Frog Rod Outer Segment Shedding In Vitro: Histologic and Electrophysiologic Observations

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Rod outer segment shedding in the frog, *Rana pipiens*, has been studied using an in vitro eyecup method. Control experiments have shown that shedding responses in vitro are comparable to those in vivo and, like the situation in vivo, shedding in isolated eyecups requires a dark period followed by light onset. We found an initial, rapid and light-evoked component of the shedding response to be critically dependent upon bicarbonate concentration, supporting the initial discovery of a bicarbonate requirement for *Xenopus* rod shedding by Besharse et al. In *Rana*, in vitro shedding occurs in the presence of 20 mM aspartate, suggesting that functional integrity of the inner retina is not a prerequisite for rod shedding. Additionally, shedding was found to be suppressed completely in the presence of the local anesthetic MS-222 and the phosphodiesterase inhibitor IBMX. In the case of IBMX, electrophysiologic recording indicated changes in photoreceptor sensitivity in the presence of the drug. Such changes may play a role in the observed inhibition of shedding. Invest Ophthalmol Vis Sci 24:277-284, 1983

Over the past ten years, a number of detailed studies have been published on rod photoreceptor outer segment shedding in intact animals. These studies have provided us with essential data on outer segment shedding and have dealt in part with the question of whether or not shedding is under intra- or extra-ocular control. However, we are still far from an understanding of the fundamental physiologic mechanisms involved in the process of outer segment shedding and phagocytosis. In order to answer these questions, a number of laboratories have turned to in vitro experimental techniques. The in vitro technique offers the general advantage of more precise control of key physiologic parameters and the possibility of exact ionic and pharmacologic manipulations. To date, two fundamental lines of approach have been employed. The first takes as the experimental material isolated pigment epithelial tissue, either in organ or cell culture, and uses various substrates (polystyrene spheres, killed or native bacteria, rod outer segment suspensions) for studying phagocytosis. The second approach, only recently developed, uses the entire isolated eye or posterior eyecup in organ culture. In particular, the work of Besharse et al. has carefully established many of the necessary conditions for in vitro studies of shedding and phagocytosis in *Xenopus laevis*. The present study is a report of frog rod outer segment shedding in *Rana pipiens* using the isolated eyecup method. In addition, electrophysiologic recordings are presented in order to analyze further the effects and site of action of chemical agents found to affect the outer segment shedding process.

Materials and Methods

In Vitro Shedding Studies

Frogs (*Rana pipiens*) were kept in constant light in 24°C incubators for two days and two nights before use. They were then killed in the light by decapitation, pithed, and the eyes rapidly enucleated and cleaned of attach muscle tissue and membranes. The eyes were opened with razor blades and the anterior portion with lens discarded. The remaining posterior eyecups were rapidly transferred to small flasks in a Dubnoff tissue shaker at 24°C. Each flask contained 2.5 ml of incubation medium per eyecup. The normal medium had the following composition; 70 mM NaCl, 2 mM KCl, 2 mM CaCl₂, 2 mM MgSO₄, 1.25 mM KH₂PO₄, 35 mM NaHCO₃, 10 mM D-glucose, and 1 mg/ml casamino acids (Difco). Medium was prepared freshly for each experiment, and before use it was bubbled with 95% O₂/5% CO₂ for at least 90 min. Humidified O₂/CO₂ gas mixture was passed through the incubation flasks throughout the course
of an experiment. The flow rate was 1.25 standard cubic feet/hr/flask.

The standard protocol for an incubation experiment was to keep eyes in the dark for 60 min, followed by transfer in the light to fresh medium. The incubation then continued in the light for 90 min. Any exceptions to this protocol are noted where appropriate. Required ionic or pharmacologic manipulations could be made at any time throughout the period of incubation and relevant details are given below. The experiment was terminated by placing the eyecups into cold 2.5% glutaraldehyde, 1% paraformaldehyde in 0.16 M sodium cacodylate buffer at pH 7.3-7.4. Following fixation for 15 min in this solution, the tissues were placed overnight in fresh fixative at 4°C.

Further processing was carried out by dividing the eyecups into rostral and caudal portions through the optic disc, postfixing the tissues in 1% OsO₄ in 0.16 M sodium cacodylate for 60 min, dehydrating through graded ethanols, and embedding in “Polybed 812” plastic (Polysciences Inc., Warrington, PA). Sections were cut at 1 micrometer on a Sorvall MT2B microtome and stained with alkaline toluidine blue. All quantitative data were obtained by counting phagosomes directly from microscope slides under a 40× objective. Counts were made of all phagosomes larger than 2 micrometers in their greatest dimension in 12 adjacent fields of constant size (one field = 430 micrometers) from sections cut in the dorso-ventral axis through the optic nerve from both rostral and caudal halves of a particular eye. The phagosome counts were then expressed as a percentage of the total number of rods present in each field and the results averaged and standard deviations computed.

Electroretinogram (ERG) Recordings

Eyecups were isolated and opened in the light as described above. The vitreous was drained carefully from the eyecup using cellulose sponges. Eyecups were then filled with three to four drops of oxygenated Ringer’s solution with the same composition as listed above for normal shedding medium, or with added effector agents as described below, and placed on pads for 5C amplifier with band passes set at 0.1 Hz to 0.03 KHz. Waveforms were displayed on a storage oscilloscope. The light source was a collimated beam from a 150 W Xenon arc lamp, and stimuli were presented to the eyecups using a fiber optic cable. All stimuli were white light that were attenuated using neutral density filters.

A 10-sec exposure of the isolated frog retina to the unattenuated stimulus resulted in bleaching of 70 ± 3% of the extractable visual pigment (n = 6, each retina homogenized in 0.5 ml of 0.04 M cetyltrimethylammonium bromide). ERGs were recorded in response to 25 msec flashes. At maximum intensity this duration of exposure produced negligible bleaching in isolated retinas (6 ± 6% of extractable visual pigment, n = 4). To observe photoreceptor activity in isolation from other ERG sources, 20 mM sodium aspartate was added to the normal Ringer’s solution. Response-intensity functions were obtained by recording responses to light flashes starting with the lowest intensity and testing at 1-min intervals up to the highest intensity used (unattenuated beam). The process was then repeated in the reverse direction. A final series was then taken beginning once again with the lowest intensity. Response amplitude was taken as the mean of the three test flashes at each intensity. Recording usually commenced about 30 min after opening and preparing the eyecup. All recordings presented were scotopic.

Results

Anatomy

Light micrographs of in vitro frog eyecup preparations are presented in Figure 1A-C. Figure 1A shows a section through the retina of a two-day constant light frog incubated for 2.5 hrs in constant light. Note the complete absence of phagosomes in the retinal pigment epithelium. Figure 1B shows the retina from an eye incubated for 1 hr in the dark followed by 1.5 hrs in the light. Darkly staining phagosomes are clearly seen contained within the pigment epithelium. Figure 1C shows a section through the retina of an eyecup incubated in the presence of 20 mM sodium aspartate and is discussed further in the following section. In each of these preparations, photoreceptor preservation is good, indicating that in vitro incubations of up to 2.5 hrs have no obvious deleterious effects.

In Vitro Shedding Experiments

The results of the in vitro shedding experiments are presented in Figures 2 and 3. Figure 2 shows a comparison of shedding levels in vivo (A) and in vitro (B). In vivo controls were frogs kept in glass containers in the same room where in vitro incubations were carried out. They had previously been kept for two...
Fig. 1. Light photomicrographs of frog retinas following in vitro incubations. A, Two-day constant light retina incubated for 150 min in constant light only. No phagosomes are present in the retinal pigment epithelium (RPE). B, Two-day constant light retina incubated for 60 min in darkness followed by 90 min in light. Note the presence of numerous phagosomes in the RPE. C, Two-day constant light retina incubated for 60 min in the dark, followed by 90 min in the light with 20 mM sodium aspartate present throughout. Note normal phagosomes in the RPE although both inner and outer plexiform layers show vacuolization (compare B).
days in constant light in the same constant-temperature incubator as frogs intended for use in in vitro experiments. Two other controls for the in vitro experiment were (C) eyes prepared in the usual fashion but kept in constant light throughout the incubation period and (D) eyes kept in constant darkness for the entire incubation period.

A number of experiments were carried out to determine the effects of external bicarbonate ion concentration on various stages of the shedding process. The results of these experiments are presented in Figure 3, which shows three experimental conditions. In A, incubation was carried out for 20 min in light and 60 min in darkness in 35 mM bicarbonate medium (normal RBG), followed by immediate transfer in the light to 25 mM bicarbonate for 90 min. In B, following the initial dark period in 35 mM bicarbonate, we incubated for 20 min with 25 mM bicarbonate in the dark, that is, prior to any light exposure. In this case light exposure therefore took place in 25 mM bicarbonate with the retinal and pigment epithelial bicarbonate concentrations presumably equilibrated to this level. The light period of the incubation (90 min) was then also carried out in 25 mM bicarbonate. Finally, in C incubation was carried out entirely in 25 mM bicarbonate; 20 min light, 60 min dark, 90 min light. Although results from a particular eyecup may depart from the trend, Figure 3 shows that shedding was clearly below normal levels in those situations where the 35 mM bicarbonate was not present in the incubation medium at the point where the lights were turned on. On the other hand, immediate exposure to light in 35 mM bicarbonate medium (less than 2 min before transfer to 25 mM bicarbonate) produces a normal level of shedding. The higher bicarbonate concentration, therefore, does not appear to be necessary for shedding once light exposure immediately following dark adaptation has taken place.

Sodium aspartate; MS-222; IBMX: A number of eyecups were incubated in the usual way in the presence of these three agents. The addition to the medium of 20 mM sodium aspartate, which is known
to abolish ERG b-wave activity, thus unmasking photoreceptor activity, had no effect upon the levels of shedding observed, having a value of 25 ± 12% (n = 6). Figure 1C shows a light micrograph of such a preparation. It can be seen that, in spite of structural damage to the inner and outer plexiform layers, which have acquired a vacuolated, “moth-eaten” appearance, normal looking phagosomes are present in the pigment epithelium. Thus, synaptic activity beyond the level of the photoreceptors themselves is not necessary for the shedding process to occur.

A total of 13 eyes were incubated in the presence of the local anesthetic ethyl-m-amino benzoate methane sulfonate (MS-222) in varying concentrations: 0.01 mM (4), 0.1 mM (4), 0.5 mM (1), 1 mM (3), and 5 mM (1). In no case was shedding observed in these experiments. Additionally, following a report that MS-222 inhibits rhodopsin regeneration in Rana,15 an experiment was carried out using a “split protocol” similar to that employed in the bicarbonate study. In this case, the first protocol involved incubating two eyecups for 60 min in the dark in normal medium, followed by 15 min in the dark plus 90 min in the light with fresh medium containing 5 mM MS-222. In the second protocol, two eyecups were incubated for 15 min in the light plus 60 min in the dark in the presence of 5 mM MS-222 followed by 90 min in the light in fresh medium without MS-222. In the first case, therefore, dark adaptation took place before the addition of MS-222. In the second case, dark adaptation took place in the presence of MS-222. Once again, in no case was shedding observed. These results suggest that the effect of MS-222 on shedding is not related to its known effects on frog rhodopsin.

Finally, six eyecups were incubated in the usual way in the presence of the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX), at a concentration of 1 mM, with the intention of trying to manipulate cyclic nucleotide levels. Absolutely no new phagosomes were observed upon examination of the pigment epithelium of these animals.

ERG Results

Since IBMX has been reported in the literature to act as a depolarizing agent on neural membranes, an investigation of its effect on the frog eyecup was carried out using electrophysiologic techniques. In the first series, 1 mM IBMX was added to the normal medium, and the peak amplitude of the ERG b-wave was recorded. As can be seen from Figure 4A, IBMX significantly reduces the amplitude of the b-wave compared to controls and shifts the response intensity function to the right along the intensity axis. On the other hand, in the presence of both 20 mM aspartate and 1 mM IBMX, a different picture emerges (Fig. 4B). In this case, the actual shape of the response-intensity function is altered, becoming reduced in amplitude at the lower intensities with the amplitude at the higher intensities actually increasing until at the highest the standard deviations of the two sets of data are beginning to diverge. These observations are in general agreement with previously reported work on the electrophysiologic effects of IBMX16 and raise immediate questions concerning the nature of the inhibitory effect of IBMX on the rod outer segment shedding process, which will be addressed below.

Discussion

The results from the present study indicate that rod outer segment shedding in Rana pipiens occurs in vitro in an essentially similar manner to the process in vivo. Average levels of shedding taken from phagosome counts show no significant differences between the two conditions (Fig. 2A vs B). Also, retinal structure at the light microscope level showed no differences between the in vivo tissue and tissue incubated for up to 2.75 hours in the normal medium. Furthermore, in vitro experiments on two-day constant light frogs incubated in constant light or two-day constant light frogs incubated in constant darkness show no incidence of phagosomes greater than what we would normally consider zero level shedding (less than 5%). This observation is of importance for two reasons. Firstly, it indicates that shedding in vitro is, indeed, primed by a period of dark adaptation that requires a subsequent period of light stimulation. Secondly, shedding was not initiated by physiologically irrelevant stimuli (such as enucleation) as has been observed in mammalian eyes in vitro. 12,17,18 The findings of zero shedding levels in these controls adds support to the idea that rod outer segment shedding in Rana is a fundamentally intraocular phenomenon that is not under any form of extraocular (eg, CNS) control.19 Stimulation of shedding by enucleation in rats may be a result of the removal of some form of extraocular inhibition. However, there exists an apparent discrepancy between our observations and those both of Besharse et al1 and of Currie et al.20 In the former case, Besharse and his colleagues observed a burst of shedding in four-day constant light Xenopus eyecups when they were transferred to constant darkness in vitro in a high bicarbonate medium. Currie et al20 observed a similar burst of shedding in ten-day constant light Rana pipiens in vivo when the animals were simply placed in darkness with no subsequent light exposure. We feel that this difference may possibly be related to the fact that these animals were
kept in constant light for four or ten days prior to the experiment, whereas our Rana were kept in constant light for only two days. We have observed an increasing tendency for spontaneous (i.e., not synchronized with changes in illumination) shedding in Rana with increasing time in constant light. In any case, the important point is that in both of the studies cited, the levels of shedding are greater in those cases where the eyecups or animals were exposed to light following the initial dark period, thus indicating that subsequent light exposure is necessary for a full shedding event.

The results of the bicarbonate experiments indicate that an initial, light-evoked component of the shedding response in Rana requires 35 mM bicarbonate for its full expression. The importance of the bicarbonate ion in maintaining the normal shedding response in vitro had previously been demonstrated using Xenopus eyes by Besharse et al., who further showed that the effect was due to the presence of the bicarbonate ion itself and not to pH changes. We are therefore able to confirm their results using Rana, a second amphibian species, and, in addition, the data that we have presented suggest the particular importance of this ion to some process associated with light-evoked stimulation of shedding. The nature of this process and its relationship to rod outer segment shedding mechanisms remains undefined. However, it should be noted that bicarbonate has repeatedly been mentioned over the years in a number of studies dealing with conditions for maintenance of retinal function in vitro. In particular, the work of Steinberg and Miller has dealt with the importance of bicarbonate for the function of the retinal pigment epithelium. These authors have recorded from single amphibian retinal pigment epithelium cells with microelectrodes and have demonstrated the presence of a transmembrane potential between the apical side of the cells and the surrounding medium that is directly proportional in magnitude to the medium bi-

**Fig. 4.** Electroretinogram recordings from isolated frog eyecups. A, B-wave amplitudes. Control (solid lines, n = 7) vs. 1 mM IBMX (broken lines, n = 4). B, Aspartate isolated receptor potential (20 mM aspartate). Control, aspartate only, (solid lines, n = 8) vs. 1 mM IBMX + aspartate (broken lines, n = 7). Error bars in B are one SD from the mean.
carbonate concentration. This effect was independent of pH, which could be varied between 6.6 and 8.3 with no effect. Removal of bicarbonate from the medium rapidly abolished the transepithelial potential. It is clear from the mechanics of the rod outer segment shedding process that the retinal pigment epithelium plays a major role, at least at the stage of phagosome capture and ingestion. It would appear that the effect of 35 mM bicarbonate observed in the present study may be related to the function of bicarbonate ions in maintaining the normal electrical characteristics of the pigment epithelium cells themselves.

We observed complete inhibition of shedding in the presence of IBMX, and our electrophysiologic observations indicate changes in photoreceptor function. Intracellular recording from toad photoreceptors in the presence of 5 mM IBMX was carried out by Lipton et al., who showed that the agent depolarises membrane potential and increases response amplitudes. However, they did not show any change in the position of the response-intensity function on the intensity axis, indicating that receptor sensitivity itself was unaffected. Our data in the aspartate + IBMX-treated preparation indicate that some loss of photoreceptor sensitivity does occur (Fig. 4B). The difference here is possibly due to differing times of incubation; our recording did not commence until 30 min after application of the Ringer's solution containing the active agent, whereas Lipton et al. recorded at incubation times of less than 15 min in all cases. Examination of the response-intensity function of control vs experimental preparations in our study indicates a shift in sensitivity of about 1.75 log units, determined by comparing the intensities necessary to elicit half maximal responses (the sigma points). Yet, it should be noted that the true difference in sensitivity is likely to be higher, since the control curve has clearly reached saturation point, whereas the IBMX-treated preparation has not. Given the observation that 20 mM sodium aspartate itself does not affect shedding and, therefore, the electrical activity seen in the control curve is adequate for normal shedding, to what do we attribute the inhibitory effect of IBMX? Three possible explanations arise. Firstly, the inhibitory effect may be attributed to the observed change in photoreceptor sensitivity. This hypothesis may be tested by exposing dark-primed, IBMX-treated eyecups to more intense levels of illumination in an attempt to overcome the sensitivity deficit. Secondly, it is possible that the site of action of IBMX is the pigment epithelium itself. For example, Miller has recently demonstrated that cyclic nucleotides alter the pigment epithelium transepithelial potential, causing it to increase. Thirdly, IBMX probably affects the cyclic nucleotide levels in photoreceptors, which in turn could alter the shedding response. Experiments are under way to determine which of these possible explanations may apply to our data. Recently, a detailed study on the effects of cyclic nucleotides and phosphodiesterase inhibitors has appeared (Besharse et al.). These authors also found an inhibitory effect of IBMX on shedding.

Finally, we observed that the local anesthetic, MS-222, also inhibits the shedding response completely. This agent has previously been shown to affect the regeneration of rhodopsin in bleached frog retinas and has been observed to inhibit ERG dark-adaptation also. However, the data obtained from our split protocol experiment indicates that shedding is still inhibited even in those eyecups where dark adaptation has been allowed to proceed before the addition of the anesthetic (ie, the condition in which incubation took place for 60 min in the dark in the absence of MS-222 prior to its addition during the rest of the incubation). It would appear, therefore, that the MS-222 effect on dark adaptation is not responsible for its inhibition of shedding and phagocytosis. A more likely explanation in this case would be the general membrane effects which such local anesthetics are known to have (see for example, Ritchie and Greenberg).

In summary, we have shown that normal Rana pipiens rod outer segment shedding responses may be obtained in vitro and that these responses are dependent upon adequate concentrations of bicarbonate in the incubation medium. Furthermore, the shedding and phagocytosis process is sensitive to the addition of chemical agents known to affect cell membrane properties, particularly those of the retinal pigment epithelium itself.

Key words: frog, rod, shedding, in vitro, photoreceptor, retina, electroretinogram

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References