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An eyecup preparation for the rat and mouse

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Abstract

The eyecup preparation has traditionally been used to study retinal physiology in lower vertebrates and in some mammals. The procedures for preparing eyecups of the rat and mouse have not been described, however. We now describe methods for preparing and maintaining viable eyecups for these two species. Eyecups were everted over a plastic dome and held in place between the two halves of a superfusion chamber. Fluid exchange in the chamber was rapid, with near total exchange occurring in 9 s. Eyecup viability was tested by monitoring light-evoked retinal responses as the preparation aged. In both rat and mouse, the amplitude of the electroretinogram (ERG) b-wave decreased slowly, declining to $\frac{1}{2}$ maximal amplitude in ≈ 70 min. Light-evoked spike activity of neurons in the ganglion cell layer remained stable for ≈ 3 h and attenuated responses were recorded for an additional 1–2 h. Eyecups were able to dark adapt. Retinal sensitivity, tested by monitoring b-wave amplitude, recovered following exposure to an adapting light.

Keywords: Retina; Superfusion chamber; Electroretinogram (ERG); b-Wave; Neuronal activity

1. Introduction

The eyecup preparation has been used to great advantage to study retinal physiology in lower vertebrates, including the frog, turtle, and skate (Dowling, 1987). Electrical recording from retinal cells and rapid exchange of superfusion solutions, which are difficult to achieve in the intact eye, are easily obtained in the eyecup. In mammals, the eyecup of the rabbit has also been used successfully (Bloomfield and Miller, 1986; Miller et al., 1986). It has proven more difficult, however, to prepare viable eyecups from the rat and mouse and there are few published studies that have employed eyecups of these species to study retinal physiology.

The techniques employed to prepare rabbit eyecups cannot be used successfully for the rat and mouse. The retinas of these smaller eyes tend to detach as the eyecup is prepared. In addition, the retinas of the rat and mouse are vascularized and thicker than the retina of the rabbit, making them more difficult to oxygenate.

The isolated retina (Meister et al., 1991, 1994; Soucy et al., 1998; Winkler et al., 1999) and retinal slice (Boos et al., 1993; Hartveit, 1996; Euler et al., 1996) preparations have been used successfully in recent years to study retinal physiology in a number of vascularized mammalian species, including the mouse, rat, cat, and ferret. The retina is separated from the retinal pigment epithelium in these preparations, preventing recovery from photopigment bleach following exposure to bright stimuli. The retinas remain responsive to light for many hours as long as exposure to intense light is avoided. These isolated retina preparations cannot be used, however, to study adaptation to bright stimuli or to monitor ion concentrations using fluorescent indicator dyes, which require bright excitation illumination. Under bright illumination, regeneration of photopigments and maintained responsiveness to photopic stimuli only occurs when the retina remains in direct contact with the pigment epithelium, as it does in the eyecup.

In recent years, with the development of transgenic and knockout strains of mouse and rat, it has become increasingly important to develop a successful eyecup preparation for these species. Genomic techniques offer unique advantages for studying retinal physiology (Goto et al., 1995; Shaaban et al., 1998), advantages

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that cannot be fully exploited without a viable eyecup preparation.

This paper describes a method for preparing eyecups that utilizes a new design for a superfusion and recording chamber. The chamber enables everted eyecups of the rat and mouse to be prepared quickly and reliably. Measurements of retinal responses indicate that eyecups maintained in the chamber remain viable for many hours.

2. Methods

2.1. Superfusion chamber

The eyecup is held in place between the lower (a) and upper (b) halves of a superfusion chamber, illustrated in Fig. 1. The eyecup (c) is everted over a dome (d) protruding from the lower half of the chamber and is held in place by a thin plastic sheet (e) with a hole in it, which holds the eyecup down against the dome (Fig. 1B). The retina of the eyecup faces upwards, exposed to the solution in the superfusate well (f).

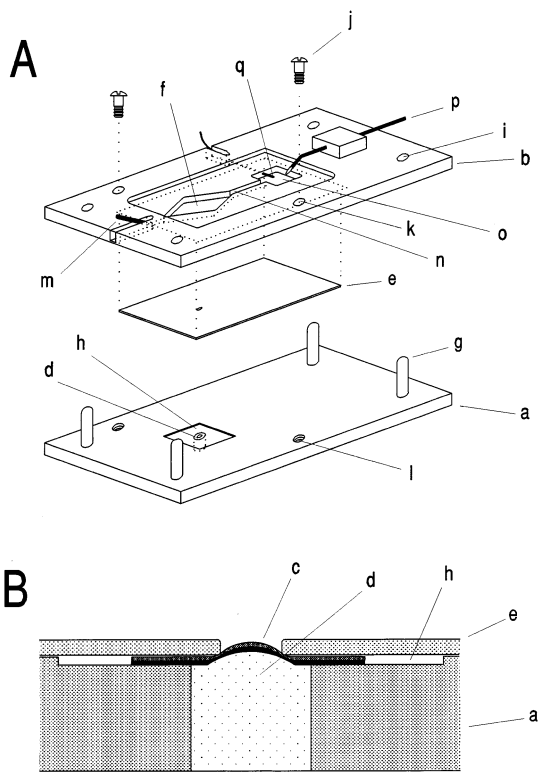

Fig. 1. Eyecup superfusion chamber. (A) Perspective view of chamber. (B) Enlarged, cross sectional view, showing the everted eyecup (c) held between the two joined halves of the chamber. The letters denoting parts of the chamber are explained in Section 2.1 of the text. Chamber dimensions are as follows: Both lower (a) and upper (b) halves are $7.62 \times 3.81 \times 0.325$ cm ($3.0'' \times 1.5'' \times 0.125''$) thick. The thin sheet (e) forming the bottom of the superfusion wells (f and o) is $4.06 \times 2.18 \times 0.406$ cm ($1.6'' \times 0.86'' \times 0.016''$) thick. The diameter of the hole in sheet (e) is 0.269 cm (0.106″) for rat and 0.165 cm (0.065″) for mouse. The dome (d) is 0.208 cm (0.082″) in diameter at its base and 0.036 cm (0.014″) high. The recess (h) in the lower half of the chamber is 0.020 cm (0.008″) deep. The upper and lowers halves of the chamber and the dome are made of acrylic. The thin sheet is polycarbonate. Detailed CAD drawings of the chamber are available on the Web at http://www.neurosci.umn.edu/faculty/newman_cad.html.

2.1.1. Lower half of the chamber

The dome (d) in the lower half of the chamber is inserted into a hole in the chamber bottom (a). This two piece construction allows different domes to be exchanged easily. A silver chloride pellet (not shown) can be inserted into a hole in the dome and serves as the scleral electrode when recording the electroretinogram (ERG). Posts (g) inserted into holes at the four corners of the lower chamber serve as guides for lowering the upper half of the chamber down onto the lower half. A recess (h) in the lower chamber around the dome prevents the eyecup from being compressed excessively.

2.1.2. Upper half of the chamber

The thin sheet (e) is seated into a recess in the bottom of the upper half of the chamber and serves as the base of the diamond-shaped superfusion well (f) and the secondary well (o). It is held in place by a film of vacuum grease. A hole is cut into the sheet so that a portion of the eyecup protrudes into the superfusion well. The alignment between the hole in the sheet and the dome in the lower chamber can be adjusted by sliding the plastic sheet within the recess in the upper chamber. The four posts (g) rising from the lower half of the chamber pass through holes (i) at the corners of the upper chamber. Two screws (j) pass through holes in the upper chamber (k) and into threaded holes (l) in the lower chamber, serving to fasten the two halves of the chamber together.

Superfusate, which is gravity-fed from a reservoir, passes into the diamond-shaped well (f) through 20 gauge stainless steel tubing (m). The superfusate leaves the well through a groove (n) machined into the top of the chamber and flows into a second, square well (o), where it is removed by suction applied through a beveled section of stainless steel tubing (p). A silver chloride pellet (q) in the square well serves as the ground electrode for neuronal recordings and as the vitreal electrode for ERG recordings.

When a water immersion (dipping) objective is used to view the retina, no coverslip is used. The superfusate forms a meniscus between the chamber and the objective. When a dry objective is used, a coverslip is placed across the top of the chamber to contain the superfusate. The coverslip should not cover the corner of the well where superfusate enters. Air bubbles in the super-

fusate escape from this open portion of the well before they are trapped beneath the coverslip. The chamber is designed so that solution in the well will not be sucked dry, even if flow of superfusate into the chamber is interrupted.

2.2. Procedure for preparing the eyecup

All procedures are preformed under normal illumination since the preparation dark adapts once placed in the dark (see Figs. 3 and 5). Animals are killed with an overdose of sodium pentobarbital (200 mg kg^{-1}) injected intraperitoneally and the eyes enucleated.

2.2.1. Rat

A small section from the back of the eye is used in preparing the eyecup. The enucleated eye is cut part way through with a razor. The cut is completed with iridectomy scissors with the eye submerged in superfusate solution. The cut through the eye is made obliquely, from the ora serrata on one side of the eye to the far side of the optic nerve. The eye segment is placed retinal side up on the dome of the bottom half of the chamber, using the open end of a Pasteur pipette for transfer. Excess fluid is pipetted from the chamber bottom. Surface tension serves to hold the eyecup flat against the dome. The eyecup is then positioned with forceps so that it is centered over the dome.

The top half of the chamber is lowered through the four guide posts onto the eyecup. As the two halves of the chamber come together, the eyecup is everted and held in place on the dome. The united chamber is then screwed together and the guide posts removed, if necessary.

Vitreous humor, which has been pushed through the hole into the well in the upper chamber, is removed by suction applied through 28 gauge stainless steel tubing. Superfusate is then added to the well and suction applied to remove additional vitreous. Care must be taken not to damage the retina when applying suction. The chamber is placed on the stage of a microscope (the chamber is not much larger than a standard microscope slide) and superfusion begun. The entire procedure, from enucleation to the start of superfusion, takes approximately 5 min.

2.2.2. Mouse

The mouse eyecup is prepared in a similar fashion to that of the rat. Because of its small size, the entire back half of the mouse eye is used. It is positioned with the optic disc centered over the dome. The hole in the plastic sheet forming the bottom of the superfusion chamber is smaller for the mouse eyecup than it is for the rat. The same size dome can be used for both preparations.

2.3. Superfusion of the eyecup

The volume of the diamond-shaped superfusion well, with a long working distance dipping objective in place, is ≈ 250 μl. In the current study, the eyecup was superfused at a rate of 2.5 ml min^{-1} with bicarbonate-buffered Ringer's solution or Ames' medium heated to 37°C. The Ringer's solution contained (in mM): NaCl, 117.0; KCl, 3.0; $CaCl_2$, 2.0; $MgSO_4$, 1.0; NaH_2PO_4, 0.5; dextrose, 15.0; $NaHCO_3$, 32; L-glutamate, 0.1. Ames' Medium (Sigma Chemical, St. Louis, MO), was supplemented with 32 mM $NaHCO_3$ and 0.1 mM glutamate. The solutions were equilibrated with 5% CO_2 in O_2 and had a pH of ≈ 7.53 at 37°C. In some experiments, rat eyecups were incubated in Ringer's solution containing collagenase/dispase (2 mg ml^{-1}; Boehringer–Mannheim) and DNase (0.1 mg ml^{-1}; Sigma) for 16 min at room temperature before beginning superfusion.

2.4. Stimuli and electrical recordings

Eyecups were stimulated by diffuse white light flashes from a tungsten source focused through a 10× objective lens above the preparation. The illuminance of the stimulus was 1200 foot-candles for ERG stimulation and adaptation and 90 foot-candles for spike recordings from neurons. The ERG was recorded differentially between vitreal (positive) and scleral (negative) electrodes and responses bandpass filtered between 0.1 and 200 Hz.

Extracellular spike activity from neurons in the ganglion cell layer was recorded with metal-in-glass microelectrodes plated with gold and platinum black (Dowben and Rose, 1953; Newman and Zahs, 1998). Signal-to-noise ratios for single unit recordings were typically 5:1 to 20:1. Recorded tri-phasic action potentials ranged from 150 to 500 μV (peak-to peak). Whole-cell patch-clamp recordings were made from astrocytes at the retinal surface as described previously (Newman and Zahs, 1997).

2.5. ERG measurement

The ERG b-wave amplitude was measured from the trough of the a-wave to the peak of the b-wave. The peaks of oscillatory potentials were disregarded when measuring b-wave amplitude. Results are given as mean ± SD with the number of samples in parentheses.

3. Results

3.1. ERG b-wave

The ERG, a sensitive measure of retinal state, was used to test the health of the everted eyecup prepara-

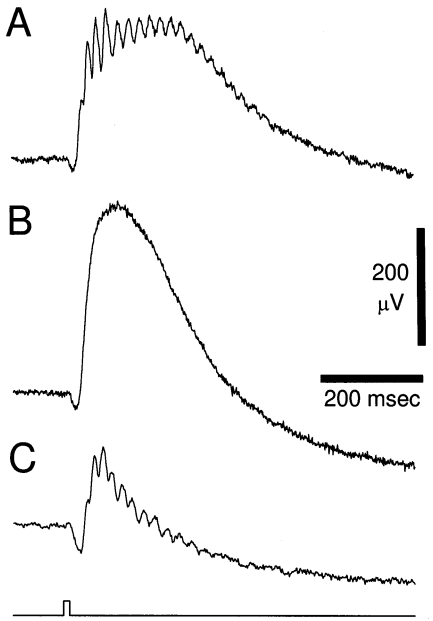

Fig. 2. ERGs recorded (unaveraged) from everted eyecups. (A) Rat eyecup superfused with Ringer's solution. (B) Rat eyecup superfused with Ames' medium. (C) Mouse eyecup superfused with Ringer's solution. The a-wave (initial negative transient), the b-wave (positive response), and oscillatory potentials (in A and C) are visible. Time course of the 12 ms light stimulus is indicated at the bottom.

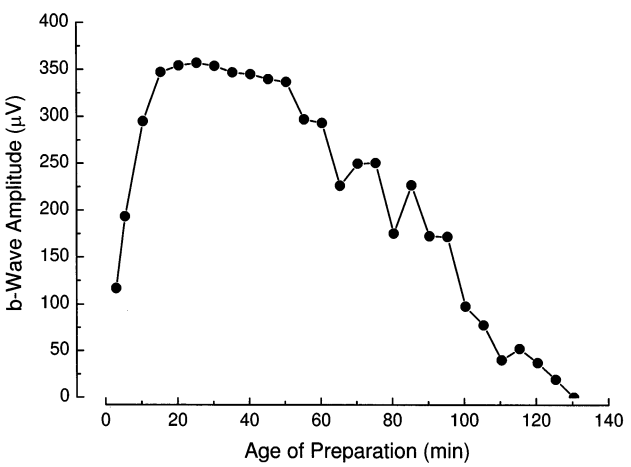

Fig. 3. Amplitude of the ERG b-wave as a function of age of the preparation. The ERG was recorded from a rat eyecup superfused with Ames' medium. The eyecup was in the dark between stimulus flashes, which were applied at 5 min intervals. The initial increase in b-wave amplitude occurred as the retina dark adapted following exposure to light during preparation of the eyecup.

tion. Both rat and mouse eyecups had prominent a- and b-waves (Fig. 2), typical of photopic ERGs evoked by brief light flashes (Reuter and Sanyal, 1984; Schaeppi et al., 1988; Goto et al., 1995). A prolonged negativity followed the b-wave in both species.

Prominent oscillatory potentials were present in rat (eight of eight experiments) when eyecups were superfused with Ringer's solution (Fig. 2A). These oscillatory potentials often lasted for 200 ms or longer, suggesting that the eyecup preparation superfused with Ringer's is somewhat more susceptible to oscillations than is the retina in vivo. The oscillatory potentials were substantially diminished when the preparation was superfused in Ames' medium (Fig. 2B). The oscillatory potentials were reduced in amplitude by more than 90% in three of ten experiments and were completely absent in the remaining seven experiments. Small oscillatory potentials were present in the mouse ERG when superfused in Ringer's solution (Fig. 2C; five of five experiments).

Mouse b-waves were consistently smaller than those of the rat. This is probably because: (1) in the mouse, the optic disc occupied a significant fraction of the stimulated region of retina while in the rat, the disc was outside the stimulated area; and (2) the shunt pathway around the edge of the eyecup (reducing ERG amplitude) was proportionally larger in the mouse because the stimulated area of the mouse was smaller than that of the rat ($\approx 38\%$ of the rat).

We have previously used a protocol of collagenase/dispase and DNase digestion in the isolated rat retina to efficiently remove the vitreous humor prior to making patch clamp recordings (Newman and Zahs, 1997). ERG measurements demonstrate that the rat eyecup is damaged by this treatment. Following enzyme incubation, both the a- and b-waves were completely absent. The loss of the ERG is as a result of enzyme-induced retinal detachment, which separates the retina from the pigment epithelium and prevents bleached photopigment from regenerating.

3.2. Superfusion solution

Several observations were made in preliminary experiments. Not surprisingly, the ERG b-wave was considerably smaller and slower at 24°C than at 37°C. At the higher temperature, used in all subsequent experiments, b-wave amplitude was greater and the eyecup remained viable for a longer time when 100 μM glutamate was added and 32 instead of 26 mM HCO_3^- was used in the superfusate.

3.3. Viability of the eyecup

The long-term viability of the eyecup preparation was determined by monitoring two light-evoked retinal responses, the ERG b-wave and neuronal spike activity, as the eyecup aged.

3.3.1. ERG

The ERG, evoked by 12 ms light flashes, was recorded at 5 min intervals. The amplitude of the ERG b-wave declined slowly over 2 h as the eyecup aged (Fig. 3). In the rat, maximal b-wave amplitude equaled 289 ± 82 μV (9). The b-wave declined to $\frac{1}{2}$ maximal

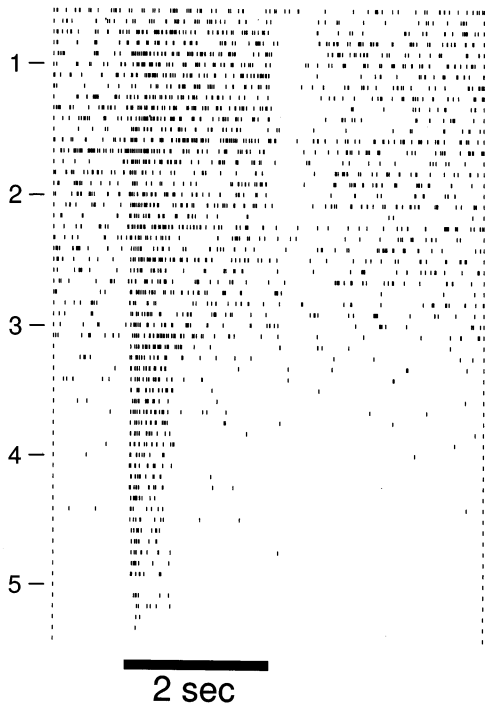

Fig. 4. Light-evoked spike activity of a single neuron in the ganglion cell layer over a 5 h period. Activity was recorded from a rat eyecup superfused with Ringer's solution. In this raster display of activity, each row represents a single 6 s stimulation trial. Within each trial, vertical line segments mark the occurrence of spikes. The beginning and end of each trial are also marked by vertical line segments. Trials were repeated at 5 min intervals. The numbers to the left indicate the age of the preparation in hours. The occurrence of the 2 s light stimulus is indicated at the bottom. During the first 3 h, the neuron possessed spontaneous activity, a sustained ON response, and a transient inhibition at light OFF. As the preparation aged, the spontaneous activity disappeared and the ON response became transient. The light-evoked response disappeared completely after 5.3 h.

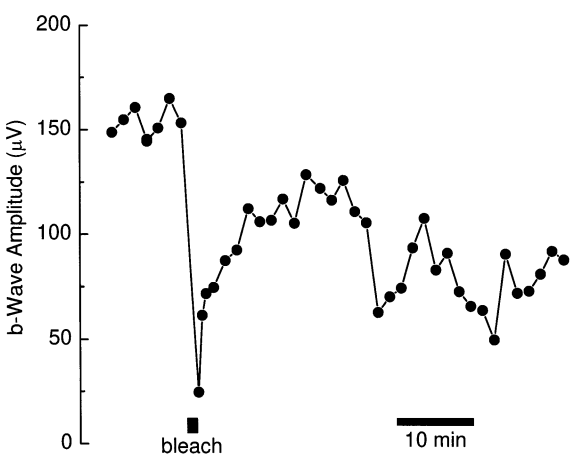

Fig. 5. Recovery of b-wave amplitude following prolonged light stimulation. b-Wave amplitude was recorded from a mouse eyecup in Ringer's solution. Following a 2 min bleaching light exposure, b-wave amplitude recovered over a 15 min period. The two dips in b-wave amplitude in the latter half of the plot occurred spontaneously.

amplitude after 77 ± 30 min (8). The ERG b-wave recorded from mouse eyecups showed a similar decay with time although it had a smaller amplitude. Maximal amplitude equaled 173 ± 57 μV (5) while the b-wave declined to $\frac{1}{2}$ maximal amplitude after 67 ± 9 min (5). In both rat and mouse, the sustained negative component of the ERG remained long after the b-wave had disappeared.

3.3.2. Neuronal spike activity

The viability of the eyecup was also determined by recording the extracellular spike activity of neurons in the ganglion cell layer evoked by 2 s light flashes. Light-evoked neuronal activity was present for a substantially longer time than were b-wave responses. Neuronal responses were present for at least 4 to 5 h. The raster display of spike activity illustrated in Fig. 4 was typical, with spontaneous activity and sustained responses dropping out after ≈ 3 h and transient responses remaining for an additional 2.3 h.

3.4. Recovery following light exposure

A critical test of eyecup viability is whether retinal responses recover following prolonged exposure to an adapting light. Recovery of retinal sensitivity was seen at the beginning of each ERG experiment. b-Wave amplitude increased as the eyecup dark adapted following the intense light exposure encountered during the preparation procedure (Fig. 3).

Recovery was also tested by adapting eyecups with a 2 min continuous light stimulus (Fig. 5). Immediately following the light exposure, the b-wave was reduced to $15 \pm 13\%$ (9) of maximal amplitude. b-Wave amplitude then recovered to near control levels after approximately 5–12 min in the rat and after 10–25 min in the mouse.

In these dark adaptation experiments, recovery of retinal sensitivity was monitored with test flashes applied at 5 min intervals. These flashes were relatively intense and prevented the retinas from dark adapting completely. Thus, our dark adaptation experiments measure the time course of the initial (photopic) phase of adaptation (Green, 1973), which is completed within minutes (Dowling, 1987), rather than the time course of adaptation to a fully dark adapted state.

3.5. Rate of superfusate exchange

A rapid rate of superfusate exchange at the retinal surface is needed to adequately oxygenate the eyecup and is advantageous when performing ion exchange and pharmacological experiments. The rate of solution exchange was assessed by recording the shift in membrane potential in astrocytes at the surface of the retina in response to changes in superfusate K^+ concentra-

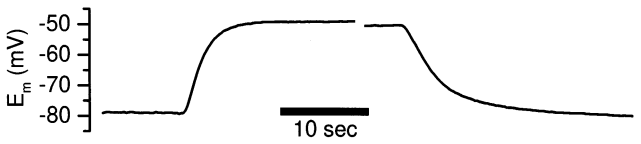

Fig. 6. Speed of superfusate exchange at the eyecup surface. The membrane potential of an astrocyte at the vitreal surface of the retina (E_m) shown as a function of time. When the superfusate was switched from a solution containing 3 mM K^+ to one containing 12 mM K^+, the cell depolarized rapidly. A rapid hyperpolarization followed the return to 3 mM K^+. A 3 min interval between the two solution changes has been removed from the trace.

tion. When superfusate K^+ concentration was raised from 3 to 12 mM, astrocytes depolarized rapidly (Fig. 6). The exchange time (for nearly complete exchange) was ≈ 9 s. The superfusate exchange time for neurons within the retina will, of course, be somewhat longer.

4. Discussion

4.1. Eyecup preparation

Our results demonstrate that eyecups of the rat and mouse can be successfully maintained for many hours in vitro. Neurons in the proximal retina remain responsive for 4 to 5 h. The eyecups are prepared easily and rapidly. Superfusate exchange in the chamber is rapid, with near total exchange at the retinal surface occurring in under 10 s.

4.2. Superfusate composition

The eyecups remained healthy longer when the superfusate was supplemented with 100 μM glutamate. A similar finding has been reported for the isolated rat retina (Winkler et al., 1999), where the addition of 250 μM glutamate prevents a decline in b-wave amplitude. It is interesting to note that glutamate levels in the vitreous humor in vivo are ≈ 10 μM (Gunnarson et al., 1987; Dreyer et al., 1996; Ambati et al., 1997). Thus, the mammalian retina normally functions in the presence of relatively high glutamate levels.

ERG b-wave amplitudes were considerably larger when the superfusate contained elevated HCO_3^- levels, 32 mM rather than the standard 26 mM. A similar finding has been observed in the isolated rat retina (Winkler, 1986). The raised HCO_3^- level, resulting in a superfusate pH of ≈ 7.53, presumably offsets an abnormally low pH within the retina arising from inadequate clearance of metabolic by-products. In hippocampal slices, 35 mM HCO_3^- Ringer's has been used to offset tissue acidification and to raise intracellular pH to normal levels (Grichtchenko and Chesler, 1994).

Eyecups superfused with either Ringer's solution or Ames' medium remained healthy for several hours. The oscillatory potentials present in Ringer's (and in vivo) were largely absent in Ames' medium, however. Glycine or other amino acids present in Ames' medium may block inhibitory interactions in the retina, suppressing the oscillatory potentials, as they do in the amphibian retina (Wachtmeister and Dowling, 1978).

4.3. Eyecup viability

The two measures of eyecup viability used in this study, b-wave amplitude and neuronal activity, yielded different assessments of how rapidly the preparation deteriorates with age. At approximately 1 h, when b-wave amplitude was reduced by half, the activity of neurons in the proximal retina showed little change. Neurons remained responsive to light stimuli for 2 to 3 h after the b-wave was abolished. A similar dissociation of the ERG from neuronal activity has been reported in the rabbit (Masland and Ames, 1975), where ganglion cell activity remained unchanged after substantial reductions in b-wave amplitude. It is not clear why neurons in the proximal retina remain responsive several hours after the b-wave, which reflects the activity of ON bipolar cells (Xu and Karwoski, 1994), has declined. The deterioration of the b-wave most likely reflects an inadequate long term oxygenation of the retina. In the rabbit eyecup, which has a significantly thinner retina and thus is easier to oxygenate, the ERG remains stable for many hours (Miller et al., 1986).

Note added in proof

Additional experiments have demonstrated that the ERG remains stable for a substantially longer time when eyecups are maintained at 30 rather than 37°C. In the mouse eyecup at 30°C, the b-wave remains healthy for hours, its amplitude declining by only 25% after 3.5–5 h.

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