Maintenance of Opsin Density in Photoreceptor Outer Segments of Retinoid-Deprived Rats

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Dietary deficiency in the retinoid precursors of the visual pigment chromophore 11-cis-retinal eventually results in selective degeneration of the photoreceptor cells of the vertebrate retina. Early effects of retinoid deficiency are depletion of rhodopsin from the retina and vesiculation of the photoreceptor outer segment disc membranes. Experiments were conducted to determine whether these early changes were accompanied by an alteration of the opsin content of the disc membranes. After being fed a retinoid-deficient diet containing retinoic acid for 26 weeks, the rhodopsin content of rat retinas was reduced by over 85%. Both the diameters and the lengths of the outer segments decreased significantly. However, immunocytochemical and freeze-fracture analyses indicated that retinoid deficiency did not lower opsin density in the outer-segment disc membranes. These findings indicate that in the rat, opsin synthesis and disc assembly are coordinated processes that remain coupled despite reduced availability of the vitamin A chromophore. The fact that disc size decreases and disc synthesis eventually ceases in retinoid-deprived rats indicates that specific retinoids are essential for disc morphogenesis. The mechanism by which these retinoids regulate disc assembly remains to be determined. Invest Ophthalmol Vis Sci 32:1968–1980, 1991

Aldehyde forms of retinoids act as chromophores for the visual pigment proteins in the photoreceptor cells of all vertebrates and invertebrates studied.1–5 Dietary deficiencies in the retinoid precursors for vitamin A aldehydes lead to substantial reductions in the visual pigment content of the vertebrate retina.6,7 In several insect species, chromophore deficiency results in specific reductions in visual pigment protein synthesis.7–11 Production of the membranes in which this protein is normally embedded continues in the photoreceptor cells of retinoid-deprived insects, but the opsin content of these membranes is drastically reduced.9,12,13 Thus, photoreceptor membrane morphogenesis and visual pigment protein synthesis do not appear to be tightly coupled in insects.

In mammals, retinoid deficiency may cause the photoreceptor cells to stop producing the entire specialized organelle (the outer segment) that contains the visual pigment.6 It is not known, however, whether retinoids directly regulate all the metabolic processes involved in outer segment synthesis, or whether cessation of visual pigment protein synthesis is the primary response to retinoid deficiency, with outer segment disappearance occurring as a secondary event. A relatively early effect of retinoid deprivation is the vesiculation of the outer segment membranes.7 This change in the membranes that contain the visual pigment suggests that retinoids act in some way to stabilize normal outer segment structure. It is possible that, as in insects, retinoid deprivation impairs incorporation of opsin into newly formed outer segment membranes. Reduced opsin density could lead, in turn, to membrane instability and thus be the cause of the observed vesiculation. Experiments were conducted to evaluate this possibility by determining whether the density of visual pigment protein in the photoreceptor outer segment membranes of rats is altered as a result of retinoid deprivation.

Materials and Methods

Animals and Diets

Male Fischer 344 albino rats were obtained at 21 days of age from Harlan Sprague-Dawley, Inc. (Indianapolis, IN). On arrival, the animals were divided into two treatment groups. One group was fed a syn-
thetic diet that contained adequate levels of all nutrients known to be required by the rat.14 This diet (+A) contained vitamin A in the form of retinyl palmitate, which can be metabolized to all of the retinoids involved in the visual process and used by other tissues. The remaining rats were fed an identical diet, except that retinoic acid was substituted for retinyl palmitate (−A).14 Retinoic acid can satisfy the metabolic requirements of most tissues for vitamin A but cannot be converted metabolically into the retinoids involved in vision. The animals were all housed in the same room under 12-hr/12-hr cyclic illumination. Illumination was provided by 75-W incandescent bulbs. Light levels were determined with a Lutron model LX-101 light meter with the probe placed face up on the bottoms of the animal cages. During the light phase of the lighting cycle, average luminance measured at the cage bottoms was 10–20 lux. This level of illumination was low enough so that no apparent light-induced damage to the retinas occurred during the course of the experiments.15,16 Total darkness was maintained during each 12-hr dark cycle. The room in which the animals were housed was maintained at a constant temperature of 20°C. All investigations were done in accordance with the ARVO Resolution on the Use of Animals in Research.

Rhodopsin Determinations

To monitor retinoid depletion from the retina, the amount of the visual pigment rhodopsin was measured in the eyes of rats fed these diets for various time intervals. Rhodopsin measurements were done with a modification of a widely used spectrophotometric technique.7 The rats were placed in total darkness for a minimum of 24 hr. All subsequent procedures were done under dim red light unless otherwise noted. The animals were killed with CO2 gas, and their eyes were enucleated immediately. The lens and vitreous were removed from each eye through a slit in the cornea. The rest of the eye was cut in several pieces into 700 μm thick sections. All operations were done under dim red light unless otherwise noted. The retinas were placed in 4% formaldehyde, 85 mM sodium phosphate, pH 7.2, and fixed for 5 hr at 4°C. After fixation, the retinas were washed three times with 85 mM sodium phosphate, pH 7.2. The tissues were then each suspended in 3.0 ml of the same buffer (made 0.2 M in HCl) to give a pH of 1.4–1.5. After 15 min of incubation in this acidic medium, 60 mg of borane dimethylamine (BDMA) was added to one sample from each animal with gentle agitation. No BDMA was added to the other retina from each rat. All retinas were then incubated in the dark for 1 hr at 4°C followed by 1 hr at room temperature. Each retina was then washed five times with 85 mM sodium phosphate, 3% sucrose, pH 7.2. The washed tissues were embedded in Tissue Tek medium and frozen. Sections of the retinas were cut at a thickness of 10 μm on a cryostat (Bright Instrument, Huntingdon, England) and mounted in 33% buffered glycerol. Fluorescence micrographs of the retinal sections were obtained using a Zeiss Photomicroscope I with an epi-fluorescence accessory equipped with a 100-W high-pressure mercury lamp. A Zeiss 487701 filter set consisting of a BP 365/11 exciter filter, an FT 397 barrier filter was used for photomicrography.

Opsin Immunocytochemistry

Immunocytochemical labeling of opsin was used to measure the density of this protein in the photoreceptor outer segment membranes. Rabbit preimmune serum and serum containing antibodies directed against human opsin were obtained from Dr. Paul Hargrave. The polyclonal antiserum was tested against cyanogen bromide peptide fragments of opsin and was found to be reactive mainly to the amino terminus of the protein.19 Immunoglobulin G (IgG) of the preimmune and antiopsin antisera was purified on a protein A-Sepharose column (Pierce, Rockford, IL) and stored at −70°C until used.
Rats were killed with CO₂ gas 2.5–3 hr after the onset of the light phase of the daily lighting cycle. Their eyes were enucleated immediately and placed in a fixative consisting of 1% paraformaldehyde, 1.25% glutaraldehyde, 130 mM sodium cacodylate, and 130 μM CaCl₂ at pH 7.4. With the eyes immersed in the fixative, the corneas, irises, and lenses were removed. The remaining portions of the eyes were kept in the fixative at room temperature and gently agitated for 2 hr. The tissues were then stored in the fixative at 4°C for approximately 20 hr.

After fixation, a region including and extending approximately 2.5 mm superior to the optic nerve head was dissected from each eyecup. The dissections were done such that one edge of each tissue block was an arc of the superior–inferior meridian that passed through the optic nerve head. The tissue blocks were embedded such that sections would be obtained along this meridian. The tissue was washed briefly with 0.17 M sodium cacodylate at pH 7.4, and then four times for 3 min each in 50 mM sodium phosphate, 7% sucrose at pH 7.4. After washing, the samples were embedded in flat embedding molds with LR White resin.²⁰

Thin sections of the retina 1–2 mm superior to the optic nerve head along the superior–inferior meridian were obtained from each sample and collected on Formvar-coated gold grids. Nonspecific binding sites on the sections were blocked by incubation with 4% bovine serum albumin in 100 mM Tris, pH 7.4, for 120 min. The grids were then incubated with either rabbit preimmune serum IgG or antiopsin IgG diluted to a protein concentration of 5 μg/ml with 100 mM Tris buffer. They were incubated for 4 hr at 4°C. After washing in 100 mM Tris, 150 mM NaCl, pH 7.4 (TBS), the sections were incubated with *Staphylococcus aureus* protein A conjugated to 10-nm gold particles (Janssen, Beerse, Belgium) as described previously.¹⁹ The sections were then washed with TBS, followed by distilled water, and stained with uranyl acetate and lead citrate. Electron micrographs of the treated sections were obtained at a magnification of 20,000 times with a JEOL 1200EX microscope. This microscope was used for all ultrastructural analyses in our study. The density of the gold particles bound to the photoreceptor outer segment membranes was determined with an Analytical Imaging Concepts computer-based image-analysis system. Morphometric analyses were done in a masked fashion; the examiner had no knowledge of the treatment group from which each micrograph was taken. A minimum of 10 μm² of outer segment membrane area from each animal was analyzed. The analyzed areas were restricted to the basal half of the outer segments.

**Freeze-Fracture Analysis**

In addition to opsin immunocytochemical labeling, freeze-fracture analysis was used to assess the effect of retinoid deficiency on opsin density in the photoreceptor outer segment disc membranes. Opsin constitutes approximately 90% of the intrinsic membrane protein in the disc membranes of vertebrate photoreceptors.²¹,²² Thus, the density of intramembrane particles (as revealed by freeze-fracture preparations) can be used to indicate opsin density in the disc membranes.

After 26 weeks of treatment rats from each dietary group were killed with CO₂ gas 2.5–3 hr after the onset of the light phase of the daily lighting cycle. Their eyes were enucleated immediately, and after removal of the corneas, irises, and lenses, the neural retinas were dissected from the eyecups. The retinas were fixed in 1% glutaraldehyde, 0.17 M sodium cacodylate, pH 7.4, at room temperature for 20 min. After fixation, the tissues were washed four times with 0.17 M sodium cacodylate, pH 7.4, and then each suspended in 4 ml of the latter buffer. Glycerol was slowly added to each sample over 2 hr until a final glycerol concentration of 30% was achieved. During the glycerol additions, the samples underwent constant gentle agitation.

With the retinas immersed in 30% glycerol, the central region of each tissue was dissected into small pieces each approximately 1-mm square. Each piece was mounted on a gold specimen holder with the photoreceptor outer segments facing up. The samples were frozen rapidly by immersion in liquid freon cooled with liquid nitrogen and then were transferred to liquid nitrogen for storage. The frozen samples were fractured, and carbonplatinum replicas were prepared using a Balzers 400 freeze-fracture apparatus. The replicas were freed from tissue in a chromic acid–sulfuric acid solution, washed with distilled water, and collected on uncoated copper grids.

Electron micrographs of regions of the replicas that represented outer segments fractured perpendicular to their long axes were obtained at a magnification of 50,000 times. Particle densities in the inner leaflets of the outer segment disc membranes were determined from the micrographs using the computer-assisted image-analysis system. Because of their proximity to one another, many particles appeared to be fused. The number of particles in each fused structure was estimated by dividing the total area of the fused particles by the average area of an individual membrane particle. A minimum of 0.26 μm² of disc membrane area per animal was analyzed to determine freeze-fracture particle densities. Particle densities were analyzed by a single observer in a masked fashion.
Photoreceptor Outer Segment Dimensions

Measurements were done to determine whether vitamin A deficiency resulted in an alteration in the dimensions of the rod outer segments. The rats were killed, and their eyes were fixed as described for opsin immunocytochemistry. The eyes were then dissected to obtain the regions on either side of the superior–inferior meridian extending between approximately 0.5 and 2.5 mm superior to the optic nerve head. These tissues were washed in 0.17 M sodium cacodylate (pH 7.4), postfixed in 1% OsO₄, and embedded in Epon-araldite resin. To determine the rod outer segment diameters, ultrathin sections of the retina were cut in a plane perpendicular to the long axes of the photoreceptors, and the sections were stained with uranyl acetate and lead citrate. Outer segment cross-sectional areas were determined from electron micrographs of these sections using a JAVA computer-based image-analysis system. Only those outer segments with circular cross-sectional profiles were included in the analyses. Tangentially sectioned outer segments were excluded by measuring cross-sectional areas of only those outer segments in which the ratio of the longest axis to the shortest axis of the outer segment profile was less than 1.20. Rod outer segment lengths were determined in the region of the retina adjacent to that used for cross-sectional diameter measurements. Ultrathin sections of the embedded tissues were cut in planes parallel to the long axes of the photoreceptors. The sections were stained and photographed as described, and the outer segment lengths were measured with the assistance of the JAVA image-analysis system. Length was determined only on those outer segment profiles that could be traced from the inner segments to the apical side of the retinal pigment epithelium. A minimum of 28 outer segment cross-sectional areas and 15 outer segment lengths were measured from each retina.

The retinas of some animals also were examined using scanning electron microscopy. The eyes were enucleated 2.5–3 hr after the onset of the light phase of the daily cycle. The retinas were dissected immediately from the eyes and placed in the mixed-aldehyde fixative described earlier. After 24 hr of fixation, the retinas were cut into several pieces, washed in 0.17 M sodium cacodylate buffer (pH 7.4), and postfixed with OsO₄ for 1 hr. The retinas were then dried using the critical-point method, mounted on stubs, and sputter coated with gold. The coated specimens were examined and photographed with a model S-570 Hitachi scanning electron microscope.

Statistical Analyses

We determined whether age and diet had significant influences on the measured parameters using analysis of variance. Individual treatment groups were compared using the Newman-Keuls procedure.

Results

Rhodopsin Depletion

Retinoid deficiency resulted in a reduction in retinal rhodopsin content by an average of 50% after 10 weeks (P < 0.001, Fig. 1). After 26 weeks of retinoid deficiency, the mean amount of retinal rhodopsin was reduced to about 14% of that present in animals fed the diet containing vitamin A precursors for visual pigment synthesis (P < 0.001, Fig. 1). The presence of this residual rhodopsin was visualized in the rod outer segments by fluorescence microscopy after treatment of the retinas with the reducing agent BDMA (Fig. 2). The intensity of BDMA-induced fluorescence in the photoreceptor outer segments was proportional to the amount of rhodopsin measured in the retinas with the spectral technique. Retinas that were not treated with BDMA had no detectable fluorescence in the outer segments.

Immunocytochemical and Freeze-Fracture Measurement of Opsin Density

The antiserum directed against human opsin reacted with apparently high affinity and specificity to opsin in the rat retina. There was heavy labeling by the antiopsin serum of the photoreceptor outer seg-

![Fig. 1. Influence of dietary vitamin A on retinal rhodopsin content. After 10 weeks, rats fed the -A diet had only about 50% as much rhodopsin per eye as did the +A animals. By 26 weeks, retinal rhodopsin content in the -A rats was reduced to a mean of 14% of that present in the +A animals.](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933388/)
Fig. 2. Fluorescence micrographs of cryostat sections of retinas that had been treated with BDMA to convert rhodopsin to a fluorescent reduced form. The retinas were from rats that had been fed the +A diet (A) or the −A diet (B) for 26 weeks. The photoreceptor outer segments of the +A rats displayed a bright rhodopsin-specific fluorescence (A), whereas the outer segment fluorescence was much weaker in the −A animals (B).

Despite the substantial reduction in retinal rhodopsin content that occurred as a result of retinoid deficiency, the density of outer segment labeling by an antiopsin antibody was not reduced in the retinoid-deprived animals (Figs. 4, 5). After 26 weeks the mean labeling density was actually about 20% higher in the rats fed the −A diet than in the rats that received retinyl palmitate in their diet ($P < 0.05$). Freeze-fracture analysis confirmed that the opsin density in the outer segment disc membranes was not reduced by retinoid deprivation. After 26 weeks of treatment, the density of intramembrane particles present in freeze-fracture replicas of the disc membranes appeared to be similar in both dietary groups (Fig. 6). Estimates indicated that after 26 weeks, 19,097 ± 240 particles per µm² were present in the disc membranes of the animals fed the +A diet. The corresponding particle density in the rats fed the −A diet was 20,105 ± 233 particles per µm². Thus, retinoid deficiency resulted in an increase in particle density of approximately 5% ($P < 0.05$).

Fig. 3. Electron micrographs illustrating the specificity of the immunogold staining technique. Retina sections incubated with antiopsin antibody and protein A-gold showed labeling only in the photoreceptor outer segments; other parts of the retina, including the photoreceptor inner segments, were unlabeled (A). Phagosomes in the RPE also were labeled with antiopsin antibody (arrow in B). When retinas were incubated with preimmune serum instead of antiopsin antiserum, protein A-gold did not bind to the photoreceptor outer segments (C). Bar in (B) indicates the magnification of the micrographs in both (B) and (C).
Fig. 4. Electron micrographs showing immunogold labeling of rod outer segment opsin in retinas from rats that had been fed the +A diet (A) or the −A diet (B) for 26 weeks.

Fig. 5. Effect of retinoid deficiency on opsin immunogold staining density of rod outer segments. The staining density remained constant throughout the experiment in the +A animals, but actually increased somewhat between 10 and 26 weeks in the −A rats. By 26 weeks, the mean staining density in the −A group was approximately 20% higher than in the +A rats.

**Outer Segment Dimensions**

Ultrastructural examination of the retinas of the rats fed the −A diet indicated that there was more space between adjacent outer segments than was present in the retinas of rats fed the +A diet (Figs. 7, 8). After 26 weeks of retinoid deficiency, no significant photoreceptor cell loss had occurred in the animals fed the −A diet, as determined by photoreceptor nuclei densities. Thus, the increase in the amount of space between outer segments was either due to a specific loss of outer segments from some photoreceptors without loss of the cell nuclei, or to a decrease in outer segment diameter. The cross-sectional areas of the outer segments were found to be substantially reduced in the animals fed the −A diet (Figs. 9, 10). After 26 weeks, the mean outer segment cross-sectional area in these rats was only about 58% of that in the animals fed the +A diet (P < 0.001). This indicates that the sizes of the discs synthesized by the photoreceptor cells were reduced as a result of retinoid deprivation. In addition, mean outer segment lengths were reduced in the rats fed the −A diet (Figs. 7, 11). After 26 weeks, the mean rod outer segment length in
the deprived rats was 27% less than in the animals fed the +A diet ($P < 0.01$).

Discussion

The present study confirmed and extended previous data indicating that, during vitamin A depletion in the rat, rod outer segments accumulate opsin in excess of available chromophore. After being fed the retinoid-deficient diet for 26 weeks, the amount of rhodopsin in the retina was reduced an average of 86%. Based on measurements in the posterior retina, freeze-fracture analysis indicated that opsin density in the disc membranes increased an average of 5%, whereas immunocytochemical techniques suggested an increase in opsin density of about 19% in the retinoid-deprived rats. In the same region, the mean volume of the outer segments decreased by 58%. Assuming all regions of the retina were affected equally by vitamin A deprivation, the amount of op-
Fig. 7. Electron micrographs of the outer segment regions of retinas from rats that had been fed the +A diet (A) or the −A diet (B) for 26 weeks. The outer segments of the −A animals were shorter, more convoluted, and less tightly packed than those of the +A rats.

Fig. 8. Scanning electron micrographs of cross-sections of the retinas from rats that had been fed the +A diet (A) or the −A diet (B) for 26 weeks. The rod outer segments of the +A animals were long and straight and were arranged in a tightly packed parallel array. In the −A rats, the outer segments were shorter, thinner, more loosely packed, convoluted, and less regular in appearance.
Fig. 9. Electron micrographs of rod outer segment cross-sections cut in planes perpendicular to the long axes of the photoreceptor cells. After 26 weeks, the diameters of the outer segments of the −A rats (B) were much less than those of the +A animals.

Fig. 10. Effect of retinoid deficiency on rod outer segment (ROS) cross-sectional area. After 10 weeks, mean ROS cross-sectional area was reduced by 13% in the −A rats. After 26 weeks, retinoid deficiency resulted in a mean reduction in ROS cross-sectional area of approximately 42%.

sin therefore was reduced by a maximum of about 53% in the deficient animals after 26 weeks. Thus, even in the dark-adapted state, at least 70% of the opsin in the photoreceptor outer segments was chromophore-free. This finding is consistent with studies of others6,7 who reported that opsin depletion from the retinas of vitamin A-deprived rats lags significantly behind the depletion of rhodopsin.

Dowling and Wald reported that vitamin A deprivation in rats resulted in a fairly rapid fall in retinal rhodopsin levels once blood levels of retinol were depleted.6 Only after a substantial reduction in the amount of rhodopsin did opsin levels begin to fall. The diets used by these authors were deficient not only in the retinoids involved in the visual process, but also in retinoic acid. As a result, not only did the retinas of the vitamin A-deprived animals degenerate, but so did supporting tissues, including the retinal pigment epithelium. Thus, it was not possible from their experiments to determine whether the lack of availability of the retinoids involved in visual transduction was directly responsible for the loss of opsin. The latter was, however, shown to be the case by
others, who included retinoic acid in the diets of their vitamin A-deprived animals. In these experiments opsin levels declined but never disappeared. From the animal studies done to date, the retina has been found to be able (at least temporarily) to synthesize outer segment membranes containing opsin in excess of the amount of chromophore available.

Observations on night blindness resulting from vitamin A deficiency in humans also suggested that a significant amount of chromophore-free visual pigment protein can be present in the outer segment membrane in dark-adapted individuals. In some subjects deprived of vitamin A until a mild night blindness occurred, oral administration of vitamin A resulted in a return of both rod and cone visual thresholds to normal within several hours. In addition, human subjects whose tissues are vitamin A deficient as a result of abetalipoproteinemia also have their greatly reduced visual sensitivities enhanced rapidly by massive oral doses of vitamin A. A similar rapid recovery of visual sensitivity has also been reported in cats after giving retinoid-deprived animals oral vitamin A supplements. In all cases, the recovery of visual sensitivity was much more rapid than could be accounted for by new visual pigment protein synthesis. Thus, it appeared that the photoreceptors were capable of incorporating more visual pigment protein into the outer segment membranes than there was chromophore available to convert this protein into functional visual pigments. The present experiments confirm that this is the case, as do observations by Engbretson and colleagues, who reported that rod outer segments grow normally with a full complement of opsin in severely vitamin A-deficient Xenopus tadpoles.

The decrease in photoreceptor outer segment length observed in the retinoid-deprived rats was similar to that reported by Carter-Dawson and colleagues. Little is known about regulation of photoreceptor outer segment length. A stable outer segment length results from a balance between rates of new disc synthesis by the photoreceptors and old disc phagocytosis by the retinal pigment epithelium. Because outer segment renewal is relatively rapid, with complete turnover occurring approximately every 10 days in mammals, a small persistent or a larger transient change in the balance between these two processes would result in the observed change in outer segment length. The rate of new outer segment disc synthesis was reported to be slowed in vitamin A-deprived rats. The decreased outer segment length therefore could occur as a consequence of vitamin A deficiency having failed to cause a corresponding decrease in the rate of outer segment phagocytosis by the retinal pigment epithelium that matched the decrease in disc synthesis either chronologically or in magnitude.

Retinoid availability is not the only factor that has been shown to affect photoreceptor outer segment length. Maintaining animals under bright cyclic light was also found to result in a significant shortening of the rod outer segments in rats. It is possible that the mechanism by which the light effect occurs is through light-induced local retinoid deficiency in the retina. Unlike retinoid deficiency, however, bright cyclic light exposure also resulted in an apparent reduction in the opsin packing density in the outer segment membranes. The latter finding suggests that light and retinoid availability regulate photoreceptor outer segment length and composition by at least partially independent mechanisms.

In a previous study, the amounts of vitamin A esters in the retinal pigment epithelium were measured in retinoid-deprived and retinoid-supplemented rats after totally bleaching the retinal rhodopsin in vivo. Based on these measurements and the rhodopsin determinations in this study, we estimate that approximately 52% of the vitamin A released during rhodopsin bleaching was captured and esterified to fatty acids by the retinal pigment epithelium of the retinoid-supplemented rats. Only 43% of the released vitamin A was esterified in the retinoid-deprived animals. This suggests that either transport of vitamin A from the retina to the retinal pigment epithelium or metabolism of vitamin A by the retinal pigment epithelium is impaired as a consequence of retinoid deprivation. It is possible that in addition to reducing disc synthesis, retinoid deficiency results in a
reduction in the synthesis of interphotoreceptor retinoid-binding protein (IRBP) by the photoreceptor cells. Reduction in the amount of IRBP, purported to be involved in the transport of retinoids between the retina and the retinal pigment epithelium, could exacerbate the effects of retinoid deficiency on the photoreceptor cells.

Freeze-fracture analysis indicated that there were an average of approximately 20,000 intramembrane particles per \( \mu m^2 \) in the outer segment disc membranes of both dietary groups. Thus, each particle with its associated lipids occupied an area of about 50 nm\(^2\). The cross-sectional area of a single opsin molecule has been estimated as approximately 7 nm\(^2\). Thus, it is possible that each particle represents an aggregate of several opsin molecules, as has been suggested. Since no effect of retinoid depletion on particle size was observed in our experiment, the observed similarity in particle densities in the retinoid-deprived and retinoid-supplemented rat outer segments indicate that the opsin densities were therefore similar. Particle density was also found to be normal in the rhodopsin-depleted outer segments of retinoid-deprived Xenopus tadpoles. The labeling density of the rat outer segments with the antiopsin antibody was much lower than the opsin density found by freeze-fracture analysis (Fig. 5). However, immunocytochemical labeling densities of insect photoreceptors with antiopsin antibodies were similar to those reported in our experiment and were proportional to the amounts of opsin measured in the photoreceptors by other independent techniques. Thus, the immunocytochemical analyses appear to provide a good quantitative comparison of relative opsin densities in the different treatment groups.

The effects of retinoid deprivation on opsin synthesis and the assembly of photosensitive membrane have been studied in several insect species. Insect photoreceptor cells contain arrays of microvilli named rhabdomeres that are analogous to the photoreceptor outer segment disc membranes of vertebrates. In insects, incorporation of opsin into the rhabdomere outer segments indicates that the opsin densities were therefore similar. Particle density was also found to be normal in the rhabdomere membrane. Chromophore-free opsin in the rhabdomere membrane would be useless in the visual process. Therefore, the requirement of chromophore to opsin already present in the photoreceptor membranes as occurs in vertebrates. The fact that retinoid deprivation reduces the opsin content of rhabdomere membranes in insects indicates that chromophore availability regulates deployment of opsin to the rhabdomeres independently of rhabdomere membrane morphogenesis. Our findings show that, in rats however, regulation of opsin deployment to the outer segment is tied closely to the control of disc membrane morphogenesis. It is possible that, in order for opsin to be inserted into nascent photoreceptor membranes in insects, the protein must have chromophore bound to it, whereas membrane bearing chromophore-free opsin may be assembled into the outer segment discs in vertebrates. Indeed, it has been reported that \(^3\)H-vitamin A is not incorporated into opsin in the photoreceptor inner segments, suggesting that the retinal chromophore is not attached to opsin until late in the disc assembly process. More recent evidence suggests that the chromophore is added to opsin in the inner segment shortly after the apoprotein is translated in the rat, but this may not be an essential step in the intracellular transport of opsin and assembly of disc membranes. Our findings do not exclude the possibility that incorporation of the chromophore into the opsin molecule is required for vertebrate photoreceptor disc membrane assembly. Since sufficient chromophore is available after 26 weeks to saturate up to 30% of the available opsin in the outer segments, there is obviously enough retinoid available to support synthesis of new disc membranes containing rhodopsin. Once the rhodopsin has been incorporated into the membrane, the chromophore can be removed easily by bleaching and can even be exchanged in the dark. Thus, sufficient free chromophore could be available for supporting new disc membrane synthesis, although at a lower than normal rate. The finding that the amount of disc membrane synthesized was reduced in the retinoid-deprived rats indicates that the chromophore is required for the overall process of disc morphogenesis.

The differences between vertebrates and insects in the effects of retinoid deprivation on opsin incorporation into photoreceptor membranes may be related to differences in the visual cycles in these two groups of organisms. Unlike the situation in vertebrates, insect visual pigments do not release their chromophores in response to light exposure, but rather form stable meta-rhodopsins. Thus these animals apparently have not evolved a mechanism to add chromophore to the visual pigment protein once it has been incorporated into the rhabdomere membrane.
ment that the retinoid chromophore be present for opsin insertion into the rhabdomere is advantageous in insects. In vertebrates, exposure of the photoreceptors to light results in the release of the retinoid chromophore from the protein component of the visual pigment. Thus, in the light a certain proportion of the visual pigment protein is always chromophore free. The visual pigment can be regenerated quickly on application of the appropriate form of vitamin A to the chromophore-free protein. Unlike the case in insects, therefore, it would be adaptive for vertebrates to incorporate visual pigment protein into the photoreceptor disc membranes even in the absence of sufficient chromophore to convert all of this protein into functional pigment.

It has been demonstrated that retinoid deficiency in rats resulted in a reduction in the amount of outer segment disc membrane synthesized without lowering the opsin density in the disc membranes. The contrasting ability of retinoid-deprived insects to synthesize rhabdomere membranes with reduced opsin content apparently reflects fundamental differences in the pathways of photoreceptor membrane assembly. In insects, chromophore availability regulates deployment of opsin to the rhabdomere but not rhabdomere membrane synthesis. In the rat, the entire process of disc membrane assembly appears to be regulated coordinately by the availability of specific retinoids. Further investigation will be required to elucidate the exact mechanisms by which these retinoids regulate disc morphogenesis.

Key words: vitamin A, rhodopsin, retina, immunocytochemistry, disc membranes

Acknowledgments

The authors thank J. Scott Christianson and Linnette Maier for assistance with the morphometric analyses, Dr. Stanley D. Carlson and Melissa Curtis for assistance with scanning electron microscopy, Dr. Paul Hargrave for supplying the antiopsin antibody, and Dr. Paul K. Brown and Dr. Ruth R. Bennett for helpful comments on the manuscript.

References


