Electrophysiological properties of a new isolated rat retina preparation

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Abstract

A piece of rat retina was mounted in an open chamber and perfused with a Ringer solution at 37°C. The electroretinogram (ERG) was recorded between an extracellular microelectrode in contact with the rod outer segments and a reference electrode under the retina. The addition of 250–500 μM of glutamate to the media prevented the b-wave from decaying in amplitude with time. Minor components of the ERG, the scotopic threshold response (STR) and oscillatory potentials (OPs), were well maintained with glutamate in the media. Experiments on the spatial properties of the recordings indicated that a small area immediately around the microelectrode contributes most strongly to the response. The similarity of ERGs recorded in vivo from the cornea to the transretinal ERGs from the isolated retina of the same animal indicated that the functional integrity of the isolated retina was well preserved in the media with glutamate. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: Isolated retina; Electroretinogram; Rat; Glutamate

1. Introduction

The motivation for the experiments reported here was the need for a preparation that would be suitable for studying postreceptor (network) mechanisms of retinal adaptation in the mammalian retina. The rat retina is well suited for these studies since it is known to show significant nonreceptor adaptation. A steady background light at an intensity that just begins to affect receptor potentials elevates b-wave thresholds by 2 log units and ganglion cell thresholds by 3 log units (Powers & Green, 1990). The pioneering studies of Weinstein, Hobson and Dowling (1967), and Winkler (1972) have demonstrated the utility and feasibility of recording a relatively normal looking ERG from the perfused isolated rat retina using a closed chamber. Since we wanted to be able to introduce microelectrodes we needed an open chamber. In our initial experiments on the isolated retina we found that after 15 min in the our open recording chamber the responses from the isolated retina began to steadily decline. As we report here, to

obtain stable recordings it was necessary to add glutamate to the perfusate. We describe the effect of glutamate on the stability of the b-wave. A series of experiments deal with the origins of the b-wave in this preparation. Finally, to establish the extent to which our responses are normal we have compared responses from perfused, isolated rat retinas to those obtained from anesthetized, intact animals.

2. Methods

2.1. Preparation of retinas

Albino rats 6–8 weeks old (Sprague-Dawley) weighing 200–250 g were dark-adapted for a minimum of 12 h. Using night vision devices (NAV-3, Intevac, Palo Alto, CA) and infrared illumination, an animal was anesthetized with intraperitoneal injections of sodium pentobarbital (30 mg kg^{-1}) and then decapitated. Both eyes were enucleated. With each eye an incision was made at the equator of the globe with a razor blade and the anterior portion of the eye removed by successively enlarging the incision with microscissors. The eyecup,

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hemisected slightly below the ora serrata, was then placed in a dish of Ringer solution at room temperature that had been bubbled with 95% O_2 and 5% CO_2. The vitreous humor and lens were removed and the retina was then detached from the retinal pigment epithelium (RPE). Just before being placed in the chamber the retina was cut into pieces. A piece of the retina ($\approx 3 \times 3$ mm) was placed receptor side up on a slightly smaller piece of Millipore filter (type SC), which had been perforated with several 1 mm holes, and placed in the recording chamber.

2.2. Perfusion system

The filter and retina were positioned horizontally in the perfusion chamber between two 12 mm diameter circular pieces of mesh, which were glued to Teflon O-rings with cyanoacrylate adhesive (Krazy Glue, Borden, Columbus, OH). The lower mesh was made from fiberglass screening and the upper mesh from a white nylon material having threads 0.2 mm diameter and approximately 1 mm grid spacing (Fig. 1). The volume of the fluid in the chamber was approximately 0.5 ml. The fluid entered the open chamber (12 mm diameter) through a small orifice above the retina on one side of the open chamber and flowed over the retina and through the mesh to perfuse both sides of the tissue. It was drained away by a small strip of tissue paper on the opposite side of the chamber. The tissue paper wicked the fluid into a second chamber where it was sucked by a vacuum pump into an accumulation reservoir.

The bathing solution entering the chamber was gravity-fed from bottles above the recording chamber. The composition of the Ringer solution was (in mM): NaCl, 110; KCl, 5; Na_2HPO_4, 0.8; NaH_2PO_4, 0.1; $NaHCO_3$, 30; $MgSO_4$, 1; $CaCl_2$, 1.8; glucose, 22, Na-glutamate 0.25–0.5. Plasma was not used since we were unable to show a noticeable effect on retinal survival or on the

ERG waveform when it was present in the perfusate. To avoid bubble formation in the chamber we found that it was necessary to preheat the perfusate to 38–39°C in the bottles (aquarium heaters were used for this purpose). In addition, using a heated Nichrome wire wrapped around approximately 1 foot of Teflon tubing which threaded under the chamber itself, the temperature inside the chamber was maintained at 36–37°C. To do this a small thermistor probe (Yellow Springs Instruments, Model 73a, Yellow Springs, OH) was inserted into the fluid in the chamber and connected to an on-off controller (YSI, Model 554). The controller switched a constant current power supply (Tektronix PS282, Beaverton, OR) that heated the Nichrome wire. The perfusate in the bottles was bubbled with a 95% O_2 and 5% CO_2 mixture using fine-pored air stones. The pH of the solution at 38–39°C was 7.45–7.55, and required no further adjustment. The retina was continuously perfused at 3 ml min^{-1}, as indicated on an in-line flow meter (Gilmont, #12, Barrington, IL).

2.3. Recordings

All recordings were performed in a light-tight Faraday cage. Light stimuli were projected onto the retina through a Zeiss operating microscope, from an optical stimulator mounted externally to the cage. Microelectrodes were advanced using hydraulic microdrives (Model 650, Kopf Instruments, Tujunga, CA) mounted on x,y,z manipulators (Narishige, Model MM3). The microelectrode entered the chamber from above and made an angle of 70° with respect to the retina (Fig. 1). The tip openings were 1.5–5.0 micron diameter. The resistance of the micropipette was 5–25 mΩ.

Electrical potentials were recorded between an Ag–AgCl macro-electrode (Type E202, IVM, Healdsburg, CA), built in the bottom of the chamber, and a glass capillary microelectrode filled with physiological saline. The potentials were amplified with a high gain amplifier (CyberAmp 320, Axon Instruments, Foster City, CA), using a bandwidth of 0–400 Hz. The noise level of the recordings was 5 μV. The variability in amplitude between successive responses to the same stimulus was of the order of ± 10 μV. Thus, except at the lowest intensity, there was no need to average responses and single flash responses were obtained for each condition. Responses were digitized at a rate of 1000 samples per second and stored using an on-line Macintosh Computer (Apple, Cupertino, CA) data acquisition system (LabView, National Instruments, Austin, TX).

2.3.1. Isolated retina

The chamber was mounted on a vertical microjack (Model EL 80, Newport/Klinger, Garden City, NY) onto which x-y translators had been mounted (Model 16021, Oriel, Stratford, CT). A dial micrometer
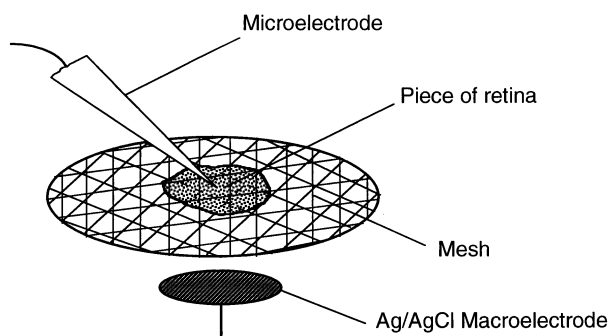

Fig. 1. Schematic diagram of the recording arrangement. A piece of the retina ($\approx 3 \times 3$ mm) was placed receptor side up on a slightly smaller piece of Millipore filter (type SC) between pieces of mesh and illuminated from above. ERGs were recorded between a microelectrode at the tips of the rod outer segments and a reference electrode 2 mm below the retina.

mounted above the chamber registered the vertical position of the chamber and allowed its height to be varied in a controlled manner. Prior to placing the retina in the chamber, the light stimuli were focused on the surface of the fluid in the chamber. By viewing through the microscope, the microelectrode tip was visualized and carefully advanced until it just touched the fluid surface. The reading on the microdrive position counter defined the location of the plane of focus. The electrode was then laterally positioned using the x-y manipulator so it intersected the plane of focus in the center of the smallest spot stimulus. The microdrive was then withdrawn several mm and the piece of retina was placed in the chamber. It took about 10 min for the temperature to stabilize. There was 1.5–2.0 mm of fluid about the retina and so the height of the chamber was raised to bring the stimulus into focus on the retina. The electrode was readvanced and the point where the electrode touched the retina was identified by the slight voltage shift which occurred when the electrode entered the retina. The reading of the depth counter on the micromanipulator was noted. If the plane of focus was more than 100 μm from the tips of the outer segments, adjustments were made to the height of the chamber to bring the stimulus into focused on the tips of the outer segments. The experimental clock was set to zero when the temperature stabilized. The electrode was then advanced until it just touched the tips of the rod outer segments.

2.3.2. Intact animal

Before each experimental session the animal was dark-adapted for at least 12 h and then anesthetized with intraperitonial injections of a saline solution containing (in μg gm^{-1} body weight) 15 ketamine, 15 xylazine, 600 urethane. Supplementary doses of anesthetic were given as needed. The surgery and setup were done under infrared illumination. The eyelids of both eyes were drawn back with silk sutures. The cornea was anesthetized with Tetracaine (0.5%) and the pupil dilated with 1% atropine sulfate applied to the cornea. The rat was laid on its side, with its head fixed in place with surgical tape. Its body temperature was maintained at 37°C with a heating pad that was switched on and off by a YSI temperature controller (Model 554) connected to a rectal temperature probe. ERGs were recorded between a silver–silver chloride cotton-wick electrode that was placed on the vertex of the cornea and a similar electrode placed in a cut in the snout. To produce uniform stimulation of the whole retina a segment of a ping-ping ball was placed over the eye.

2.4. Stimulation

The stimulus ($\lambda = 500$ nm) was delivered as either a 40 ms shuttered flash or as a brief electronic flash (less

than 1 ms duration). The shutter (Uniblitz, Model 325B) was placed in a beam of light derived from a 150 W xenon arc lamp (Osram, XBO150). The electronic flash was derived from a commercial photoflash unit. The intensity and color of the stimuli were controlled by placing calibrated neutral-density and interference filters in the stimulus beams. The timing of the stimulus presentation was under computer control. Data were collected using a fixed stimulus set in which the intensity increased progressively in 0.5 log steps. Low intensity flashes were interposed between brighter flashes to assure there was sufficient time between flashes for the prior sensitivity to be restored. A 12 s interstimulus interval (ISI) with low intensity responses and a 60 s ISI with the highest intensity responses were sufficient for the measured response amplitudes to be unaffected by earlier flashes. The peak amplitudes of the recorded ERGs were measured by fitting a second-order polynomial to a segment of the record containing the peak (Green, Herreros de Tejada & Glover, 1991). The amplitude of the first negative peak defined the a-wave amplitude. The voltage measured from the negative peak (a-wave) to the first positive peak defined the b-wave amplitude.

2.5. Stimulus calibration

In the isolated retina at 500 nm the unattenuated xenon stimulus was estimated to produce 3.74 log quanta/rod/flash. This estimate was obtained as follows. The photon flux ($\lambda = 500$ nm) incident on the retina from a xenon arc lamp was directly measured using a calibrated radiometric detector (United Detector Technology, S370, Hawthorne, CA). To estimate the stimulus intensity in the intact animal the photon flux falling on the eye was measured and this was converted to retinal units by assuming a 4.0 mm diameter entry pupil and distributing the total light entering through the pupil over the whole 40 mm^2 of retina. The flux per rod was calculated by assuming half the light is absorbed (Hagins, Penn & Yoshikami, 1970; Penn & Hagins, 1972) and each rod has a cross-section area of 2.3 μm^2 (Cone, 1963). The electronic flash was calibrated by equating it with the shuttered flash. That is, at low intensities equal b-wave responses to the shuttered and electronic flashes were assumed to occur when the retina had absorbed equal numbers of quanta/flash.

3. Results

3.1. Survival

Fig. 2 shows b-wave response amplitude versus time from five representative experiments using a Ringer
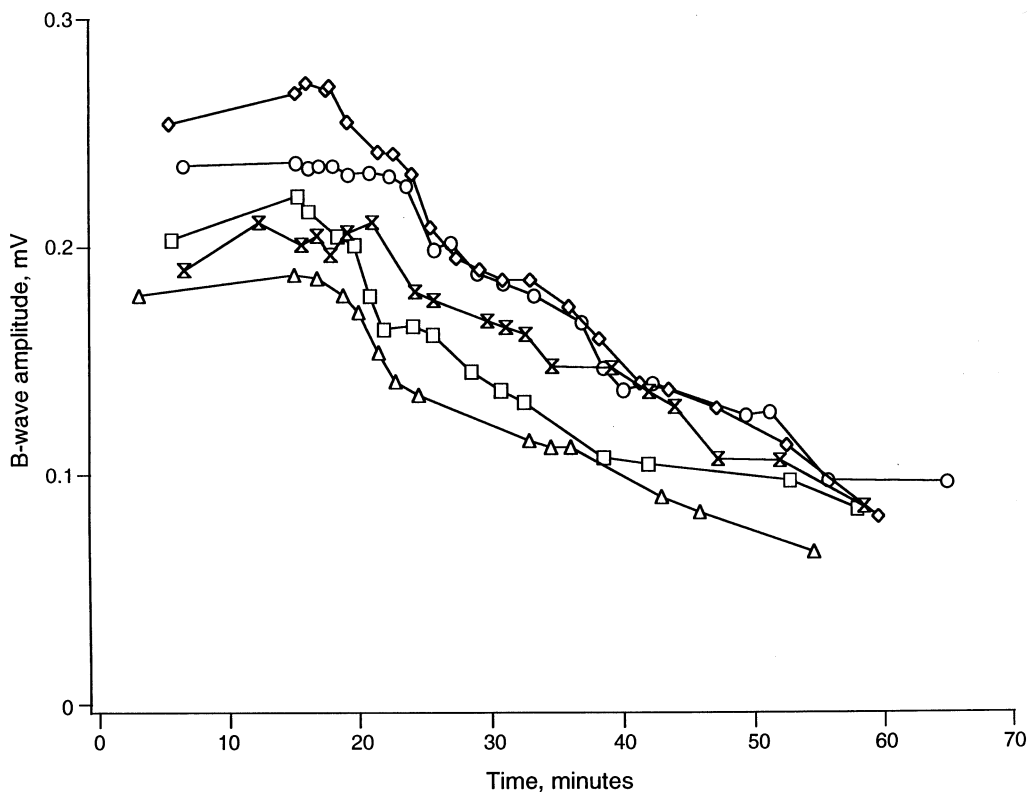
Fig. 2. B-wave amplitude as a function of time, with the retina perfused with a Ringer lacking glutamate. The points show the amplitude of the response elicited by a flash of intensity 0.18 log (quanta/rod/flash). Each different symbol indicates a different retina.

solution containing inorganic salts and glucose. The responses were evoked by a dim flash (0.2 log quanta/rod/flash). A total of 15 min after the temperature stabilized, response amplitudes began to steadily decline so that after 40 min the amplitudes averaged 42% of their initial values. In 14 other experiments the b-wave amplitude after 1 h was $47.5 \pm 9.7\%$ (SD) of its initial value. Responses to higher intensity flashes showed that the b-wave had decayed more than the a-wave. The deterioration of the b-wave relative to the a-wave suggested that synaptic transmission from receptors to bipolar cells was compromised. Given the high release of glutamate from photoreceptors in the dark it seemed possible that perfusion might be washing away glutamate before it could be recycled.

To test this idea we added glutamate to the media after the b-wave had deteriorated. Concentrations of 250–500 µM of glutamate had striking and dramatic effects. They reversed the decline and partially restored the response amplitude ($n = 7$). Fig. 3(A) shows an example of such an experiment. After 1 h of recording without glutamate, the b-wave was 40% of control (open symbols). When 250 µM glutamate was added to the medium (solid symbols) the process of b-wave decay was reversed and the b-wave response began to grow in amplitude. Fig. 3(B) shows responses obtained at different times during the experiment. At the start

(time point #1) the stimulus (0.2 log quanta/rod/flash) evoked a 200 µV b-wave. A total of 30 min later, without glutamate, this response had decreased to 80 µV (time point #2). Substituting a medium with glutamate for 30 min (time point #3) increased the response to 150 µV. Fig. 3(C) shows responses to a brighter stimulus (2.7 log quanta/rod/s) at these same time points. Both a- and b-waves are evident in the records. The record at time point #2 shows b-wave deterioration, with some associated a-wave reduction, but the recovery with glutamate at time point #3 is without any change in the a-wave relative to time point #2. Fig. 3(D) plots the b-wave response-intensity response functions at the three time points and Fig. 3(E) shows these functions after being normalized by the response amplitudes at an intensity of 0.7 log quanta/rod/flash. Neither the b-wave decay (time point #2) nor the recovery with glutamate (time point #3) seemed to be associated with a general shift in the intensity-response curves. There was a dip in the curves at a log intensity of 2.0. The likely reason for the dip is that measuring the b-wave from the peak of the a-wave to the peak of the first positive response grossly underestimates the true amplitude of this process. In this regard, it is interesting to note that there is a larger dip at 55 min (time point #2) than at 5 min (time point #1) or at 90 min (time point #3). This is presumably
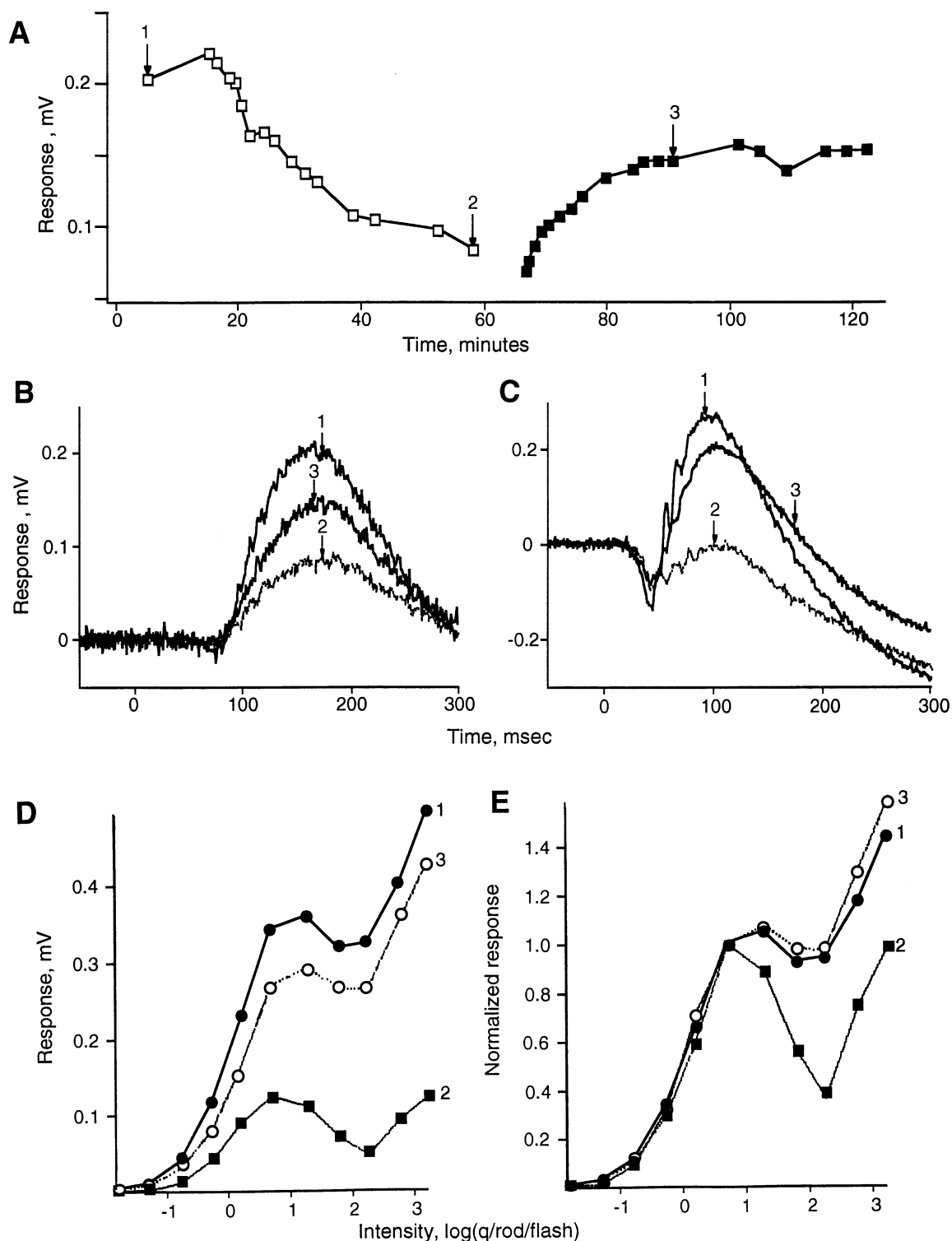
Fig. 3. Adding glutamate to medium after 1 h without glutamate. (A) Open squares show the amplitudes of the responses elicited by a flash of intensity 0.18 log (quanta/rod/flash) without glutamate in the medium. The filled squares were determined after switching to a medium with 250 μM glutamate. When glutamate was added to the medium the b-wave response began to grow in amplitude. The numbers identify three time points at which a series of flashes of variable intensity were presented. (B) Sample responses evoked by a flash of intensity 0.18 log (quanta/rod/flash) at the three time points. (C) Responses evoked by a flash of intensity 2.7 log (quanta/rod/flash) at the three time points. (D) Amplitude of the b-wave as a function of intensity before and after glutamate (times labeled 1, 2 and 3 in Fig. 3A). (E) Normalized response intensity functions. The responses to a 0.7 log (quanta/rod/flash) were normalized to one.
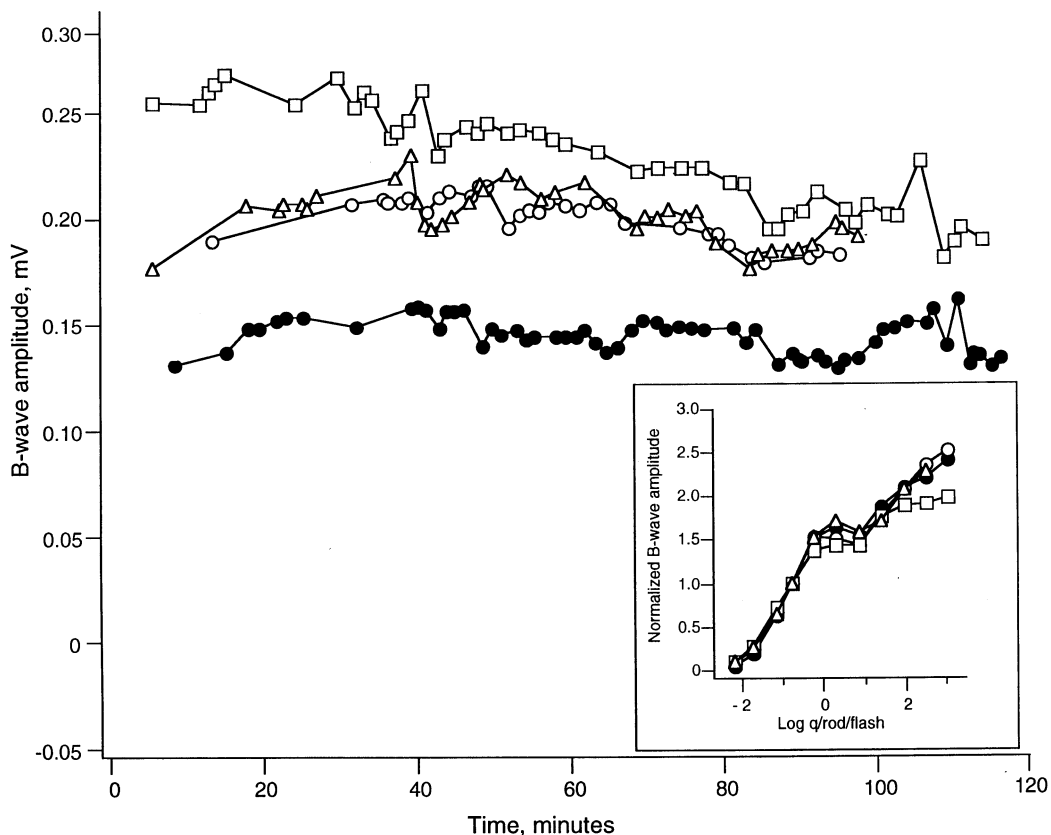
Fig. 4. Survival of b-wave amplitudes in solution containing glutamate. (Upper panel) Amplitudes of the response elicited by a flash of intensity 0.18 log (quanta/rod/flash) as a function of time. The perfusate contained either 250 (triangles) or 500 µM of glutamate (circles and squares). (Lower panel) Response-intensity relationships for the four retinas. The responses are normalized by the amplitude of the response evoked by the stimulus intensity 1.3 log (quanta/rod/flash). The symbols are the same as those used in upper panel.

because the decay of the b-wave results in a larger a-wave/b-wave interaction.

Given the findings outlined above we did experiments in which varying amounts of glutamate were added to the media. We found that by having 250–500 µM glutamate in the media responses could be maintained over extended period of times. Representative results from four retinas are shown in Fig. 4. In the upper panel in Fig. 4 the amplitudes of the b-wave evoked by a single dim flash (0.2 log quanta/rod/flash) are plotted over a 2 h period. It is of interest to note that the data shown with circles (○, ●) are from one of the rare instances where recordings were made from the left and right eyes of the same animal. Recordings from one piece were made immediately after dissection (●). The second piece (○) which had been kept in a dish of oxygenated Ringer solution containing glutamate for 2 h prior to recording, had the larger responses. These responses remained stable in amplitude for an additional 2 h. The lower right panel in Fig. 4 shows plots of the response amplitude as a function of stimulus intensity for each of the four pieces. For 19 retinas (including the four retinas shown in Fig. 4), the average amplitude after 2 h was $92.8 \pm 11.5\%$ (SD) of its initial

value. For the six retinas in 250 µM the amplitudes were $97.8 \pm 7.0\%$ (SD) of control after 2 h and for the 13 retinas in 500 µM glutamate the amplitudes were $90.5 \pm 12.3\%$ (SD) of control after 2 h. Concentrations of 125 µM and lower, slowed the decay process but did not completely eliminate it (see Fig. 6). Since it is well known that concentrations greater than 2 mM glutamate can block synaptic transmission from photoreceptors to second order cells in the retina, presumably by elevating the concentrations of glutamate in the synaptic cleft and interfering with synaptic transmission, it seemed possible that the addition of 250 µM glutamate might elevate extracellular glutamate sufficiently to alter synaptic transmission from receptors to bipolar cells. Consequently, we decided to carefully examine the effects of these concentrations of glutamate on kinetics of the b-wave and its sensitivity. In four retinas we compared responses obtained without glutamate to those obtained after glutamate had been added to the media. This comparison was possible because there was little or no deterioration in the response during the first 20 min without glutamate in the recording chamber (see Fig. 2). Fig. 5(A) shows a family of responses to flashes of increasing intensity from these four retinas recorded
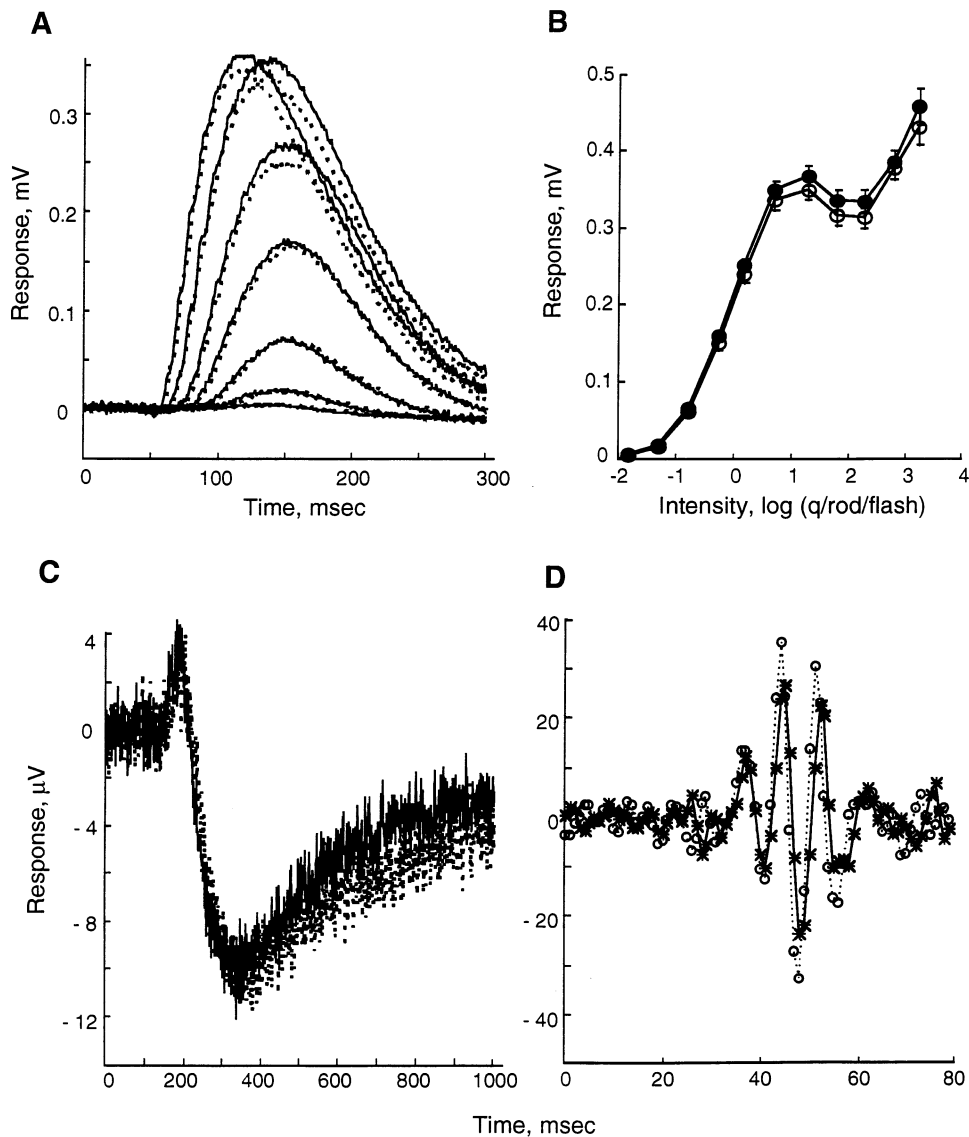
Fig. 5. Effects of glutamate on response components. (A) Family of b-wave responses as a function of intensity with and without glutamate. The responses plotted with dotted lines were recorded during the first 20 min, when the medium lacked glutamate. The responses plotted with solid lines were obtained after adding 250 μM glutamate to the medium. Each curve was obtained by averaging the responses from four different retinas. The stimulus intensities ranged from −1.8 to 1.3 log (quanta/rod/flash), in 0.5 log unit steps. (B) Comparison of intensity-response functions with (●) and without (○) glutamate in the media. The results are from two groups of retinas. In one group the retina was prepared and perfused without glutamate ($n = 23$) and in the other the retina was prepared and perfused with 250 μM in the media ($n = 21$). The points plot the peak amplitude ($\pm$ SE) as a function of the stimulus intensity. (C) STRs with and without glutamate (solid and dotted lines, respectively). The two curves are the lowest intensity records in part A, on different response and time axes. (D) The OPs isolated from high intensity responses (2.8 log quanta/rod/flash) by bandpass filtering (75–300 Hz). Records obtained before glutamate (dotted curve with open circles) and with glutamate (solid curve with stars). The OPs are from one of the animals used to obtain the records in part A.

in the absence of glutamate (dashed lines) and after glutamate had been added to the media (solid lines). The two sets of responses are very similar in their amplitudes and kinetics. To examine a larger group of animals two groups of retinas were examined. In one group ($n = 23$) the retina was prepared without glutamate and the other ($n = 21$) with 250 μM glutamate in the media. The points in Fig. 5(B) show a comparison

of response-intensity functions from these two groups recorded during the first 20 min with and without glutamate in the perfusate. The standard errors of the measurements overlap, but there is a systematic tendency for the responses in glutamate to be slightly larger in amplitude at all intensities, an effect we attribute to deterioration which occurred in the absence of glutamate.

3.2. Minor components

Low intensity stimuli evoked slow, relative to the b-wave, negative responses called the scotopic threshold responses (STRs) (Sieving, Frishman & Steinberg, 1986). The STR is not of photoreceptor origin but rather reflects the activity of amacrine or ganglion cells (Sieving et al., 1986; Naarendorp & Sieving, 1991; Robson & Frishman, 1996). Fig. 5(C) shows examples of STRs. These records are actually the lowest intensity responses from the series shown in Fig. 5(A), where glutamate was added to the medium during the course of the experiment. In the group of 21 experiments where glutamate was present in the media during the whole experiment the average STR amplitude to a flash of -1.8 log quanta/rod was 11.9 ± 0.9 μV (SE), as compared to an amplitude of 9.1 ± 0.5 μV (SE) in the 23 experiments where glutamate was absent from the media. Thus STR was significantly larger ($P = 0.001$) with glutamate in the media.

In response to a bright flash our b-waves contained wavelets or oscillatory potentials (OPs), high frequency components superimposed on the leading edge of the b-wave. It is uncertain which cells generate these responses, but there is general agreement that OPs are of inner retinal origin (Heynen, Wachtmeister, & van Norren, 1985; Sandberg, 1994). The OPs can be easily isolated from the total ERG by bandpass filtering the signal at 75–300 Hz. In Fig. 5(D) we show an example of OPs, from a single experiment, recorded before and after glutamate. We have not averaged these responses since with 1 ms sampling the variability in the exact timing of the OPs from one experiment to another tended to remove this component from the averaged response. Consequently we filtered individual records and measured the large amplitude OPs at about 45 ms. In the 21 retinas with 250 μM glutamate in the media the average amplitude from the baseline of the OPs in response to a flash of intensity 2.8 log quanta/rod was 36.1 ± 2.7 μV (SE) compared to 26.7 ± 4.1 μV (SE) in the 23 retinas without glutamate. The OPs in glutamate were significantly larger than without glutamate ($P = 0.01$). The larger STR's and OPs in the presence of glutamate shows that there was greater activity evoked in third order neurons and suggests to us that these retinas were in better physiological condition.

Fig. 6 shows the dose response curve for the ability of glutamate to prevent decay of the b-wave. Without glutamate the b-wave response amplitude fell by about 50% in 1 h. Only when the glutamate concentration was 250 μM was there no significant decay in the amplitude of the b-wave over time.

3.3. Receptive field of the recordings

To address the question of how large an area of the retina contributed to the responses, we measured responses to spots of increasing diameter (Fig. 7A). The smallest spot that we could sharply image on the retina had a diameter of 0.125 mm. When the stimulus is sufficiently small that it illuminates an area around the electrode within which all the cells contribute equally to the extracellular response, the response amplitude should increase linearly with stimulus area (the line in Fig. 7A). Our measured extracellular b-wave never grew linearly with area, indicating less than perfect areal summation.

When we checked the centering of our spots by moving them in each direction, we discovered that moving a small spot by as little as 0.2 mm from the centered position led to a large fall in response amplitude. Fig. 7(B) shows measurements from three such experiments where great care was taken to ensure well focused stimuli (see Section 2). The results show that the response fell even when the spot was moved by as little as 0.1 mm from being centered on the tip of the electrode. To characterize the spatial dependency of the response, the fall-off with distance, $S(x)$, was fit with a mathematical function. We found that the data were reasonably well fit by the function,
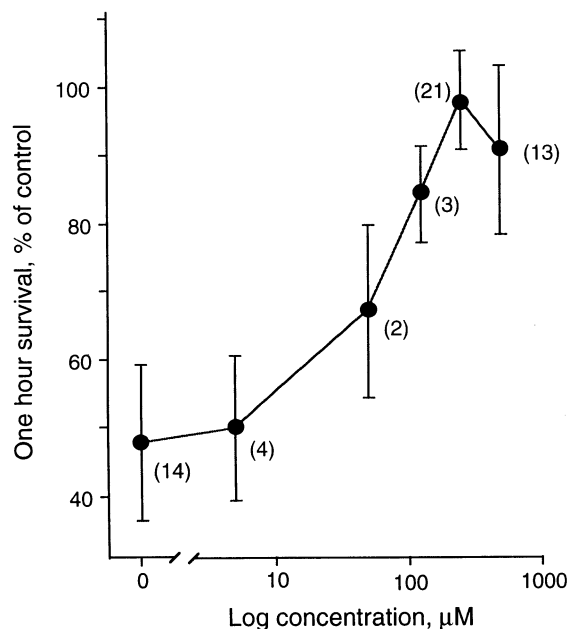

Fig. 6. Dose response curve for the ability of glutamate to prevent decay of the b-wave. The amplitude of the b-wave measured 1 h after the start of experiment is plot against the concentration of glutamate in the media. The b-wave amplitude is plotted as a percentage of its starting value. Each point is the mean with bars indicating the standard deviations. The number beside each point indicates the number of experiments.
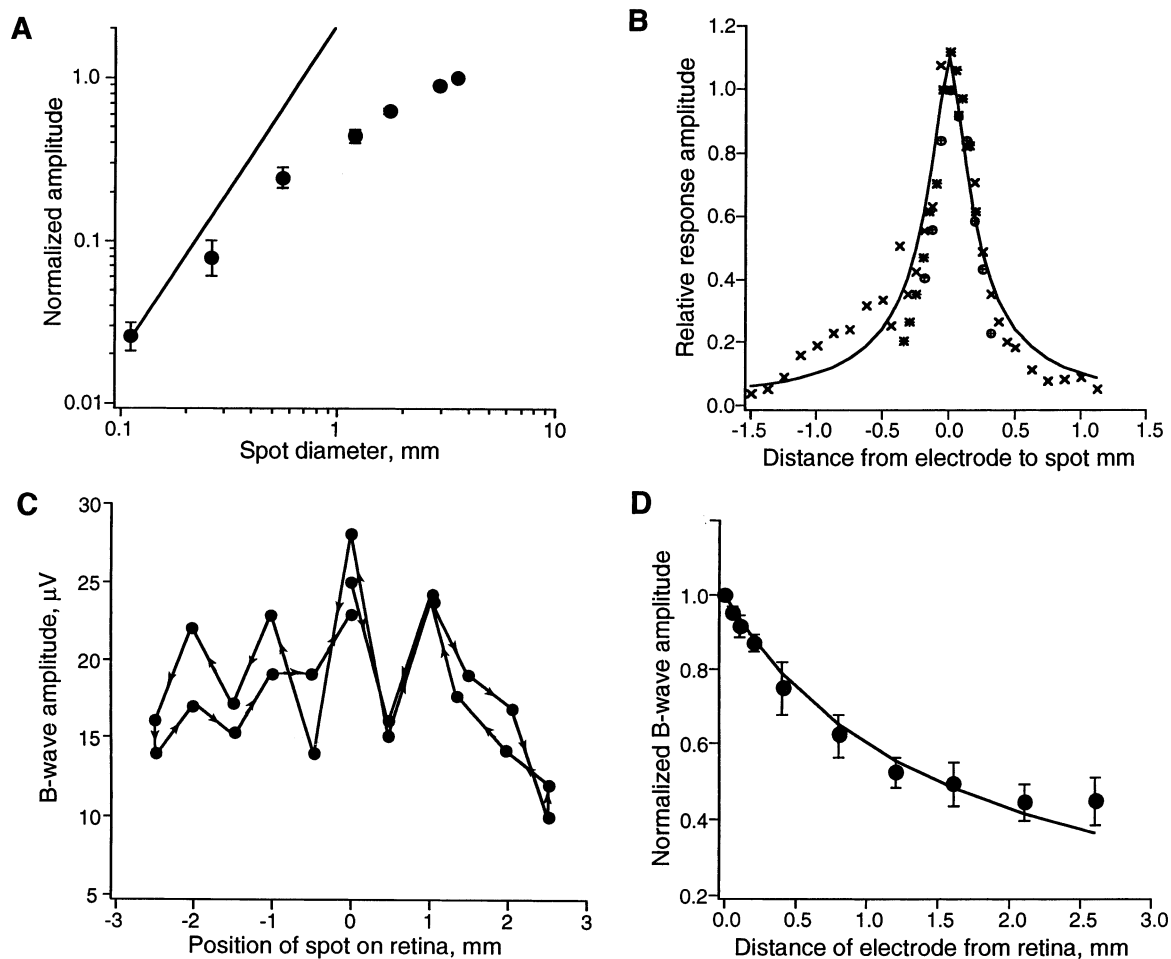
A

B

C

D

Fig. 7. Spatial properties of the responses. (A) The amplitude of the b-wave evoked by spots of different diameters centered on the recording electrode. The points plot the means $\pm$ SD ($n = 4$). The straight line plots the relationship to be expected if response were a linear function of the stimulus area. (B) Response receptive field. The amplitude of the response evoked by a small spot as a function of its distance from the recording site. After normalization to the same peak amplitude of response, the results of three separate experiments were plotted with different symbols. The solid curve drawn through the points is the function $S(x) = 0.11/(0.1 + |x|^{3/2})$. (C) Effect of position of stimulus on the piece of retina. An electrode and associated centered spot were imaged onto various parts of a piece of retina. The points are the amplitudes of responses to a single flash. The arrows indicate the sequence of movements over the piece of retina. (D) Effect of distance of electrode from surface of the retina. Amplitude of the b-wave as the recording microelectrode was moved away from the tips of the rod outer segments. The points, which have been normalized to one at zero distance, plot the means $\pm$ SD ($n = 5$). The solid curve is the function $1/(1 + x/1.5)$, where x is distance from the retina in millimetres.

$$S(x) = \frac{0.11}{0.1 + |x|^{3/2}}$$

(the solid curve in Fig. 7B), where x is distance in mm. The responses were reduced to half amplitude when the spot was displaced by 0.2 mm, though small responses continued to be measured out to the edges of the retina. In total 16 other experiments in which focus was not as well controlled yielded receptive fields that were as narrow or slightly broader than those in Fig. 7(B). All of the above indicate that the recorded b-wave comes from a relatively small area around the recording electrode (see Section 4).

3.4. Electrode position on retina

In most experiments the electrode was positioned so it was close to the center of the piece of retina. To determine how critical this placement was and whether cutting the retina had damaged the edges of the piece, responses were measured with an electrode in different positions on a piece of retina. Larger than normal pieces ($\approx 3 \times 5$ mm) were used. A 0.5 mm diameter spot of light was carefully centered on an electrode. To move the electrode and centered spot to a new position on the retina the electrode was withdrawn and the

recording chamber was displaced horizontally in steps of 0.5 mm. After each displacement, the electrode was then lowered to its previous depth and responses from a new portion of retina were measured. Fig. 7(C) shows sample results and the directions that the electrode moved between each recording. The responses were small in amplitude due to the dim stimulus that was used. In this experiment and in five other experiments using different retinas, the responses were more or less independent of the position of the electrode on the retina. Some of the variation in amplitude as the light spot and the electrode marched across the retina was due to screening of the light by the nylon mesh covering the retina. When the piece whose responses are shown in Fig. 7(C) was examined in the light after the experiment, it was clear that the stimulus fell on the translucent nylon mesh that covered the retina in positions -0.5 and $+0.5$ mm. When the responses from the six retinas were averaged, there was nothing systematic in the data to suggest that the edges of the retina had been damaged by being cut.

3.5. Distance from retina

In recording the ERG from the isolated retina we tried to position the microelectrode at the tips of the outer segments. When the electrode entered the retina there was a shift in the recorded baseline each time the electrode was advanced a few microns into the retina. The presence or absence of these shifts, presumably due to the standing dark current that exists across the outer segments (Penn & Hagins, 1969), was used as the criterion for establishing when the electrode was positioned at the tips of the outer segments. Independent confirmation that we were at the tips came from the reduction in the transretinal a-wave as the electrode was advanced into the retina. To assess the effects of small differences in electrode placement a series of experiments were conducted in which the electrode was systematically moved away from the retinal surface. Fig. 7(D) shows the results of such an experiment and indicates the dependency of the b-wave amplitude on the distance of the microelectrode from the retinal surface. Thus the response fell only slightly if the electrode was not exactly at the tips of the outer segments. The electrode had to be moved 1500 µm from the retinal surface before b-wave response amplitudes were halved.

3.6. Shunting

Only one recording electrode was in direct contact with the retina. The reference electrode was on the bottom of the chamber about 2 mm below the retina. This was done intentionally so that there would be space for the perfusate to flow under and over the

retina. Given this recording arrangement responses were surely shunted by the surrounding fluid. To provide some indication of the magnitude by which the responses were shunted a second microelectrode was advanced several hundred microns beyond the first microelectrode, so it passed through the full thickness of the retina. Under these conditions the two microelectrodes spanned the retina. Such responses were compared with those obtained using the single microelectrode at the tips of the outer segments, referenced to the large macroelectrode 2 mm below the retina. We found that the shapes of the responses were similar, but the amplitude of the response measured between the microelectrodes was about twice as large (responses not shown). The macroelectrode is 2 mm below the retina, which is consistent with the fall-off with distance shown in Fig. 7(D).

3.7. Intact eye

To compare the responses from the isolated retina with those from the intact eye we measured ERGs with corneal wick electrodes on a group of animals, several days prior to euthanizing the animals and removing their retinas. Fig. 8 shows typical corneal responses measured from the retina of an anesthetized animal (panel A) and the responses we obtained from a piece of this same retina when it was placed in a perfusion chamber (panel B). To allow the two sets of data to be compared (see Section 2 for the details of how quantum fluxes were calculated) the intensities have been converted to quanta/rod/flash and the b-wave response amplitudes were normalized to one at an intensity of 1.0 log quanta/rod/flash. The same normalizing factor was applied to a- and b-waves alike, so that the a-wave/b-wave ratio remained unchanged. Fig. 8(C) shows comparisons of the intensity-response functions for a-waves and b-waves in the isolated retina ($\bigcirc$) and the intact eye ($\bullet$). Fig. 8(D) shows how the latency of the b-wave varied as a function of stimulus intensity in isolated retina and the intact eye. The latency was defined as the time from the electronic flash to the first sign of the b-wave. The agreement between the two curves in Fig. 8(D) shows how similar responses in the isolated retina were to those of the intact eye. The only other systematic difference between the intact and isolated retina was that the intensity-response curves for the intact animal were approximately 0.3 log units to the left of those for the isolated retina, suggesting that the isolated retina maybe a factor of two less sensitive. There are however too many assumptions in the calculations that underlie this comparison to be confident that this difference actually exists.
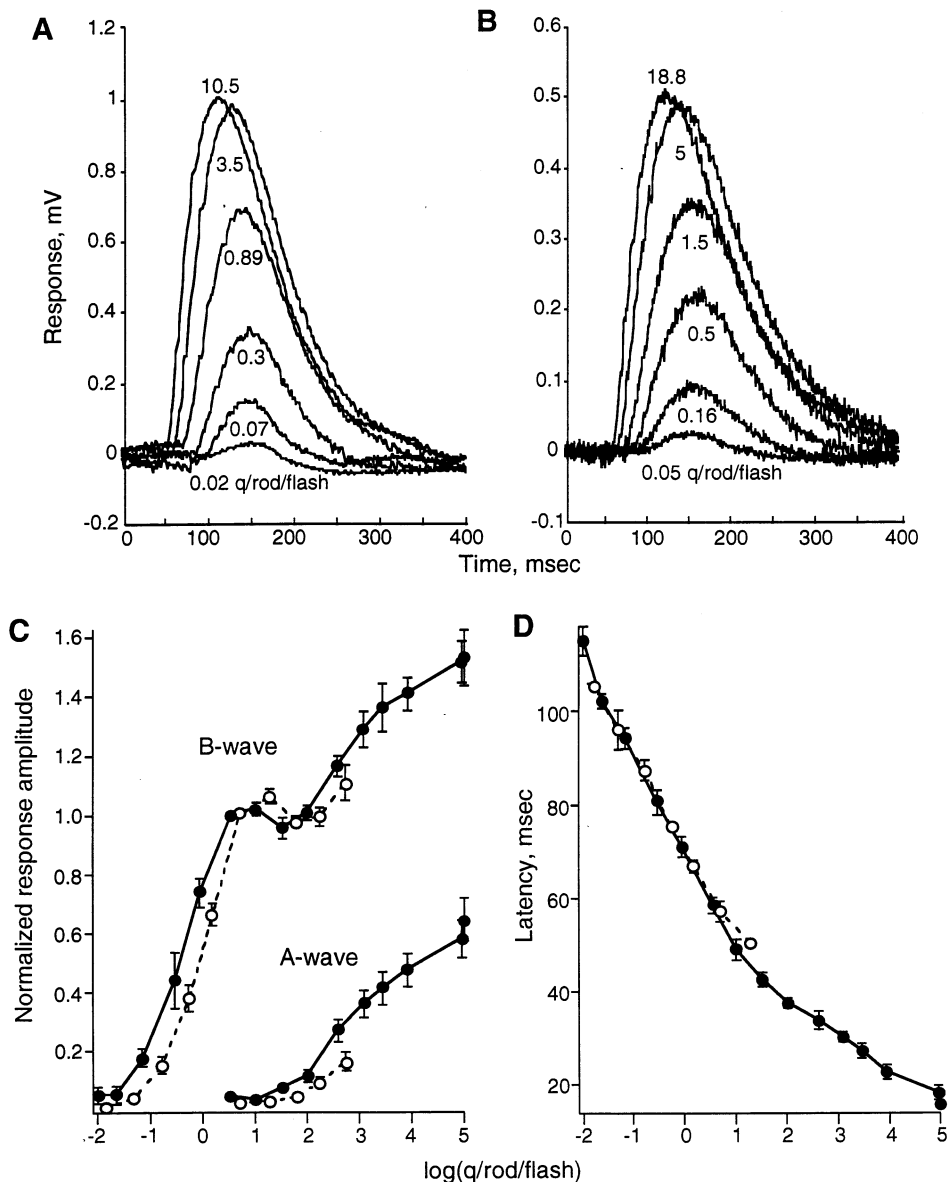
Fig. 8. Comparison of responses from intact eye and isolated retina. (A) ERGs measured from the intact eye. The numbers give the estimated intensity of the stimulus in quanta/rod/flash. (B) Records from a piece of the same retina as shown in part A after it has been isolated and placed in the recording chamber. The numbers give the estimated intensity of the stimulus in quanta/rod/flash. (C) V–log I and latency comparison from intact animals (filled circles) and from their isolated retinas (open circles). The amplitudes of the b-waves are plotted on the left and of the a-wave on the right. Both a- and b-wave amplitudes have been normalized by the b-wave response amplitudes at an intensity of 1.0 log quanta/rod/flash. The points plot the means ± SD ($n = 3$). (D) B-wave response latency as a function of stimulus intensity from the same eyes as in part A.

4. Discussion

One of the objectives of these experiments was to establish conditions under which responses from an isolated rat retina at 37°C would remain stable over many hours. We have been successful in reaching this goal and found that using 250–500 μM of glutamate in the media was the key to preventing responses from slowly running down over time (Fig. 2). Typically the amplitude of the b-wave decayed by less than 5%/hour. In reviewing the literature we found that two groups working on the isolated rat retina had previously re-ported using glutamate in their media. Weinstein, Hobson and Dowling (1967) stated that they used 5 mM glutamate, but this seems doubtful since we and others (Ames & Gurian, 1963) have found that this concentration of glutamate completely blocks post-receptor activity. Others (Doly, Isabelle, Vincent, Gaillard, & Meyniel, 1980; Doly, Bonhomme, Braquet, Chabrier & Meyniel, 1987; Cluzel, Doly, Bazan, Bonhomme & Braquet, 1995) had used 500 μM in the perfusate and referred to Weinstein et al. (1967). None of these publications addressed the importance of glutamate in maintaining b-wave responses.

The rundown of the b-wave without glutamate in the media suggested that glutamate was leaching out of the retina. The reduction in the b-wave may be a consequence of sufficiently lowering the intracellular stores so as to compromise synaptic transmission from the receptors to second order cells. A change in amplitude of the b-wave without a shift in the V–$\log I$ function is consistent with depletion of glutamate in vesicular stores. That is, scaling of amplitudes without a shift along the intensity axis is expected if one assumes that the amount by which vesicular release is reduced at each flash intensity remains the same over time, but the amount of glutamate contained in each vesicle changes over time.

At least 250 μM glutamate was required in the media if responses were to be maintained for several hours. While this concentration may seem high, the reported concentrations of endogenous glutamate in vitreous of the rat ranged from 9 (Gunnarson, Jakobsson & Sjostrand, 1987) to 400 μM (Heinamaki, Muhanen & Piha, 1986). When ERGs recorded in glutamate-free solutions were compared with those in 250 μM (Fig. 5) we could detect no changes that resulted from having glutamate in the medium. Glutamate at a concentration of 250 μM maintained the b-wave over many hours without interfering with the STR or OPs, components believed to come from third-order neurons.

In order to use an open chamber into which microelectrodes could be inserted, we simply placed a piece of retina on a Millipore filter surrounded by physiological solution (Fig. 1). The retina was not mounted as a membrane between guard rings, as others have done. Thus the fluid around the piece of retina must shunt the response. Nonetheless we found that b-waves of 500 μV or larger could be obtained. These responses are as large as those obtained by others who have mounted the retina as a membrane between two compartments (Arden & Ernst, 1972; Winkler, 1972; Doly et al., 1980; Kapusta & Zak, 1994). Several lines of evidence suggest that the responses we measured were shunted by at least a factor of two. Firstly, there is the factor of two difference in response amplitude when two electrodes were in contact with the retina as compared with when only one electrode contacted the retina. Secondly, the responses from the isolated retina had to be scaled by about a factor of two to agree in amplitude with the responses recorded from the cornea of the intact eye.

The responses to spots of increasing size centered on the recording electrode did not grow linearly with stimulus area suggesting that the recording originated in a rather local area of retina. Lateral inhibition might produce similar effects, but experiments in which a small spot was moved away from the recording electrode failed to reveal the presence of an inhibitory surround. The size of the receptive field in Fig. 7(B)

indicate that our measured responses are dominated by signals that come from the area about 0.2 mm in diameter close to the recording site. In several other studies of isolated rat retina the whole 40 mm² of retina was used (Weinstein et al., 1967; Arden & Ernst, 1972; Winkler, 1972; Kapusta & Zak, 1994; Hanitzsch, Lichtenberger & Mattig, 1996) rather than a 3×3 mm piece of retina. Cutting out a piece doesn't seem to have damaged the retina since a small spot imaged onto different parts of a piece of retina evoked responses of the same amplitude close to the cut as in the middle of the piece. Because light that falls on areas of the retina at a distance from the electrode contributes little to the measured response, there is no particular advantage in using the whole retina.

The isolated retina differs from the retina in vivo in that it has been separated from the pigment epithelium and is exposed to an artificial medium. To establish the degree to which the responses from the isolated retina were normal, we compared the ERGs from the intact animal with those recorded from the isolated retina. Normality was assessed by using the actual shapes of the responses, the relationship between intensity and response amplitude, and the dependency of the b-wave response latency on stimulus intensity. We found that not only were the major components similar, but the minor components of the ERG, not readily seen in Fig. 8, were present in both sets of records. At intensities below b-wave threshold the STRs were recorded in both the isolated retina and the intact animal. OPs appeared on the rising edge of the b-waves responses to bright flashes. Thus, even though our preparation is separated from pigment epithelium the cellular integrity of the retina seems to be well preserved. There is reasonably close agreement between the sensitivity of the responses recorded in the isolated retina and those from an intact animal. More importantly, the waveforms of the responses from the isolated retina appear to be like those found in vivo, which provides evidence that retinal function is normal even with glutamate in the media.

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