A Diurnal Rhythm in Opsin Content of *Rana pipiens* Rod Inner Segments

Alan C. Bird,** John G. Flannery,**† and Dean Bok**††

Quantitative electron microscope immunocytochemistry, employing an antibody specific to opsin, was used to evaluate the amount and location of opsin in *Rana pipiens* rod photoreceptors throughout a 24 hr light/dark cycle. We found a distinct diurnal rhythm in the density of anti-opsin labeling of the rough endoplasmic reticulum (RER) and Golgi apparatus in the myoid region of the rod inner segment. Opsin labeling of these organelles was lowest at light onset, increasing thereafter by three- to four-fold, and remained high until 2 hr into the dark phase. A fall in labeling density occurred within the following 4 hr, and remained low for the remainder of the dark phase. Our finding of a diurnal rhythm regulating inner segment opsin transport in *Rana pipiens* contrasts with published observations on outer segment membrane turnover, since it has been shown that the rates of disc formation and disc shedding are governed by environmental lighting alone in this species. These results imply that there is opsin pooling in the inner segment during the first 14 hr of a 24 hr light/dark cycle; thereafter the loss of inner segment opsin due to mobilization of this protein from the Golgi exceeds the rate of formation of new opsin. There was no evidence of accumulation of opsin-containing vesicles near the cilium or in the ellipsoid just prior to light onset. At light onset, prominent opsin labeling was identified at the proximal portion of the outer segment in regions separate from the disc stack. In two separate experiments, additional groups of frogs were killed around the time of light onset and were examined by conventional transmission electron microscopy. Disordered disc membranes were seen at the base of the outer segment which were not in register with the disc stack. These disordered membranes were observed as early as 2 hr before light onset, and were no longer observed by 1 hr after light onset. We suggest that these disordered membranes reflect a step in the biogenesis of new discs, serving as a pool of membrane that forms during the later part of the dark cycle. It appears that light onset triggers the ordering of neatly registered discs from this new membrane, rather than assembly of new membrane from pooled transport vesicles in the inner segment. Invest Ophthalmol Vis Sci 29:1028-1039, 1988

Two different mechanisms have been demonstrated to regulate disc shedding from rod outer segments in vertebrates. Both of these mechanisms are cyclic, being regulated by light, or an intrinsic circadian rhythm. The assembly of new discs at the base of the outer segment has been shown to occur with similar periodic characteristics. The cyclic regulation of biosynthesis of opsin, the major disc membrane intrinsic protein, has been examined in two amphibian species. Opsin biosynthesis was reported to be constant throughout the light/dark cycle in *Xenopus laevis* eyecups maintained in vitro by Hollyfield et al. By contrast, opsin synthesis was found to vary in *Rana pipiens* by Matsumoto and Bok. In the latter study, the specific activity of newly synthesized protein changed by as much as 13-fold, although changes in outer segment opsin-specific activity suggested only a two-fold increase in synthesis.

The disparity in results between *R. pipiens* and *X. laevis* may reflect major differences in the regulatory mechanisms controlling opsin metabolism in these two species. Alternatively, the changes in nascent opsin-specific activity in *R. pipiens* could be attributed to fluctuations in either opsin biosynthesis or the pool size of inner segment opsin during the diurnal cycle. Enhanced levels of messenger RNA controlling opsin synthesis following light onset have been reported recently in fish and toads. Taken together with the observed variation in the specific activity of outer segment opsin during the diurnal cycle, this suggests strongly that part of the change in nascent

From the **Institute of Ophthalmology, Moorfields Eye Hospital, London, England, and the †Jules Stein Eye Institute and ‡Department of Anatomy, UCLA School of Medicine, Los Angeles, California.

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Reprint requests: Dean Bok, Jules Stein Eye Institute, UCLA School of Medicine, Los Angeles, CA 90024.
opsin-specific activity is due to fluctuations in synthesis. The results would also be more compatible if there were large diurnal changes in the opsin pool in the inner segments of *R. pipiens* at certain times in the light/dark cycle but not in *X. laevis*. The possibility of inner segment opsin pooling in *R. pipiens* has been suggested,\textsuperscript{20} but has not been examined to date. In order to test this hypothesis, we used electron microscopy immunocytochemical localization to quantitate the synthesis and delivery of opsin from the rough endoplasmic reticulum (RER) to the outer segment.

The results of this initial study caused us to undertake a second series of observations on the relationship of outer segment membrane assembly to disc production around the time of light onset.

**Materials and Methods**

**Experimental Animals For Immunocytochemical Analysis and Standard Electron Microscopy**

Two separate and complete groups of animals were prepared for immunocytochemical examination. In each experiment, adult *R. pipiens* were entrained to a 12 hr light/12 hr dark cycle for a 3 week period, with light onset occurring at 7 AM. The frogs were maintained under cyclic illumination of 32 foot-candles provided by incandescent bulbs, and were kept at a constant temperature of 20°C. The exact time of light onset and offset were confirmed by a separate photocell and chart recorder. The frogs were fed live mealworms weekly, and were treated in accordance with the ARVO Resolution on the Use of Animals in Research.

In the first experiment, one frog was killed at each of six time points during the light dark cycle: 7 AM, 7:30 AM, 8:30 AM, 11 AM, 3 PM and 9 PM, which are 0, 0.5, 1.5, 4, 8, and 14 hr after the onset of light, respectively. One eye of each frog was processed for electron microscopic immunocytochemistry.

In the second experiment, two frogs were killed at 7 AM, one at 7:30 AM, two at 8:30 AM, one at 11 AM, one at 3 PM, one at 9 PM, two at 11:30 PM, and two at 2:30 PM, which are 0, 0.5, 1.5, 4, 8, 14, 17, 20, hr after light onset, respectively. In this experiment both eyes were used from each animal.

In the final series of observations, two groups of frogs were entrained in an identical manner. In the first group two frogs were killed at 2 and 1 hr before, and 0.5 and 1.5 hr after light onset. In the second, one animal was killed at each of the following time points: 2 hr and 1 hr before light onset, at light onset and 1 and 2 hr after light onset. Both eyes of each animal were processed for standard transmission electron microscopy.

In all experiments, the enucleation and initial processing were undertaken in deep red safelight conditions when frogs were killed during the dark phase.

**Preparation of Affinity-Purified Rabbit Antibodies to Frog Opsin**

Rod outer segments were isolated from 40 fresh frog eyes according to the method of Papermaster and Dreyer.\textsuperscript{22} Opsin was separated from other proteins on a 4 mm thick sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) according to the method of Laemmli,\textsuperscript{23} and the position of the opsin band determined by scanning the gel at 280 nm. The region corresponding to opsin was excised from the gel, and the protein was extracted and further purified by hydroxylapatite chromatography according to previously published methods.\textsuperscript{24} Subsequent SDS-PAGE analysis of the extracted opsin and silver staining of the gel showed no contaminants other than opsin multimers. One hundred micrograms of purified opsin in complete Freund's adjuvant was injected intradermally into each of two rabbits on a weekly basis for 6 weeks. Animals were bled from the marginal ear vein on the fifth, sixth and seventh weeks. The presence and specificity of opsin antibodies were determined by labeled antibody staining of nitrocellulose replicas (Western blots) obtained by electrophoretic transfer of solubilized outer segment proteins from SDS gels.\textsuperscript{25}

Staining of outer segment proteins on Western blots was limited to opsin and its multimers. Specific anti-opsin IgG was affinity-purified from immune serum by coupling purified bovine opsin to cyanogen bromide-activated Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ) following instructions provided by the manufacturer, except that NaCl was omitted from the buffer that was used to bind the antigen. After the serum sample was loaded onto the affinity column and washed to remove unbound proteins, specific IgG was eluted with 4 M MgCl\textsubscript{2} in phosphate-buffered saline (PBS; 10 mM sodium phosphate in 0.9% NaCl). One milliliter fractions were eluted into 5 ml volumes of PBS in order to achieve rapid dilution of the MgCl\textsubscript{2}. The specific IgG was dialyzed against PBS and concentrated prior to use.

**Tissue Preparation and Immunocytochemistry**

At the times specified, frogs were decapitated, the eyes were enucleated and opened, and the tissue was fixed in 2.5% glutaraldehyde/2% formaldehyde for 2 hr, and transferred to 2% formaldehyde for 4 days to enhance antigenicity before standard infiltration and embedding in LR White epoxy resin (Polysciences,
Fig. 1. Electron micrographs of longitudinal sections of *Rana* rod photoreceptor inner segments labeled with rabbit antibody to frog opsin from a frog sacrificed at light onset (a), and 14 hr into the light/dark cycle (b). The density of labeling of Golgi apparatus (arrows) and rough endoplasmic reticulum is much smaller at light onset than at 14 hr. The inner segments are short at light onset. They become progressively longer and narrower during the following 14 hr due to photomechanical movement, after which time they return to the light onset form (×12,500).

Inc., Warrington, PA). Polymerization of LR White was performed at 60°C for 12 hr. Ultrathin sections were mounted on carbon-stabilized formvar-coated copper grids, and incubated on drops of 0.1 M Na phosphate (pH 7.4) containing 5% bovine serum albumin (BSA) for 5 min to block nonspecific binding.
Grids were then labeled for 1 hr using affinity-purified rabbit antibody to frog opsin diluted to 0.1 o.d. in the first experiment, and 0.06 o.d. in the second (A_{280}, 1 cm light path) in 0.1 M Na phosphate containing 0.1% BSA. Grids were washed and transferred for an additional hour to 20 mM Tris buffered saline, (pH 8.2) containing goat anti-rabbit IgG absorbed to 10 nm gold colloid (Janssen Pharmaceuticals, Beerse, Belgium), 0.1% BSA and 2 × 10^{-2} M Na azide. Lastly, grids were contrasted with 5% aqueous uranyl acetate. Grids from all sacrifice times were labeled simultaneously in each experiment.

Ultrathin sections were examined with a JEOL 100C electron microscope (JEOL Inc., Tokyo, Japan) and micrographs were printed to a final magnification of ×15,000. A minimum of two 1 mm² areas of retina were examined for each sacrifice time. The appropriate rod inner segment regions were identified on electron micrographs, their areas measured using a digital planimeter (Laboratory Computer Systems, Cambridge, MA) and the gold particles counted. The colloidal gold particle density over specific organelles was derived from these measurements. Grating replica grids (Ernest Fullam, Inc., Schenectady, NY) were photographed, printed and measured identically in order to calibrate the area measurements.

Quantitative data were analyzed using a VAX 11/780 computer (Digital Equipment Corp., Maynard, MA) with the SAS (Statistical Analysis System) and SAS/Graph software packages (SAS Institute, Cary, NC). The labeling densities were compared by pairwise t-tests between each of the sample times. Errors between each sample time were minimized using K-ratio t-test and the multiple range test for analysis of variance to the α = 0.01 stringency.
Results

Immunocytochemical Studies

The appearance of the inner segments changed during the light/dark cycle. At light onset the inner segment was short and broad (Fig. 1a), and became more elongated as the light cycle progressed (Fig. 1b). Return to the light onset form occurred progressively as the dark phase progressed. These changes were accompanied by movement of pigment granules into epithelial processes after light onset, and their withdrawal into the cell body after light offset. This acted as an internal control for the light and dark adapted state.

Labeling of the Rough Endoplasmic Reticulum (RER)

In both groups of *Rana*, we found the density of labeling over the RER to vary significantly over the course of the light/dark cycle (Fig. 2), being lowest at the time of light onset and rising rapidly soon thereafter to high levels at 4 hr. Highest levels were reached at 14 hr into the L/D cycle before falling again. The exact timing of the downturn in labeling density was not resolved in the first experiment due to our choice of sacrifice times (Fig. 2a). In the second experiment we were able to make an informed choice of sampling times, and the data indicate that the reduction in labeling occurs early in the dark phase of the light/dark cycle (Fig. 2b).

Labeling of the Golgi Apparatus

The Golgi apparatus appeared as a longitudinal labeled membrane system in the myoid region at those time points at which the inner segments were elongated (4 to 17 hr into the cycle) (Fig. 1b). At times when the inner segments were short and squat, part of the Golgi was identified as a transverse structure parallel to the border of the nucleus (Fig. 1a). The Golgi was most heavily labeled 14 hr into the L/D cycle in both experiments, and thereafter the density of labeling fell. The major fall was between 14 hr and light onset in the first experiment (Fig. 3a), and between 14 and 17 hr in the second (Fig. 3b). The lowest levels of density were at light onset, and the main rise at 1.5 to 4 hr.

Labeling of the Ellipsoid Region

Our observation of a rapid reduction in labeling density in the second half of the light/dark cycle for

Tissue Preparation For Ultrastructural Examination

In groups of frogs for standard ultrastructural examination the eyes were fixed in 2% formaldehyde and 2.5% glutaraldehyde in 0.1 M Na phosphate buffer at pH 7.4 for 2 hr, post-fixed in 4% OsO4, dehydrated in a graded series of ethanol, infiltrated in propylene oxide and embedded in Araldite 502 epoxy resin (Ciba Products Co., Summit, NJ). At each time point the number of rods with disordered basal membrane and the number of phagosomes/rod was counted.

Fig. 4. Electron micrograph of a longitudinal section of the ciliary region from a frog fixed 14 hr after light onset (a) and at light onset (b). Vesicles labeled with rabbit antibody to frog opsin antibody (arrows) are observed at both timepoints (X21,000).
both Golgi and RER led us to search for the new locus of the mobilized protein. It was considered that the protein might be found in high concentration in opsin transport vesicles in the ellipsoid region, particularly near the cilium.\textsuperscript{29} Labeled vesicles were found in this region at all times in the cycle (Fig. 4a, b), but were most numerous at the time of high labeling concentration in the Golgi rather than corresponding with the fall in Golgi labeling density. The labeled vesicles appeared to be restricted to a specific linear pathway rather than being evenly distributed with the ellipsoid (Fig. 5).

We measured antibody density over the ellipsoid in order to quantify this observation (Fig. 6). In both groups of frogs these results showed highest labeling at 14 hr, which declined thereafter, being lowest at light onset, before rising again.

**Labeling of the Outer Segment Membrane**

A novel phenomenon was observed concerning receptor membrane at the base of the outer segment at the time of light onset in both experiments. Linear membrane-associated labeling was observed which was separate from the registered disc stack (Fig. 7). In some rods this membrane appeared to form pouches which protruded away from the long axis of the outer segment, and in others it appeared to be invaginated into the inner segment. This membrane configuration was seen in a more restricted form 30 min after light onset, but not thereafter.

**Standard Electron Microscopy**

In order to investigate the significance of this ectopic labeled membrane, two additional groups of frogs were killed around light onset and examined by standard transmission electron microscopy. In some rods, apparently redundant disc membrane was seen at the proximal outer segment which was parallel to, and separated from, the outer segment in some receptors, while in others the membrane appeared to be invaginated into the distal portion of the inner segment (Fig. 8). The number of receptors with ectopic membrane was counted around the time of light onset, and phagosomes were counted as an internal control (Fig. 9). The number of receptors with ectopic

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**Fig. 5.** Electron micrograph of the longitudinal section of a receptor from the time point 4 hr into the light phase labeled with rabbit antibody to frog opsin. The Golgi is labeled (solid arrows), and a track of labeled vesicles (open arrow) extends from the myoid to the cilium (×17,500).
Discussion

Changes in Opsin Concentration

Our results indicate that regular changes in the concentration of opsin in the RER and Golgi apparatus occur during the 24 hr light/dark cycle. Our two experiments are in close agreement on this principle.

We were not able to make a quantitative estimate of the amount of opsin in individual organelles, since the techniques available do not allow an accurate measure of the absolute number of antigens in a given area of a section. Estimates by Griffiths and Hoppeler suggest that the immunogold labeling efficiency over Lowicryl sections of RER and Golgi was 18.4% and 6.6%, respectively. As suggested by Griffiths and Hoppeler, this effect may be due to a difference in affinity of the antibody for the antigen in the different organelles or a differential effect of fixation on the two organelles. However, this does not invalidate measurements of the relative density of an antigen in the same organelle at different time points.

Comparison of Autoradiographic and Immunocytochemical Results in R. pipiens

The observation that the opsin pool in the RER and Golgi apparatus was small in the early stages of the light/dark cycle is compatible with predictions derived from the results of Matsumoto and Bok. These investigators observed a 13-fold increase in inner segment opsin specific activity following light onset. A small opsin pool at light onset which increases with time would have caused the specific activity to be high at early points in the cycle, and would account for a fall thereafter, even if incorporation of labeled amino acids into opsin were constant.

Our conclusions agree qualitatively with those of Matsumoto and Bok in reference to changes in the concentration of opsin in the inner segment. In their experiments the change in specific activity during the course of the diurnal cycle was 13-fold, whereas our measurements only showed changes of three- to four-fold. The quantitative disparity may reflect differences in techniques. It may not be justified to compare immunocytochemical techniques, which analyze only the surface of the tissue (a two-dimensional display), with a technique which allows access to three dimensions, such as autoradiography or chemical extraction.

Other factors may be relevant. At the hours when the tissue was only lightly labeled by the antibody in our study, it was more difficult to define the limits of the Golgi apparatus and it is likely that some unlabelled Golgi was not recognized in these sections. The absence of this unlabelled area would decrease the measured area in comparison with the true area and cause a falsely high calculated density. Overestimation of the density at the low points in the L/D cycle would reduce the identified difference between the observed minimum and maximum densities. For this reason it is possible that our results underestimated the magnitude of variation in the quantity of opsin stored.
in the Golgi apparatus throughout the light/dark cycle.

It is only at the times later than 14 hr into the light cycle that our results do not agree with the predicted behavior of the opsin pool in the Golgi apparatus. We did not expect the level of opsin in the Golgi and RER to fall until much later in the light/dark cycle, perhaps close to the time of light onset. The reduction of opsin pool size identified in this experiment, and the previously reported low specific activity occurring at this time, could only be explained by a lower rate of incorporation of protein into the inner segment during the dark period. The density of label in the RER may give some indication of the rate of opsin formation, although it is clear that this density will represent a balance between rates of formation and mobilization. If the marked lowering of RER labeling observed after 14 hr implies a reduction in opsin
Fig. 8. Electron micrographs of longitudinal sections of the junction of inner and outer segment of osmicated, Araldite-embedded tissue at light onset. Ectopic membrane appears to be invaginating the inner segment (a, c, d) or parallel to the outer segment disc stack (b) (x21,000).
synthesis, this would help explain the dilemma. It is also consistent with lower levels of opsin messenger RNA reported recently for toads and fish.21

Comparison With Autoradiographic Results in X. laevis

Our results support the primary conclusion of Hollyfield and coworkers18 that the increased rate of membrane assembly at light onset is not dependent upon an enhanced rate of opsin synthesis. There is, however, some conflict with the conclusion that protein synthesis is constant during the 12 hr light/dark cycle.19 The results of our study together with the specific activity measurements of Matsumoto and Bok21 imply that the rate of opsin incorporation in the inner segment varies over the course of the light/dark cycle in R. pipiens.

A plausible explanation for the different results is that there is a major difference in opsin metabolism between R. pipiens, on which our observations were made, and X. laevis with which Hollyfield et al.19 worked. These two species differ in their regulatory mechanisms of outer segment shedding and membrane synthesis; in R. pipiens it is believed to be governed by light alone,29 whereas in X. laevis there is a well established intrinsic circadian rhythm.30

However, a major difference exists between the studies in that much of the work on X. laevis was undertaken in vitro. The in vitro situation differed from the in vivo studies in that the 3H-labeled precursors added to the culture medium were continually available for protein synthesis throughout the incubation. This is in contrast to the “pulse” of 3H-precursor which occurs when the 3H-label is injected into whole animal. It is also possible that there is some damping of the intrinsic circadian characteristics in vitro.

That prolonged alteration of lighting conditions may affect the rate of opsin synthesis is not in dispute; several days of prolonged darkness have been shown to cause reduced opsin synthesis in X. laevis,19 and light may enhance opsin incorporation after prolonged darkness in R. pipiens.31

The Destination of the Mobilized Opsin

We sought the site of opsin pooling immediately prior to light onset. There is evidence that opsin is transported in vesicles from the Golgi, and that those vesicles fuse with the apical inner segment plasma membrane prior to being incorporated in the outer segment.28 We therefore expected that the apical inner segment would be a likely pooling site for opsin. We searched for collections of labeled vesicles near the cilium during the later part of the dark phase, but were unable to demonstrate any such accumulation. In addition, examination of the ellipsoid region of the rod at the time when the density of labeling in the Golgi was decreasing did not show any increase of labeled material. The presence of labeled vesicles at all sample times suggests that movement of opsin to the outer segment is continuous, although the rate may vary during the light/dark cycle. The path of delivery through the myoid appears to be along well defined channels.

It seems credible to us that opsin, once transported to the ciliary region, becomes incorporated into outer segment membrane some hours prior to formation of completely ordered discs. Support for this concept was given by our observation of ectopic and misoriented plasma membrane associated with the proximal portion of the outer segment, which was seen only in retinas fixed just prior to light onset or slightly afterward. The appearance of this region in both immunocytochemical and conventional electron mi-
croscopy implies the presence of "redundant" membrane, which is then taken up by the formation of new discs soon after light onset. This membrane appears to consist of double thicknesses of membrane which is continuous with the membrane within the disc stack. It is likely that it serves as a reservoir of membrane for the "burst" of new disc formation which occurs at light onset. If this interpretation is correct, the ordering of disc membrane into its final form is a process distinct from the assembly of disc membrane, the latter occurring during several hours prior to light onset whereas the former is light-activated.

The implication that the mechanism initiating disc ordering is light-activated and resides in the outer segment is in close accord with the results reported by Hollyfield et al. In their experiments with X. laevis, an enhanced rate of outer segment membrane assembly occurred during the early light phase of the diurnal rhythm in the absence of an increase in the rate of opsin synthesis. Their observations imply that disc formation does not require the biosynthesis of new protein, and are compatible with the notion that the newly formed discs are constructed from protein already assimilated by the outer segment. Furthermore, other investigators have observed that incorporation of opsin by rod outer segments is only moderately reduced in R. pipiens maintained for 24 hr in darkness. The lack of a light cue apparently does not adversely affect the movement of protein to the outer segment.

A process of disc morphogenesis by evagination of the ciliary membrane, as proposed by Steinberg et al., is compatible with our observations and suggests that evaginations proceed beyond the physical limits of ordered discs formed at an earlier time. The unordered membrane may be "reeled in" subsequently by some contractile mechanism as part of a process of placing new discs in the proper register. In support of this, Chaitin and coworkers have localized actin to the site of disc evagination. Furthermore, Williams et al. have shown that cytochalasin D produces disordered membrane similar to that observed in the current study.

The amount of "redundant" plasma membrane which we observed in the artificial lighting conditions to which the R. pipiens were entrained may be in excess of that which normally occurs in the process of disc morphogenesis. The absolute light onset/light offset employed in this study is certainly a more abrupt perturbation of the diurnal rhythm than the lighting gradient that leads to morning and evening in the natural environment. The all-or-none cue provided by laboratory conditions may result in an exaggerated demarcation of the individual processes of disc morphogenesis. Nonetheless such observations facilitate the formulation of concepts concerning the cyclic cellular activity.

Key words: rhodopsin biosynthesis, electron microscope immunocytochemistry, diurnal rhythm, retina, photoreceptors

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References


