Exp. Eye Res. (1990) **50**, 231–233

LETTER TO THE EDITORS

Shedding of Cone Photoreceptor Disk Membranes in Lizard Eyecups Maintained In Vitro

In 1967, Young reported that rod photoreceptors turn over their disk membranes. Later, it was found that cone photoreceptors do the same (Hogan, Wood and Steinberg, 1974; Anderson and Fisher, 1975). The establishment of conditions in which to keep isolated frog eyes or eyecups in vitro, so that rod disk turnover occurs normally (Flannery and Fisher, 1979; Besharse, Terrill and Dunis, 1980; Heath and Basinger, 1983), has greatly facilitated analyses of the mechanisms regulating rod disk shedding and assembly. In this preparation, eyes or eyecups from *Xenopus* or *Rana* are kept in a balanced salt solution, including Ca^{2+}, Mg^{2+}, and at least 30 mM bicarbonate, plus amino acids (Besharse et al., 1980; Heath and Basinger, 1983; Greenberger and Besharse, 1983; Besharse and Dunis, 1983). The rod outer segments (ROS) must remain in intimate contact with the retinal pigmented epithelium (RPE) in order to permit a normal rod disk shedding response (Williams and Fisher, 1987), although disk assembly seems to be only slightly affected by short-term detachment (Hale, Matsumoto and Fisher, unpubl. res.).

Despite the usefulness of in vitro frog eyecups in the study of turnover of rod disks, the turnover of cone disks has not been investigated in vitro. Consequently, much less is known about the regulation of disk shedding and assembly in cones than in rods. The choice of an in vitro system in which to study cone disk turnover is not straightforward, however. Frog retinas are dominated by rod photoreceptors. Moreover, disk shedding may not be normal in in vitro preparations of eyecups from other animals. For example, when posterior segments of rat eyes are placed in vitro, rod disks shed spontaneously at abnormally high levels (Goldman and O'Brien, 1978; Tamai, Mizuno and Chader, 1982).

The lizard, *Sceloporus*, like most lizards and snakes (Walls, 1942), has only cone photoreceptors (Young, 1977; Bernstein, Breding and Fisher, 1984). We have previously described a preparation for maintaining eyecups of this animal in organ culture (Williams et al., 1986). Additionally, its cone disk shedding in relation to time-of-day and ambient lighting has been characterized in vivo (Young, 1977; Bernstein et al., 1984). In the present study, we have examined *Sceloporus* eyecups in vitro in order to determine if normal cone disk shedding is sustained.

Adult western fence-lizards, *Sceloporus occidentalis*, were caught in southern California (under permit from the State of California, Department of Fish and Game). They were kept in a vivarium at 30°C on a light/dark cycle (lights-on at 0700 hr, lights-off at 1900 hr) for at least 1 month prior to use. Animals were decapitated, double-pithed, and one eye from each animal was enucleated. For in vitro incubation, eyecups were prepared 1–2 hr before the time of

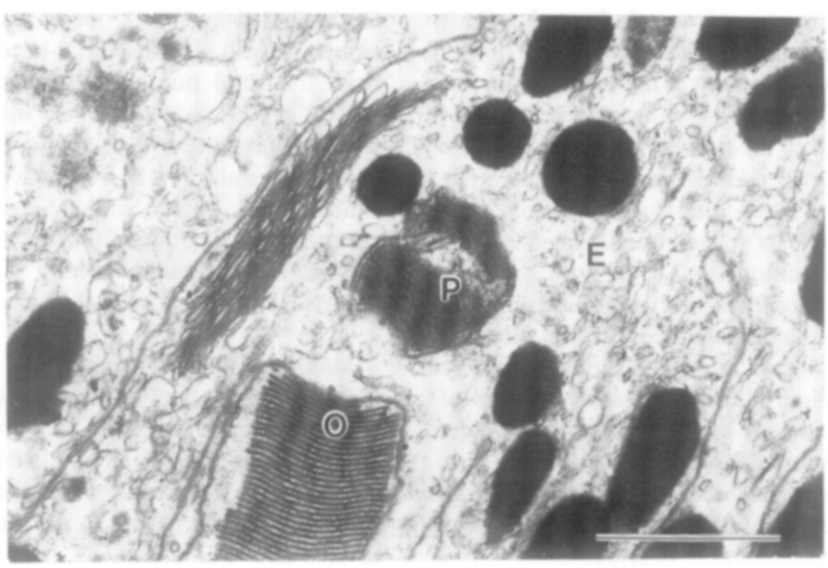

FIG. 1. Electron micrograph from an eyecup maintained in vitro and fixed 2 hr after lights-off. It shows the distal end of a cone outer segment (O), and a phagosome (P) of COS disks within the retinal pigmented epithelium (E). The phagosome contains disks that are still just distinguishable, so that it would have been counted as a fresh phagosome. The morphology appears normal. Scale bar = 1 μm.

0014–4835/90/020231+03 $03.00/0

lights-off by cutting away the cornea and lens. This dissection requires considerable care for the cone outer segments (COS) are fragile and easily damaged. Eyecups were collected in culture medium A, containing balanced salts (122 mM NaCl, 5 mM KCl, 1·8 mM $CaCl_2$, 0·8 mM $MgSO_4$, 1 mM NaH_2PO_4, 20 mM $NaHCO_3$, 15·5 mM D-glucose) + 1 g l^{-1} casamino acids (Difco), or medium B, containing the same except with $NaHCO_3$ increased to 35 mM and NaCl decreased to 107 mM. Medium B was gassed with 95% O_2/5% CO_2 to give a pH of 7·4. Medium A was gassed with 100% O_2 for 5–10 min and then 95% O_2/5% CO_2 until the pH also reached 7·4. The media were kept in stoppered flasks at 30°C. After each eyecup was swiftly added, the flask was restoppered. When all eyecups were collected, the pH was checked to confirm that it had not drifted from 7·4. The stoppered flasks were placed in a gently rotating waterbath at 30°C under 500 lux of cool fluorescent lighting. Live animals for the in vivo samples were kept under similar lighting also at 30°C. Some eyecups and animals were kept in light until fixation; others were placed in total darkness at the normal time of lights-off. Eyecups from the incubation media and eyecups freshly dissected from animals were placed in fixative (2% glutaraldehyde + 2% formaldehyde in 86 mM phosphate buffer) 2 hr and 12 hr after the normal time of lights-off. They were later osmicated, dehydrated and embedded for electron microscopy. Ultrathin sections were prepared from a region of each retina approximately midway between the fovea and the periphery, with the COS aligned longitudinally. One section per eye was examined by electron microscopy, and the extent of COS shedding was quantified by counting the number of phagosomes in the RPE over an area that included at least 100 COS. Phagosomes were only counted if the individual disk membranes could still be seen, so that we measured only the presence of recently formed or fresh phagosomes. In the samples taken 12 hr after lights-off, however, we also took note of the total number of phagosomes. The number of phagosomes was expressed in proportion to the number of COS in the area examined. Data were pooled from three different experiments. A one-tailed *t*-test was employed to determine the probability of no significant difference between different conditions.

The COS and phagosomes of in vivo and in vitro retinas appeared indistinguishable in the electron microscope. An example of a fresh phagosome is shown in Fig. 1. Figure 2 illustrates the counts of phagosomes obtained after exposure to different light and bicarbonate treatments.

Most phagosomes were observed after 2 hr dark. At this time, no significant difference ($P > 0·05$) was determined between the number of phagosomes found in vivo (mean ± 1 S.E.M. = 18·8 ± 1·6 phagosomes per 100 COS) and that found in vitro, with either concentration of bicarbonate (mean = 15·5 ± 2·3 and 16·4 ± 2·0 phagosomes per 100 COS with 20 mM and 35 mM $NaHCO_3$, respectively). These results indicate that the night-induced shedding response, which has been described previously in vivo (Young, 1977; Bernstein et al., 1984), occurs normally in vitro. Our counts of fresh phagosomes 2 hr after lights-off are less than the total number of phagosomes reported by Young (1977), but similar to the number of phagosomes still retaining a lamellar appearance reported by Bernstein et al. (1984). That the shedding response is similar with 20 mM and 35 mM bicarbonate is in contrast to the in vitro ROS shedding response in frog retinas, which requires at least 30 mM bicarbonate in order to match the in vivo response (Besharse et al., 1980).

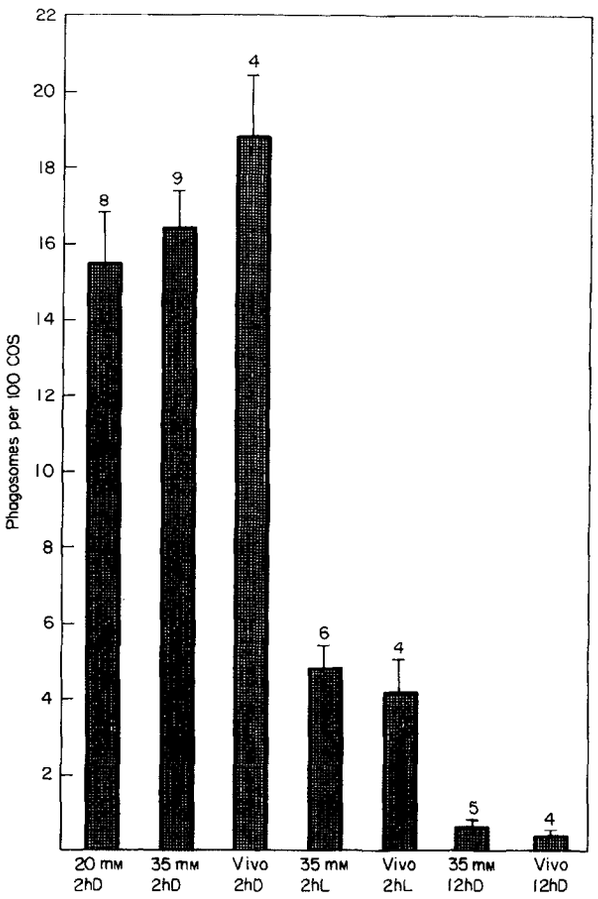

FIG. 2. Bar graph showing the mean (+S.E.M) number of fresh phagosomes per 100 COS found under different conditions tested. The number above each bar represents the number of eyes sampled.

Maintained light reduced the shedding normally observed 2 hr after lights-off to 4·2 ± 0·8 and 4·8 ± 0·6 phagosomes per 100 COS in vivo and in vitro, respectively. Bernstein et al. (1984) reported a similar result in vivo. Our results show that the suppression of shedding by light occurs to the same extent in vitro as in vivo ($P > 0·05$).

By the time of lights-on, both in vivo and in vitro retinas contained less than one phagosome of any sort (fresh or condensed) per 100 COS, indicating the absence of shedding at this time and that the

phagosomes formed just after lights-off had all been digested. This observation once again agrees with the in vivo observations of Bernstein et al. (1984) and implies that the degradation of the phagosomes is not hindered in vitro.

In summary, in vitro and in vivo shedding were similar according to all three aspects tested: (i) the amount of shedding occurring 2 hr after lights-off (i.e. when the shedding response is greatest), (ii) the extent of suppression of this night-induced shedding response by maintenance in light, and (iii) the absence of phagosomes 12 hr after lights-off, indicating a lack of shedding at this time and that the phagosomes formed earlier had been digested. Thus, the present report describes a preparation that should be suitable for in vitro analysis of the mechanisms regulating night-induced cone disk shedding.

Acknowledgments

We are grateful to Chester Wilson and Nansi Colley for some technical assistance. The work was supported by NSF grant BNS 8796252 and NIH grants EY 07042 and EY 00888.

DAVID S. WILLIAMS*

KENNETH A. LINBERG
STEVEN K. FISHER

School of Optometry and Institute for Molecular and Cellular Biology, Indiana University, Bloomington, IN 47405, U.S.A.
Neuroscience Research Program and Department of Biological Sciences, University of California, Santa Barbara, CA 93106, U.S.A.

*For correspondence at: School of Optometry, 800 East Atwater, Indiana University, Bloomington, IN 47405, U.S.A.

(Received 24 May 1989 and accepted in revised form 3 August 1989)

References

Anderson, D. H. and Fisher, S. K. (1975). Disc shedding in the rodlike and conelike photoreceptors of tree squirrels. *Science* **187**, 953–5.

Bernstein, S. A., Breding, D. J. and Fisher, S. K. (1984). The influence of light on cone disk shedding in the lizard, Sceloporus occidentalis. *J. Cell Biol.* **99**, 379–89.

Besharse, J. D. and Dunis D. A. (1983). Rod photoreceptor disc shedding in eyecups: relationship to bicarbonate and amino acids. *Exp. Eye Res.* **36**, 567–80.

Besharse, J. D., Terrill, R. O. and Dunis, D. A. (1980). Light-evoked disc shedding by rod photoreceptors in vitro: relationship to medium, bicarbonate concentration. *Invest. Ophthalmol. Vis. Sci.* **19**, 1512–17.

Flannery, J. G. and Fisher, S. K. (1979). Light-triggered rod disc shedding in *Xenopus* retina *in vitro*. *Invest. Ophthalmol. Vis. Sci.* **18**, 638–42.

Goldman, A. I. and O'Brien, P. J. (1978). Phagocytosis in the retinal pigment epithelium of the RCS rat. *Science* **201**, 1023–5.

Greenberger, L. M. and Besharse, J. C. (1983). Photoreceptor disc shedding in eyecups: inhibition by deletion of extracellular divalent cations. *Invest. Ophthalmol. Vis. Sci.* **24**, 1456–64.

Heath, A. R. and Basinger, S. F. (1983). Frog rod outer segment shedding in vitro. *Invest. Ophthalmol. Vis. Sci.* **24**, 277–84.

Hogan, M. J., Wood, I. and Steinberg, R. H. (1974). Phagocytosis by pigment epithelium of human retinal cones. *Nature* **252**, 305–7.

Tamai, M., Mizuno, K. and Chader, G. J. (1982). In vitro studies on shedding and phagocytosis of rod outer segments in the rat retina: effect of oxygen concentration. *Invest. Ophthalmol. Vis. Sci.* **22**, 439–48.

Walls, G. L. (1942). *The Vertebrate Eye and its Adaptive Radiation*. Cranbrook Press: Michigan.

Williams, D. S., Colley, N. J., Anderson, D.H., Farber, D. B. and Fisher, S. K. (1986). In vitro maintenance of a pure-cone retina. *Invest. Ophthalmol. Vis. Sci.* **27**, 666–73.

Williams, D. S. and Fisher, S. K. (1987). Prevention of rod disk shedding by detachment from the retinal pigment epithelium. *Invest. Ophthalmol. Vis. Sci.* **28**, 184–7.

Young, R. W. (1967). The renewal of photoreceptor outer segments. *J. Cell Biol.* **33**, 61–72.

Young, R. W. (1977). The daily rhythm of shedding and degradation of cone outer segment membranes in the lizard retina. *J. Ultrastruct. Res.* **61**, 172–85.