative at liquid nitrogen temperature; the vials were subsequently warmed to -80°C . Best results were obtained by following the substitution protocol proposed by Bridgman and Reese (1984) without hafnium chloride staining. Briefly, the specimens were kept for 24 hr in a solution of 10% acrolein, 0.2% tannic acid in acetone at -80°C , then overnight in 1% OsO_4 in acetone at -20°C , for 12 hr in 10% glutaraldehyde in acetone/methanol at 4°C , and for 1 hr in 1% uranyl acetate in methanol. After embedding, the mica squares were removed and the cells were thin-sectioned and stained for transmission electron microscopy.

Tracer experiments. For each experiment, a cell suspension from a single dissociation was divided equally between 2 test tubes. The cells were kept at room temperature in an atmosphere of 5% CO_2 /95% O_2 . Under ambient light, native ferritin (22 mg/ml; Miles Scientific, Elkhart, IN) that had been dialyzed for 48 hr against Ringer's was added to the cell suspensions; one test tube was then exposed to dark and the other was illuminated. Light was delivered via a fiber-optic cable from a tungsten/halogen lamp. The maximal transmittance of a 2.2% ferritin solution in Ringer's, measured with a spectrophotometer, occurred at 620 nm. Taking into account the effects of both the glass of the test tube and the tracer solution, the irradiance at the specimen was adjusted in 2 different experiments to approximately 5 mW m^{-2} and 700 mW m^{-2} . At 620 nm, the number of photons delivered was about 2×10^2 photons $\text{sec}^{-1} \text{ cm}^{-2}$ and 2×10^{14} photons $\text{sec}^{-1} \text{ cm}^{-2}$, respectively. The spectral sensitivity of rabbit rods peaks at 502 nm (Rushton et al., 1955; Weale, 1964). The experiment was terminated after 15 min by the addition of fixative fluid to the suspension.

Cell culture. Wells were created in small petri dishes as previously described (Bray, 1970), using either glass, Thermanox (Miles Scientific), or mica coverslips to form the bottom surface. To test for optimal culture conditions, dishes were then treated in 2 ways: (1) coverslips were coated with a substrate by allowing molecules to precipitate from solutions placed into the well for 2 hr; (2) collagen gels were polymerized within the wells and then, in some cases, treated with a culture substrate. Before the cell suspension was added, the dishes were carefully washed with PBS. Thus, cells were placed either onto untreated coverslips, coated coverslips, untreated gels, or coated gels (see Table 1). The following substrates were used: rabbit vitreous (made by passing vitreous body diluted 1:1 with culture medium through a Millex-GV filter; Millipore, Bedford, MA), human plasma fibronectin (100 $\mu\text{g}/\text{ml}$; Bethesda Research Laboratories, Gaithersburg, MD), laminin (100 $\mu\text{g}/\text{ml}$; Bethesda Research Labs), polylysine (1 mg/ml; Sigma P-0879 type II), chondroitin sulfate (1 mg/ml; Sigma C-3254 grade III), and hyaluronic acid (1 mg/ml; Sigma H-1876 grade III-C). Gels were made by polymerization of Vitrogen (3 mg/ml; Flow Laboratories, McLean, VA) with ammonium hydroxide vapors, followed by 2.5% glutaraldehyde for 1 hr and several rinses with PBS (M. Rutten, personal communication). The culture medium was Dulbecco's modified Eagle's medium and Ham's F-12, 1:1, with 15 mM bicarbonate, 40 mM glucose, MEM vitamin solution and trace element mix (each 1% of final medium; Gibco Laboratories, Grand Island, NY), ascorbic acid (10 mg/ml), penicillin G (300 U/ml), gentamicin (15 $\mu\text{g}/\text{ml}$), and 10% NuSerum. Cells were maintained in a humidified atmosphere of 5% CO_2 /95% O_2 at room temperature in ambient light.

Results

Fine structure

As seen with the electron microscope (De Robertis, 1956; Sjöstrand and Nilsson, 1964; Bunt, 1978), rabbit rod cells consist of an outer segment, which contains a tightly packed stack of membranous disks independent of the plasmalemma, and an inner segment, where the usual cell organelles are contained, notably longitudinally arrayed mitochondria, cisterns of the rough endoplasmic reticulum, branching and anastomosing tubules of the smooth endoplasmic reticulum, multivesicular bodies, and the Golgi apparatus. An outer fiber connects the inner segment with the cell body, which contains the nucleus, and this, in turn, is connected by an inner fiber to the synaptic ending, or spherule. Depending on the position of the cell body, either fiber may be

Table 1. Substrates for culture of isolated rabbit photoreceptors listed in order of effective adhesion

1. Glutaraldehyde-fixed Vitrogen gel
2. Rabbit vitreous on glass/Thermanox/mica
3. Mica
4. Plasma fibronectin on glutaraldehyde-fixed Vitrogen gel
5. Laminin on glass
6. Polylysine on glass
7. Plasma fibronectin/chondroitin sulfate/hyaluronic acid on glass
8. Vitrogen gel
9. Glass/Thermanox

Most adhesive substrate for rod cells = 1; least adhesive = 9. Adhesion was determined by examining cultures 1, 2, 12, 18, and 24 hr after plating. Unlike the case with rod cells, laminin (5) was the most effective substrate for Müller cell culture.

of variable length or absent (Fig. 1A). The synaptic terminal is filled with synaptic vesicles and contains a synaptic ribbon. In order to compare photoreceptors with isolated rod cells, 2 features need to be emphasized. (1) In the intact retina, the inner and outer segments of the rod cell are connected only by a thin bridge of cytoplasm, the connecting cilium (Fig. 1B); furthermore, calycal or microvillar processes (Bunt, 1978) arise from the apex of the inner segment and surround the base of the outer segment. (2) The spherule of the rod in the intact retina exhibits a single synaptic invagination (Fig. 1C) that contains processes from horizontal and bipolar cells. The presynaptic active zone of the spherule is characterized by a pentalaminar synaptic ribbon surrounded by a halo of vesicles; the ribbon is moored by an arciform density to the plasma membrane at the apex of a wedge-shaped projection of the surface of the ending, the synaptic ridge.

Enzymatic digestion, followed by mechanical trituration of the retina, can yield a mixed population of retinal neurons; however, using the technique described in Materials and Methods, with a minimum of trituration one could obtain suspensions of cells that consisted almost exclusively of isolated rods. Examination with the light microscope demonstrated that the majority of these cells were intact, with outer and inner segment, connecting fibers, cell body, and spherule (Fig. 2). At the electron microscope, conventionally fixed cells appeared structurally well-preserved (see Figs. 3A, 4A, 5A). In the outer segment, the disks were parallel, regularly stacked and, as in the intact retina, they remained separated from the plasmalemma; swelling, a common reaction of the disks to a variety of injuries, was absent. A normal complement of cell organelles was contained in the inner segment and the fibers and cell body were identical to their counterparts in the intact retina. The spherule was filled with synaptic vesicles, round vacuoles, and coated vesicles. The synaptic ribbon was surrounded by vesicles and remained associated with the cell membrane at the apex of the synaptic ridge via an arciform density; coated invaginations of the cell membrane occurred at some distance from the synaptic ridge. The synaptic invagination was retained after dissociation, although the processes of second-order neurons had been removed. In places, the surface of the terminal gave rise to short processes that projected into the cavity of the synaptic invagination; these appeared as isolated islands of cytoplasm in cross section (Fig. 5A). In fortuitous sections it was possible to see that the invaginations opened into extracellular space; this was probably a common feature of all endings because, in experiments with

Figure 1. Intact retina. *A*, Single rod cell of the rabbit retina seen by Golgi impregnation. The cell body (*CB*) is connected to the inner segment (*IS*) and spherule (*Sp*) by an outer (*OF*) and inner (*IF*) fiber, respectively. *OS*, Outer segment. $\times 1000$. *B*, The inner and outer segments (*IS*, *OS*) of the rod cell are connected by a thin stalk, the connecting cilium (*CC*). The outer segment is filled with an orderly stack of membranous disks, whereas a full complement of cell organelles, including mitochondria, are present in the inner segment. Note the presence of large vesicles near the base of the connecting cilium (periciliary vesicles, *arrowheads*). $\times 42,500$. *C*, The synaptic terminal or spherule of the rod cell contains a single invagination (*arrowheads*) of its surface filled by processes of second order neurons, the horizontal and bipolar cells. The presynaptic active zone of the rod cell is characterized by a pentalaminar ribbon (*rb*) surrounded by vesicles, and an arciform density (*ad*) attaching the ribbon to the membrane at the apex of the synaptic ridge. $\times 50,000$.

extracellular ferritin, the invaginations were consistently filled with the tracer. The only anomaly observed in isolated rods occurred at the junction between the inner and outer segments. In about half of the rod cells, the apex of the inner segment had fused with the base of the outer segment, so that these regions were now in broad continuity with one another (Fig. 4*B*). In the remaining cells, connection between inner and outer segments was restricted to the cilium (Fig. 4*A*).

Quick-freezing

After dissociation, rapidly frozen, freeze-substituted rod cells were similar in general morphology to conventionally fixed cells, but they exhibited a number of interesting cytological differences. In the outer segment (Fig. 3*B*), the membranous disks were ordered in a perfect crystalline array. The intradisk space had disappeared, so that the disk acquired a pentalaminar appearance; the interdisk space had a constant width of 30 nm and was occupied by flocculent material. The edge of the disks and the plasmalemma were connected by fine filaments, similar in size and arrangement to those described in deep-etched and rotary shadowed frog, toad, and cattle outer segments (Usukura and Yamada, 1981; Roof and Heuser, 1982). As with conventional fixation, some rod cells showed a broad continuity between the inner and outer segments; thus, this plasmalemmal alteration was not a fixation artifact. In cells in which the inner and outer segments remained separated by the connecting cilium, a pattern of fine serrations was observed along the outer surface of the cilium (Fig. 4*A*, inset). This structure, also visible after conventional fixation, has been attributed to the ciliary necklace described from freeze-fracture replicas (Röhlich, 1975). In the inner segment (Fig. 6), the density of the cytoplasmic and mitochondrial matrix was greatly increased. In the ellipsoid region of the inner segment, a collection of large (200–400 nm) vesicles was consistently located near the basal body. Microtubules and prominent bundles of intermediate filaments coursed among the longitudinally arranged mitochondria; as described for rapid-frozen axons (Schnapp and Reese, 1982), mitochondria were occasionally attached to microtubules by an array of short filaments (Fig. 6, inset *A*). In the myoid region, the Golgi apparatus, multivesicular bodies, and cisterns of rough endoplasmic reticulum with dense flocculent contents were present (Fig. 6, insets *B*, *C*). However, branching and anastomosing profiles of the smooth endoplasmic reticulum were conspicuously absent from the entire inner segment. The fibers and cell body were unremarkable, except for the increased density of the matrix. The most striking finding in the spherule was the great variability in the number of synaptic vesicles and large vacuoles: some endings were very poor in membranous organelles and the ribbons were at times completely denuded of their halo of vesicles (Fig. 5*B*).

Tracer experiments

Cells that were placed in the dark in the presence of anionic ferritin endocytosed the tracer. After the 15 min incubation, ferritin particles were found in coated vesicles, smooth vesicles and vacuoles, and synaptic vesicles (Fig. 7*A*): up to 64% of the vesicles and vacuoles were labeled. The results of the experiments on tracer incubation in the light varied, depending on the intensity of illumination. Thus, with an irradiance of 5 mW m⁻² during the incubation period, no difference was seen between dark- and light-exposed cell suspensions: in both instances, vesicles and vacuoles had incorporated ferritin. With

Figure 2. Isolated rod cell. The rod photoreceptors remain intact after isolation. Nomarski optics highlight the longitudinally arrayed mitochondria in the inner segment. CC, Connecting cilium; OLM, site of the outer limiting membrane. $\times 3500$.

Figure 3. Isolated rod cell. *A*, Conventional fixation. The membranous disks of the outer segment remain regularly and tightly packed after dissociation. $\times 45,000$. *B*, Quick-freezing. With quick-freezing and freeze substitution, the membranes of the disk are closely apposed, nearly obliterating the intradisk space. Amorphous material occupies the interdisk region. Wispy filaments are present between the edges of the disks and the plasmalemma (arrowheads). $\times 123,000$.

an irradiance of 700 mW m^{-2} , however, uptake of the tracer was completely inhibited in three-quarters of the rods: although ferritin was present along the surface of the endings, tracer-filled vesicles were not seen within the spherule (Fig. 7*B*). For the remaining endings, some ferritin was observed within synaptic vesicles, but the proportion of labeled vesicles never exceeded 22%.

Cell culture

Culture conditions were developed for the maintenance of the isolated rod photoreceptors. Variations were made in media, the relative proportion of CO_2/O_2 , temperature, and culture substrate. Initial parameters were chosen on the basis of the successful *in vitro* maintenance of the intact retina in the rabbit eyecup preparation (Miller and Dacheux, 1973; Dacheux and Raviola, 1982). The cells were exposed to bicarbonate-buffered mammalian Ringer's with 10% fetal calf serum, then gassed with 5% $\text{CO}_2/95\% \text{ O}_2$, and maintained at 37°C . Under these conditions, rod cells appeared viable for about 4 hr, as judged from their morphology at the light and electron microscopes. For longer culture, however, it was necessary to resort to the more complete medium described in Materials and Methods, and to place the sensory neurons on an adherent substrate. The rod cells were very poorly adhesive to standard culture substrates (see Table 1); furthermore, attachment to the culture surface was slow, and was often delayed until 1–2 hr after plating. However, photoreceptors did adhere to coverslips coated with rabbit vitreous, but did best on Vitrogen gels fixed with 2.5% glutaraldehyde (Fig. 8). Cells attached to glutaraldehyde-fixed gels and cultured in a complete medium were successfully main-

tained for over 48 hr. Also present in these cultures were numerous small, unidentified cell bodies and large Müller glial cells, many of which had become rounded in shape.

Like the sensory neurons, the Müller cells did not adhere well to most substrates, with one exception, laminin. Interestingly, their adhesion to laminin was specific to the endfoot region of the cell, i.e., the basal process normally in contact with the inner limiting membrane or basal lamina of the intact retina. After 40 hr, cultures using laminin as a substrate contained numerous elongated Müller cells but virtually no recognizable retinal neurons.

Discussion

Using techniques developed for the salamander retina, a suspension rich in solitary rod photoreceptors can be obtained from the adult rabbit retina. Light- and electron-microscopic examination revealed that these rod cells are intact; furthermore, the fact that their synaptic terminals endocytosed ferritin in the dark demonstrates that they are healthy and remain synaptically active after the dissociation process. Finally, they can be maintained in culture for at least a 48 hr period when provided with a rich medium and an adhesive substrate. Thus, these cells should provide a good system for the study of mammalian photoreceptor cell biology.

The fine structure of solitary rabbit rod cells is remarkably similar to its counterpart in the intact retina, with one exception: in about half of the isolated cells, a broad fusion was observed between the apical surface of the inner segment and the basal surface of the outer segment. Although it is generally accepted that the inner and outer segments communicate only at the

Figure 4. Isolated rod cell. *A*, Conventional fixation. The inner (*IS*) and outer (*OS*) segments are joined by the connecting cilium (*CC*). The cilium has an array of ridges along its surface, especially prominent after quick-freezing (*inset*, *arrowheads*). In the ellipsoid of the inner segment is found the normal complement of organelles: mitochondria, microtubules, ribosomes, and anastomosing tubules of smooth endoplasmic reticulum (*arrows*). $\times 64,000$. *Inset*, quick-freezing, $\times 32,000$. *B*, Conventional fixation. About half of the rod cells show a broad continuity between the inner (*IS*) and outer (*OS*) segment after dissociation. As shown here, the membranous disks and cell organelles are in contact with one another. Elongated profiles, oriented parallel to the connecting cilium (*CC*), may represent vestiges of former plasmalemma (*arrowheads*). $\times 32,500$.

connecting cilium in the intact retina, broad continuity between the inner and outer segments of rod cells has been described in a variety of mammalian retinas (Richardson, 1969). A similar phenomenon occurs when rod cells are isolated from the salamander retina (Townes-Anderson et al., 1985). In this instance, however, fusion is observed in all intact cells; furthermore, sal-

amander preparations yield a number of rods missing their outer segments. It is possible that during trituration of the salamander retina, outer segments that are not fused to their inner segments are torn from the rest of the cell, but in the case of the rabbit retina, these unfused cells survive the trituration intact. The junction between the inner and outer segments is the site for

Figure 5. Isolated rod cell. *A*, Conventional fixation. After dissociation, the spherule retains its invagination, even though the postsynaptic processes have been removed. Blunt processes of the surface of the ending, however, do appear within the invagination (*asterisks*). The elements characteristic of the active zone in the intact retina, the synaptic ribbon (*rb*), synaptic vesicles, and arciform density (*ad*), continue to be present after isolation. Coated vesicles occur along the surface of the invagination at some distance from the synaptic ridge (*arrowheads*). $\times 62,000$. *B*, Quick-freezing. In this ending, there are surprisingly few membranous organelles; a few synaptic vesicles are present (*arrowheads*), but the synaptic ribbon (*rb*) looks denuded of vesicles. An arciform density (*ad*) is barely visible between the plasmalemma and the ribbon. $\times 97,000$.

Figure 6. Isolated rod cell; quick-freezing. The cytoplasmic matrix of the inner segment appears dense after quick-freezing and freeze substitution. In the myoid are the Golgi apparatus (*Ga*), multivesicular bodies (Inset *A*), and the rough endoplasmic reticulum (Inset *B*, arrowheads). In the ellipsoid region are longitudinally oriented mitochondria (asterisks) and prominent bundles of cytoskeletal elements (*cs*); mitochondria are occasionally seen attached to microtubules by an array of short filaments (Inset *C*, arrowheads). Near the base of the connecting cilium is a group of periciliary vesicles (*pv*). $\times 30,000$. *A* and *B*, $\times 104,000$; *C*, $\times 85,000$.

addition of new opsin-containing plasma membrane and the formation of new disks; since both processes involve membrane fusion (Steinberg et al., 1980; Papermaster et al., 1985), this region of the cell may be especially prone to membrane rear-

rangements. It would be of interest to know what factor(s) control this fusion phenomenon.

With the quick-freezing technique, only a 10–15- μm -thick layer at the surface of the specimen (Heuser et al., 1979) is free

Figure 7. Isolated rod cell. *A*, Conventional fixation. Cells placed in the dark with anionic ferritin endocytose the tracer. Ferritin particles are present within synaptic vesicles and larger vacuoles (arrowheads). Coated pits also contain ferritin (arrow). Note that some tracer-containing vesicles are associated with the synaptic ribbon (*rb*). $\times 84,000$. *B*, Conventional fixation. Light (700 mW m^{-2}) inhibits the uptake of ferritin by the spherule. Ferritin particles (arrowheads) line the entire surface of the ending, including the membrane of the invagination, but no ferritin appears within synaptic vesicles. *rb*, Synaptic ribbon. $\times 56,000$.

Figure 8. Rod cells in culture. *A*, Dissociated rod cells placed on uncoated glass coverslips do not survive more than a few hours in culture: at 20 hr, no identifiable rods remain. *B*, Rod cells placed on glutaraldehyde-fixed Vitrogen gels attach to the substrate and many appear normal with phase-contrast optics after 20 hr. $\times 200$.

of the distortions caused by large ice crystal formation; thus, rods in the intact retina cannot be adequately preserved throughout their length. This problem was circumvented by using isolated cells and allowing them to settle out from a suspension onto a flat coverslip. We can now exploit the time resolution afforded by the quick-freezing method to study the synaptic events in the terminals of mammalian photoreceptors. Frozen and freeze-substituted rods showed a remarkably dense cytoplasmic and mitochondrial matrix; presumably this technique preserves the granular or proteinaceous material that is extracted by conventional methods of specimen preparation for microscopy (Bridgman and Reese, 1984). After chemical fixation, the rod inner segment contains 4 systems of membranes: rough endoplasmic reticulum and Golgi apparatus in the myoid, branching and anastomosing profiles of smooth endoplasmic

reticulum throughout the inner segment, and a cluster of tubules and vesicles at the base of the connecting cilium (periciliary vesicles). It has been suggested that the smooth endoplasmic reticulum serves as a shuttle system to transport membrane components from the Golgi apparatus to the periciliary vesicles; these vesicles, in turn, deliver opsin to the plasma membrane at the base of the cilium (Papermaster et al., 1985). Of these systems of membrane, one is missing after quick-freezing, the smooth endoplasmic reticulum. This agrees with observations made on growth cones, where both anastomosing smooth endoplasmic reticulum and mounds of small vesicles were absent after quick-freezing and freeze substitution (Rees and Reese, 1981; Cheng and Reese, 1985). It is noteworthy that even in amphibian photoreceptors, which contain an elaborate reticulum in their inner segment, a large proportion of the membranes

of this organelle neither binds anti-opsin nor incorporates significant amounts of ^3H -glycerol, ^3H -choline, or ^3H -leucine, and thus at present has no known function (Mercurio and Holtzman, 1982; Pappermaster et al., 1985). It is possible that these branching and anastomosing profiles of smooth membrane are an artifact of aldehyde fixation; if so, their origin is unclear, because no obvious precursor could be identified in quick-frozen inner segments.

It is interesting that in quick-frozen specimens there is such variability in the number of vesicles in the synaptic terminals. It has been suggested that the precise number of vesicles seen after aldehyde fixation does not reflect the *in vivo* situation (Smith and Reese, 1980): a burst of miniature endplate potentials can be recorded in innervated muscle for several minutes after glutaraldehyde and/or formaldehyde fixation; numerous events of exocytosis occur at the presynaptic active zones of aldehyde-fixed neuromuscular junctions; and pits are seen at some distance from the active zone, possibly indicating endocytosis. Thus, in the neuromuscular junction, chemical fixation appears to stimulate synaptic vesicle membrane fusion and turnover, thereby simulating an increase in synaptic activity. Moreover, for chemically fixed central nervous system synapses, Smith and Reese (1980) have noted that the reported structural differences between stimulated and unstimulated synapses tend to be small.

If chemical fixation stimulates vesicle exocytosis, one would expect larger vesicle numbers in quick-frozen specimens, but the opposite is actually true: vesicle numbers are quite variable and depleted endings are often seen. The variations in membranous organelles in quick-frozen cells either reflect differences in synaptic activity among the endings or result from cell injury during the dissociation procedure. If the rapidly frozen profile conforms better to the state of affairs *in vivo*, then chemical fixation somehow shifts membrane to the vesicular compartment. We are currently freezing rods after controlled periods of exposure to light and dark. This may clarify this important point.

In the spherule of solitary rods, both the organization of the presynaptic active zone and the synaptic invagination are retained after dissociation. Perhaps the postsynaptic processes are not essential for the structural stability of the synapse, and the cytoskeleton of the ending plays a major role in maintaining its complex architecture over the short periods of time involved in this study.

Synaptic vesicle recycling in photoreceptors has been repeatedly investigated in the intact retina of cold-blooded vertebrates using ultrastructural tracers and conventional electron microscopy (Ripps et al., 1976; Schacher et al., 1976; Schaeffer and Raviola, 1978). This morphological approach has corroborated the physiological evidence that transmitter release by vesicle exocytosis occurs in the dark (Trifonov, 1963; Dowling and Ripps, 1973; Cervetto and Piccolino, 1974; Dacheux and Miller, 1976) because the compensatory membrane retrieval by endocytosis also takes place in the dark. This study extends these observations to the mammalian rod cell using an alternative tracer, native ferritin. Solitary rabbit rod cells endocytose ferritin particles when placed in the dark and, as in other species, the tracer is found in coated vesicles, vacuoles, and synaptic vesicles. The relative amount of uptake in solitary rabbit and salamander rods is difficult to assess. Although the rabbit spherule contains only one synaptic ribbon (Sjöstrand and Nilsson, 1964; Bunt, 1978) and thus only one presynaptic active zone, in some

cases more than half the synaptic vesicles were labeled with ferritin after a 15 min incubation at 22°C in the dark. In solitary salamander rod cells, with about 6 available active zones and a proportionally larger synaptic pedicle, a maximum of 40% of the synaptic vesicles was labeled after a 30 min incubation with HRP at 10°C in the dark (Townes-Anderson et al., 1985). The important qualitative finding, however, is that in both cases this uptake was reduced by light; thus, these isolated neurons are able to recycle synaptic vesicles and probably release transmitter by exocytosis in the dark.

In contrast to young neurons, mature retinal neurons adhere poorly to standard culture substrates. This is true not only for isolated rabbit photoreceptors but also for retinal neurons dissociated from the mature salamander (MacLeish et al., 1983). For culture of mammalian rod cells, the most effective substrate was glutaraldehyde-fixed collagen gel. Most probably, glutaraldehyde works as a cross-linking agent, binding to amino groups on both collagen and the rod cell plasmalemma (Habeeb and Hiramoto, 1968). Other more physiological agents, such as fibronectin, chondroitin sulfate, and hyaluronic acid, were ineffective as adhesive agents, even though some of them have been reported to be present in the interphotoreceptor matrix of mammalian retinas (Bach and Berman, 1971; Edwards, 1982; Porrello and LaVail, 1984). Either rod cells do not possess membrane receptors for these extracellular molecules or the density of receptors is too low to provide adequate adhesion. In contrast, a solution of vitreous humor, which contained a variety of extracellular matrix components, especially hyaluronic acid (Boruchoff and Woodin, 1956), did provide moderate adhesion. Finally, the endfeet of Müller cells bound selectively to laminin, a component of basal laminae (Foidart et al., 1980). In the intact retina, these expanded glial processes lie adjacent to the inner limiting membrane, a layer of dense material resembling a basal lamina that separates the retina from the vitreous body. Thus, the binding of dissociated adult retinal cells correlates well with their topographical relationships as observed *in vivo*. It also underscores the differences between embryonic and mature retinal neurons: when laminin is used as a culture substrate for immature retinal neurons, it promotes both adhesion and process growth (Rogers et al., 1983; Smalheiser et al., 1984). In the adult retina, the selective association of laminin with Müller cells could provide a means for obtaining pure glial or neuronal cell populations.

Our results confirm the importance of adequate cell-substrate adhesion for the maintenance of adult central nervous system neurons in culture. In other systems, monoclonal antibodies have been used successfully as substrates in studies on growth of dissociated retinal neurons *in vitro* (MacLeish et al., 1983; Leifer et al., 1984). In the future, it may be expedient to develop antibodies as substrates for the culture of adult mammalian retinal neurons.

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