Light-evoked disc shedding by rod photoreceptors in vitro: relationship to medium bicarbonate concentration. JOSEPH C. BESHARSE, RUFUS O. TERRILL, AND DAIGA A. DUNIS.

Although studies on the control of photoperiod-related shedding by rod photoreceptors in frogs suggest that the mechanisms which initiate shedding are intrinsic features of the eye, attempts to observe light-evoked shedding in opened eye cups in balanced salt solutions have been unsuccessful. We have examined disc shedding in eye cups kept in a complex tissue culture medium. Eye cups were prepared in red light from dark-adapted animals or in white light from constant light-treated animals. Light-evoked shedding did not occur in the standard medium with ~20 mM sodium bicarbonate, but a large light-evoked response was consistently seen when medium bicarbonate was raised to ~30 mM. In high-bicarbonate medium, light-evoked shedding was comparable in magnitude, size distribution of phagosomes, and time course to similar responses observed in intact animals. Preliminary analysis of culture conditions which support shedding suggests that bicarbonate ion concentration is of critical importance. However, lack of light-evoked shedding in a high-bicarbonate Ringer solution suggests that some additional unidentified factor(s) in the complex medium is also necessary. The data indicate that both light- and dark-dependent processes involved in the control of photoperiod-related shedding are sustained in appropriate culture medium.

Turnover of rod photoreceptor outer segments (ROSs) involves new disc assembly at the region of the ROS base, shedding of groups of discs from the ROS distal tip, and degradation of discs within the lysosomal system of pigment epithelial cells. Disc loss at the ROS distal tip normally occurs 1 to 2 hr after light onset each day. Although shedding is influenced by an endogenous rhythm in rats and Xenopus laevis kept in constant darkness, light stimulation after a period in darkness is a major factor initiating shedding in amphibians. In animals kept in constant light for several days, disc shedding is reduced to a low level but is again initiated when animals are subjected to a brief period (1 to 3 hr) of darkness. Such observations suggest that some unidentified dark-dependent process is a prerequisite for light-evoked shedding. Whole animal experiments further suggest that shedding may be initiated within the eye in the absence of systemic influences.

Despite the obvious utility of an in vitro system for analysis of the control mechanisms of shedding, several attempts to establish culture conditions that support the light-evoked response in opened eye cups have not been successful (unpublished observations from this and other laboratories). Because the balanced salt solutions used in those studies did not support shedding, we recently began an analysis of eye cups maintained in a more complex tissue culture medium. In this paper we report that light-evoked shedding comparable to the in vivo response occurs in bicarbonate-buffered tissue culture medium adjusted to an osmolality comparable to X. laevis serum. In addition, we have found that the response does not occur at low bicarbonate concentration or in high-bicarbonate balanced salt solutions. The data indicate that both the light- and dark-dependent processes associated with disc shedding are sustained in vitro and suggest that bicarbonate ion and some additional unidentified component(s) in the medium play an essential role in the mechanism of the light-evoked response.

Materials and methods. To study light-evoked shedding, we prepared eye cups in dim red light from explanted eyes of dark-adapted X. laevis (postmetamorphic, 2.5 to 3.5 cm in length) that had been maintained a minimum of 1 month in cyclic light (12L:12D). After surgical removal of the cornea, iris, and lens, eye cups were transferred to the amphibian tissue culture medium (ATM) of Wolf and Quimby obtained from Grand Island Biological Co. (~240 mOsmol/kg) or to the same medium supplemented with additional sodium bicarbonate (ATMB) and adjusted to 240 mOsmol/kg by addition of water. In addition to balanced salts and complex components (10% fetal bovine serum, 10% whole egg ultrafiltrate, amino acids, and vitamins), ATM contained ~17 to 20 mM NaHCO₃ and maintained a pH of 7.5 ± 0.1 in equilibrium with air. ATMB contained ~30 mM sodium bicarbonate, and in equilibrium with 95% O₂-5% CO₂ the pH was 7.5. Osmolality of the medium was monitored by freezing point depression (Osmette; Precision Instruments, Inc.)

Groups of 15 to 26 eye cups were prepared and transferred in groups of three to five to glass vials or plastic culture dishes for individual treatments. One eye cup from each animal was exposed to light from a tungsten halogen lamp delivered through a fiberoptic system (~650 lu/m² incident at the level of culture dishes), and the remaining eye cup from the same animal was kept in dark-
Fig. 1. Photomicrographs illustrating the photoreceptor–pigment epithelial complex of *X. laevis* eye cups that had been kept in ATMB for 2.5 hr. Eye cups were prepared in dim red light and incubated in darkness (A) or light (B). Large phagosomes (open arrows) resulting from ROS disc shedding were abundant in light (B) but were rare in darkness (A). Small phagosomes ≤2 μm in diameter (small filled arrows) were present in both light and darkness in frequencies not significantly different from those reported for in vivo preparations. The overall size-frequency distribution of phagosomes was within the range of that reported in vivo. Since light-evoked shedding in vitro involved phagosome profiles >2 μm in size, only those profiles greater than this size were included in the quantitative analysis in this paper.
sections of each eye cup. Criteria for identification of phagosomes and the method for counting them were like those used formerly for in vivo preparations.

Results. In all experiments the RPE at the beginning of the incubation period (baseline) contained few phagosomes of criterion size (Figs. 1 and 2), and incubation for 2.5 hr in ATM in equilibrium with air did not significantly modify the content of phagosomes in RPE (Fig. 2). However, in ATMB maintained at the same pH by equilibration with 95% O₂-5% CO₂, a large burst of shedding, comparable in magnitude to the previously reported in vivo response of 21 to 28 phagosomes/1000 μm of RPE, occurred in all eye cups examined (Figs. 1 and 2). The size-frequency distribution (data not shown) of phagosome profiles (including those ≤2 μm) in eye cups incubated in light for 2.5 hr was within the range of that reported for in vivo preparations at 2 hr. Furthermore, the time course of the light-evoked response (data not shown) followed a pattern similar to that of in vivo preparations. The phagosome content in the RPE of eye cups in light increased in a nearly linear fashion to a peak during the first 2.5 hr and then gradually declined to a level less than half the peak value during a subsequent 9.5 hr in culture.

The light-evoked shedding response in ATMB did not depend on the gas mixture used to maintain pH, since a comparable response was observed in ATMB equilibrated with air or 100% O₂.

Fig. 2. Experimental observations which suggest that an increase in the concentration of sodium bicarbonate permits shedding in vitro. The baseline group represents the background level of large phagosomes observed in three experiments before the beginning of incubations. Each of the pairs of columns represents the average shedding response in light (open column) or darkness (stippled column). The number at the bottom of each column is the number of eye cups examined, and the vertical bars correspond to 1 S.E.M. All incubations were carried out for 2.5 hr under the culture conditions indicated along the abscissa. The columns for the baseline, ATM in equilibrium with air, and ATMB in equilibrium with 95% O₂-5% CO₂ represent the pooled data from these separate experiments in which the individual experimental results were not significantly different. Note that light-evoked shedding comparable to the in vivo response occurred in ATMB regardless of the gas mixture used but failed to occur in ATM equilibrated with 95% O₂-5% CO₂ or 100% O₂.
Compound Gas

Oxygen
Sodium isethionate
Choline bicarbonate
Oxygen + CO₂
Sodium isethionate
Air
Oxygen + CO₂

Fig. 3. Experimental observations which suggest that the light-evoked shedding response is related to an increase in HCO₃⁻ ion concentration. Medium was prepared in a fashion like that used to make ATMB except that sodium isethionate or choline bicarbonate was substituted for the increased sodium bicarbonate on an equimolar basis. Data are plotted as in Fig. 2 for two separate experiments for each compound. The compound and the gas used are indicated on the abscissa. The pH in each experiment was 7.5 ± 0.1. In a separate experiment (data not plotted) sodium methylsulfate was used; phagosome counts for both light and darkness were within the standard error for sodium isethionate. The light-evoked response in choline bicarbonate averaged slightly lower than in ATMB (Fig. 2), but the mean values were not significantly different from those for ATMB. Note that the choline bicarbonate experiments were identical but that these with sodium isethionate differed in the gas used to equilibrate the medium.

(Fig. 2) even though the pH was increased to 8.2 in both conditions. Furthermore, shedding was increased only slightly in ATM equilibrated with 95% O₂-5% CO₂ (pH 6.9) or 100% O₂ (pH 7.5). These observations indicate that the increased concentration of sodium bicarbonate in ATMB is of critical importance for the light-evoked response. In an attempt to distinguish between the effects of increased Na⁺ and HCO₃⁻ ion in ATMB, we increased HCO₃⁻ by addition of choline bicarbonate and Na⁺ by addition of sodium isethionate. In those experiments the medium was prepared to be identical to ATMB except for the ion substitutions (Fig. 3). Although some shedding was seen in the sodium isethionate medium, the magnitude was not markedly different from that seen in ATM gassed with 95% O₂-5% CO₂ or 100% O₂ (compare Figs. 2 and 3). However, a large light-evoked response was seen in the choline bicarbonate medium, which was not significantly different from that seen in ATMB (Fig. 3). The data indicate therefore that the large, light-evoked response observed in these experiments was made possible by an increase in the concentration of HCO₃⁻ ion.

Previous in vivo studies of ROS disc-shedding in amphibians have suggested the existence of a dark-dependent process that occurs as a necessary prelude to light-evoked shedding.⁷ To determine whether the dark-dependent process also occurs in vitro, we examined shedding in eye cups prepared from animals that had been maintained in constant light for 4 days. Eye cups were prepared in room light and then maintained in light, darkness, or darkness followed by light (Fig. 4). The experiment was designed to parallel previous in vivo experiments in which a large shedding response was initiated in Rana pipiens eyes when constant light–treated animals were returned to darkness. In those experiments substantial shedding occurred during a 3 hr period in darkness.
However, an even greater response was seen in animals kept in darkness for 1 hr followed by a return to light for 2 hr. A repeat of this experiment with *X. laevis* revealed a similar in vivo response (Fig. 4); the only difference was that darkness alone was also very effective in promoting shedding, although less so than the 1 hr dark–2 hr light treatment. Exposure of eye cups in ATMB to light for 3 hr did not elicit disc-shedding (Fig. 4). However, a large shedding response comparable to that occurring in vivo was seen in those eye cups incubated in darkness for 3 hr or in darkness for 1 hr followed by 2 hr of light (Fig. 4). As in cyclic light-treated eyes prepared in red light, shedding did not occur in the low-bicarbonate medium (ATM, Fig. 4). The close quantitative correspondence of in vivo and in vitro results suggests that ATMB (but not ATM) supports a dark-dependent process that leads to ROS disc shedding in constant light–treated eyes.

**Discussion.** The phenomenon of ROS disc shedding involves a complex series of cellular events that probably involve detachment of discs, recognition of shed fragments, and phagocytosis by RPE. The observations reported here establish conditions for light-evoked shedding in vitro which should prove useful in the analysis of those cellular mechanisms. Although the similarity of the data to those obtained in comparable in vivo experiments suggests that normal processes are
activated, the nature and site of the effects remain unresolved. The apparent need for high bicarbonate is of interest because it suggests a possible link between shedding and metabolic or electrical activity of the retina. In isolated mammalian retinas, bicarbonate is necessary for optimal glucose and oxygen utilization as well as maintenance of electrical activity, whereas in an isolated RPE preparation from frogs, bicarbonate hyperpolarizes the apical plasmalemma of RPE cells. The latter observation is particularly intriguing because bicarbonate is one of only two ions (the other is K⁺) that alter RPE membrane potential. Whether bicarbonate plays a permissive role by providing a more favorable environment or a more specific role in the mechanism of shedding remains to be determined.

In vivo experiments on both rats and frogs have suggested that the basic mechanisms that initiate light-evoked shedding are intrinsic features of the eye and do not depend on systemic influences. In X. laevis this view is supported by the observation of shedding in isolated, intact eyes. Our data extend previous observations by showing that both light- and dark-dependent processes, which are essential for normal disc shedding, occur in the absence of the usual systemic influences. The data do not rule out the possibility, however, that systemic factors may normally influence shedding. For example, we have recently confirmed our previous observation that an endogenous rhythm influences shedding in constant darkness. Furthermore, we have not detected light-evoked shedding in vitro in a variety of balanced salt solutions (including the balanced salts of ATMB) comparable to those used by others for metabolic and electrophysiological studies of the photoreceptor–pigment epithelial complex. The fact that such salt solutions contained 25 to 35 mM sodium bicarbonate suggests that increased bicarbonate alone is not sufficient to support shedding in vitro. We are not yet able to fully define the additional factor(s) that permits shedding in the presence of high bicarbonate. However, preliminary data from an analysis of the effects of culture medium components on shedding (Besharse, unpublished data) suggest that amino acids are an essential component of the medium.

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