Visual Neuroscience (1990), 4, 53–62. Printed in the USA.

Effects of calcium ions on L-type horizontal cells in the isolated turtle retina

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(RECEIVED June 19, 1989; ACCEPTED October 17, 1989)

Abstract

A technique by which the retina can be isolated from the turtle eye is described. Scanning electron microscopy revealed morphological variability between preparations and also between regions of the same one. Large areas were often totally free of any pigment epithelial cells, yet contained a high proportion of photoreceptors with complete outer segments. However, adjacent regions may contain photoreceptors without outer segments or with fragmented ones. The physiological properties of the horizontal cells also demonstrated large variability between different preparations. In all cases, lowering calcium concentration from 2 mM to 0.1–0.5 mM depolarized the horizontal cells and augmented the amplitude of the maximum photoresponses. However, these effects were accompanied by changes in the photoresponse kinetics and by a reduction in the horizontal cell sensitivity to light. Moreover, prolonged exposure to low calcium induced permanent damage to the retina as was indicated by the reduction in the response amplitude after superfusion with 2 mM calcium solution had been resumed. The toxic effects of low calcium were most apparent when superfusion with 0.1–1.0 μM calcium concentration was performed. These solutions induced complex time-dependent effects on the resting potential of horizontal cells and on the amplitude and kinetics of the photoresponses. We conclude from these observations that the normal concentration of extracellular calcium in the turtle retina is in the 2 mM range.

Keywords: Retina, Calcium, Horizontal cell

Introduction

The role of calcium in modulating the function of retinal cells has been revealed through the application of single-cell recording techniques to either intact retina preparations or to isolated cultured neurons of lower vertebrates that have been superfused with solutions containing various calcium concentrations. Calcium ions have been demonstrated to play a vital role in many retinal mechanisms directly related to the visual process such as phototransduction (Hagins, 1972; Brown & Pinto, 1974; Yau & Nakatani, 1985; Nakatani & Yau, 1988) and synaptic transmission (Cervetto & Piccolino, 1974). In addition, calcium ions are involved in many general cellular mechanisms and also influence several types of membrane ionic channels (Piccolino & Gershenfeld, 1978, 1980; Lasater, 1986; Maricq & Korenbrot, 1988).

The intricate and pervasive involvement of calcium ions in retinal physiology requires an accurate knowledge of the concentration of free calcium ions in the retinal extracellular milieu. Otherwise, experiments designed to study retinal mechanisms (other than the role of calcium) may generate erroneous conclusions if superfusion is performed with other than normal calcium levels. A direct measurement of extracellular calcium with calcium-sensitive microelectrodes has revealed a value of 1.2–1.4 mM for the carp retina (Kaila et al., 1984). Physiological studies may also be utilized to assess the calcium concentration in the retinal extracellular space. In most experiments on vertebrate eyecup preparations, solutions containing about 2 mM calcium concentration have been used to superfuse the preparation (Cervetto & Piccolino, 1974; Marshal & Werblin, 1978; Lasater, 1982; Bloomfield & Dowling, 1985; Normann et al., 1986; Wu, 1986). Under these conditions, large-amplitude photoresponses could be recorded for extended periods of time from photoreceptors and horizontal cells. The amplitudes and kinetics of these photoresponses were similar to those recorded in non-superfused eyecups (Baylor & Fuortes, 1970; Simon, 1973; Lasansky & Vallerga, 1975; Normann & Perlman, 1979). This is in contrast to studies in isolated retina preparations,

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where large discrepancies exist between different studies. Some researchers used calcium levels of about 2 mM for superfusion (Ishida & Fain, 1981; Mangel et al., 1985; Miyachi & Murakami, 1989; Yang & Wu, 1989), while others lowered the calcium concentration in the superfusate to 50–100 μM (Rowe & Ruddock, 1982; Hankins et al., 1985; Mangel & Dowling, 1985; Rowe, 1987; Yang et al., 1988). Furthermore, it has been claimed that large-amplitude photoresponses could only be recorded from horizontal cells in low (50–100 μM) calcium concentrations (Rowe & Ruddock, 1982; Hankins et al., 1985; Rowe, 1987). Exposure to 2 mM calcium solutions hyperpolarized these cells and virtually eliminated their photoresponses. A physiological explanation of the differential effects of 2 mM calcium in the fish eyecup *versus* the isolated retina has yet to be offered.

We have developed a simple technique by which the turtle retina can be isolated from the eyecup and pigment epithelium. The retina, isolated with this procedure, contains small patches where the photoreceptor outer segments are either missing completely or are fragmented. However, most of the preparation consists of regions that contain a full complement of photoreceptors with intact outer segments. Large-amplitude photoresponses could be recorded for long periods of time from horizontal cells when the retina was superfused with "normal" solutions containing 2 mM calcium. Lowering calcium to 0.1–0.5 mM depolarized the horizontal cells, augmented their maximum photoresponse, but decreased their sensitivity to light. Moreover, low calcium concentrations produced irreversible damage to the retina which was manifested in the physiological properties of the horizontal cells. This toxic action of low calcium became most apparent when calcium concentration was lowered to 0.1–1.0 μM. We, therefore, suggest that 2 mM is the likely calcium concentration in the extracellular space of the turtle retina.

Methods

Successful isolation of the turtle retina from the eyecup was achieved after maintaining the eye for a specific period at low temperature (10°C). This period of refrigeration facilitated the detachment of the pigment epithelium from the outer segments. Anatomical and electrophysiological experiments were conducted on retinas that were prepared following various periods of refrigeration at 10°C to assess the optimal duration which produced the best preparations. A short refrigeration period (1–3 h) usually prevented good isolations of the retina and resulted in either a preparation contaminated with pigment epithelium or one that contained too many broken outer segments. A prolonged period (longer than 12 h) in low temperature usually resulted in an excellent preparation based on anatomical considerations, but the physiological properties of the retinal cells were significantly compromised. We found that a period of from 4–8 h was optimal in facilitating retinal detachment from the pigment epithelium, yet maintaining the functional integrity of the retinal cells.

The preparation

After decapitation and pithing, the head, wrapped in a moist paper towel, was refrigerated at 10°C for a period of between 4–8 h. After the refrigeration period, the dissection procedures were performed under very dim illumination to minimize pigment bleaching. The eye was enucleated and then hemisected. The vitreous humor was gently pulled with fine mouse tooth forceps and snipped with iridectomy scissors. This process was continued until no more vitreous humor could be readily removed. The eyecup was bisected along the vertical meridian adjacent to the optic disk. One half of the eyecup (without the optic disk) was further bisected along the horizontal meridian (parallel to the visual streak) to produce two triangular pieces about 5 mm on a side. One of these pieces was then placed vitreal side down on a 1-cm diameter piece of Millipore filter (0.45 μm pore size). The retina and filter were enclosed in a 5 ml volume for 3 min, a period chosen to promote adhesion of the retina to the Millipore filter. The sclera, choroid, and pigment epithelium were then detached as a unit from the retina by grabbing one corner of the sclera and gently peeling it back from the retina.

The superfusion system

The superfusion system consisted of an array of containers which maintained constant superfusion pressure and flow. This array was attached to a valving manifold which allowed fast switches between solutions. The manifold was connected to a small caliber inlet tube which was positioned directly above one corner of the retina. The flow rate of the superfusion system was about 0.6 ml/min. The composition of the "normal" superfusion solution (in millimolar) was as follows: NaCl, 110; KCl, 2.6; $MgCl_2$, 2; $CaCl_2$, 2; D-glucose, 10; $NaHCO_3$, 22. Low-calcium solutions were prepared either by omitting appropriate amounts of calcium chloride (for 0.1 mM and 0.5 mM) or by mixing EGTA and calcium chloride (Rowe, 1987). The pH of the solutions was maintained at 7.4 by constant bubbling of 95% O_2/5% CO_2.

The photostimulation and recording systems

White light stimuli of 500-ms duration were used in this study to investigate the physiological properties of the isolated turtle retina and to examine the effects of various calcium concentrations on the resting potential of horizontal cells and on the amplitudes and kinetics of their photoresponses. The intensity of the light stimuli was calibrated in terms of effective quanta (633 nm)$s^{-1}\mu m^{-2}$ (Normann & Perlman, 1989).

The membrane potential of retinal cells was monitored intracellularly with micropipettes which were pulled on a modified Livingston microelectrode puller. When filled with 3.0 M potassium acetate, the microelectrodes had resistances of about 200 MΩ. All photoresponses were displayed on an oscilloscope and tape recorded for off-line analysis.

Scanning electron microscopy

Specimens were fixed for 4 h in 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M phosphate buffer. They were then washed in 0.1 M sodium phosphate buffer, pH 7.3, twice for 10 min. Following dehydration in a graded series of ethanols, 50, 70, 80, 95, and 100%, they were dried by the critical point drying method in a Polaron Critical Point Drier, using carbon dioxide as the transition fluid. Specimens were sputter coated with gold and photographed in a JEOL JSM-35 scanning electron microscope at 15 kV.

Results

Anatomical observations

In order to evaluate the consequences of our isolation procedure on the condition of the outer segments, scanning electron microscopic observation was used to examine the photoreceptor side of all regions of six preparations of the turtle isolated retina. The anatomical integrity of the preparation differed between retinas and also between different regions of the same retinal piece. Figure 1 illustrates the morphological variability between two adjacent retinal areas. The transition between these

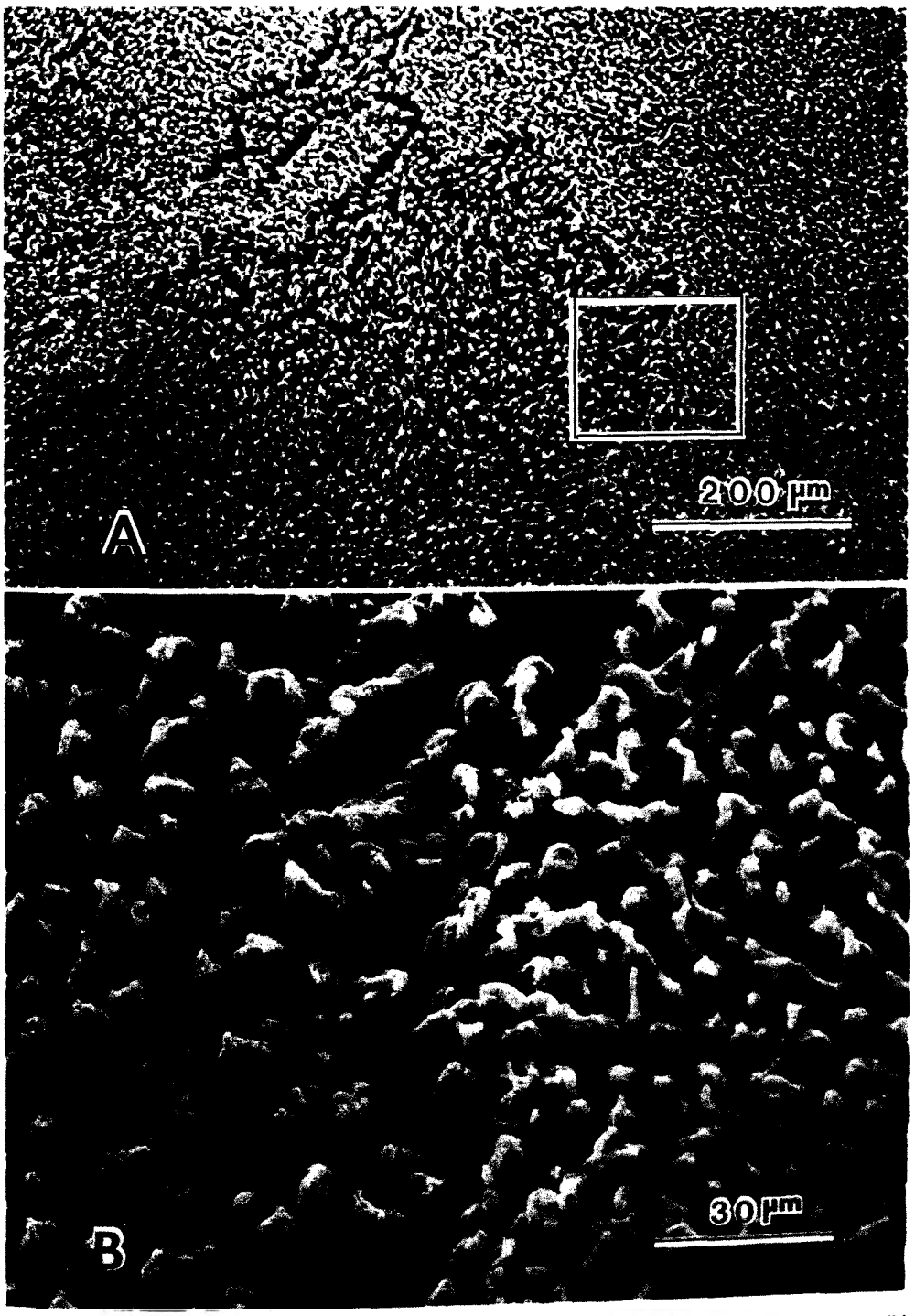

Fig. 1. Scanning electron micrographs of the isolated turtle retina preparation. A, an area of damaged retina, with visible photoreceptor inner segments, is surrounded by intact retina. B, higher magnification of the area enclosed in the box of Fig. 1A. Most of the photoreceptors in the damaged region (left) lack whole outer segments. Most of the photoreceptors in the intact region (right) appear to have whole outer segments.

two areas can be readily identified with low-power magnification (Fig. 1A). Although inner segments appeared complete over the entire retinal piece, the two areas differed with regard to the presence of outer segments. The damaged area contained photoreceptors without outer segments or with fragmented ones, while the adjacent patch contained cells with complete outer segments (Fig. 1B). The retinal piece shown in Fig. 1 was obtained during the early phases of the development of our isolation procedure. In subsequent isolations, we were able to obtain retinal pieces which contained much larger regions with intact outer segments. However, even after considerable experience, the retinal pieces still contained small regions with damaged outer segments.

Photoresponses in the isolated retina

In successful retinal preparations that contained a full complement of outer segments, large amplitude photoresponses could often be recorded from luminosity-type horizontal cells and occasionally from red cones and chromaticity-type horizontal cells, even when the superfusate contained 2 mM calcium. Figure 2 shows photoresponses of a red cone (A) and an L-type horizontal cell (B) which were recorded in such an isolated retina preparation. Photoresponses from a single L-type horizontal cell could often be recorded for over 1 h, and large-amplitude photoresponses could often be recorded from other horizontal cells impaled in a given retina for periods as long as 4 h. The photoresponses, shown in Fig. 2, are virtually identical to those typically recorded in the turtle eyecup preparation (Normann et al., 1986) suggesting that the isolation procedure, described above, produces little retinal damage. Furthermore, it is clear that micromolar concentrations of calcium are not a prerequisite for the genesis of large-amplitude photoresponses by horizontal cells in the isolated turtle retina. However, because the isolated retina does not contain pigment epithelium, the sensitivities of the horizontal cells in the isolated retina preparation were somewhat reduced (by a factor of about 10) compared to values typically recorded in the eyecup preparation, due to a small amount of photopigment bleaching that occurred during the isolation and experimental procedures.

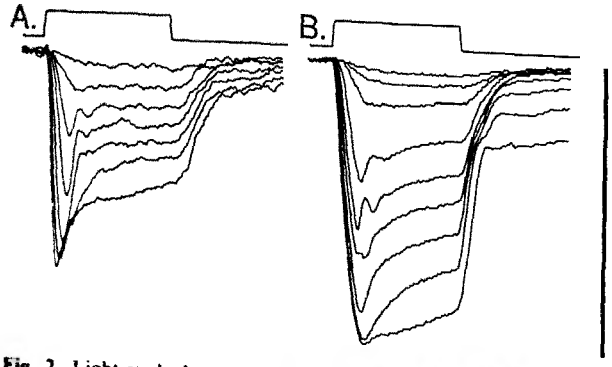

Fig. 2. Light-evoked responses recorded from neurons of the outer plexiform layer in the isolated turtle retina superfused with 2 mM calcium solution. Each set of responses was evoked by light stimuli of 500-ms duration. A, photoresponses recorded from a red cone photoreceptor. The intensities of the light stimuli used to elicit these photoresponses were 2.5, 3.1, 3.6, 4.0, 4.5, 5.1, and 5.6 log effective quanta (633 nm)$s^{-1}\mu m^{-2}$ and are separated by about 0.5 log units. B, photoresponses recorded from a luminosity type horizontal cell. These responses were evoked by light stimuli of the following intensities: 1.9, 2.5, 3.1, 3.6, 4.0, 4.5, 5.1, 5.6, and 6.2 log effective quanta (633 nm)$s^{-1}\mu m^{-2}$ and are separated by about 0.5 log units. Vertical calibration bar in A = 25 mV; B = 50 mV.

The effects of calcium on horizontal cell photoresponses

The responses of the red cone and L-type horizontal cell, shown in Fig. 2, were indistinguishable from those recorded in the eyecup preparation. However, not every cell impaled in every isolated retinal preparation demonstrated such robust responses. Similar to the variability in the morphological integrity of isolated retinas (Fig. 1), we observed a variability in the physiological properties of the L-type horizontal cells. We studied 34 cells which were characterized by dark membrane potentials ranging from −76 to −26 mV. The amplitudes of the maximum photoresponse of these varied between 5–40 mV. The intracellular recordings from most of these cells were stable enough to examine the effects of lowering the free extracellular concentration of calcium ions from 2 mM (our "normal" solution) to concentrations of 0.5 mM, 0.1 mM, 0.5 μM (1 mM EGTA + 0.75 mM calcium chloride) and 0.1 μM (2 mM EGTA + 0.75 mM calcium chloride). We studied the effects of these solutions on the dark resting potential, maximum response amplitude, intensity-response curve, and response kinetics.

Effects of 0.1–0.5 mM calcium

In most of our experiments, we employed modest reductions in calcium to preclude the toxic effects of very low calcium superfusion which will be described in a subsequent section. Figure 3 shows the effects of reducing calcium concentrations from 2 to 0.5 mM on two horizontal cells. Figure 3A shows a typical experiment on a horizontal cell which had a large (hyperpolarized) dark potential and a small maximal amplitude photoresponse. Lowering the calcium concentration depolarized the horizontal cell by about 12 mV and augmented the saturating photoresponse amplitude from 27 to 38 mV. Thus, low-calcium solutions transform horizontal cells with small maximum photoresponses into ones with a much more "normal" amplitude photoresponse. The 0.5 mM calcium solutions also altered the intensity-response relation of the cell (Fig. 3C). Responses to dim stimuli decreased while responses to bright stimuli were augmented. The data points were fitted to a hyperbolic function (Naka & Rushton, 1966) with the parameters given in the figure legend. The effect of low calcium on the small-amplitude photoresponses, elicited by dim light stimuli, can be appreciated from the increased value of σ, the semisaturating light intensity, needed to fit the data points to the hyperbolic function. It should be noted that every cell studied ($N = 26$) under low-calcium (0.1–0.5 mM) superfusion, exhibited a significant reduction in the amplitude of the responses elicited by dim light stimuli. Although it may not be apparent in Fig. 3C, the degree of reduction was typically larger than a factor of 2. The effects of reducing calcium concentrations from 2.0 to 0.5 mM on horizontal cells with large-amplitude photoresponses was minimal (Fig. 3B). Accompanying the slight depolarization (6 mV in the cell shown) was a subtle decrease in the amplitude of the responses to dim flashes and a small increase (from 42 to 45 mV) in the maximal response amplitude (Fig. 3D).

The effects of 0.1–0.5 mM calcium on horizontal cells are

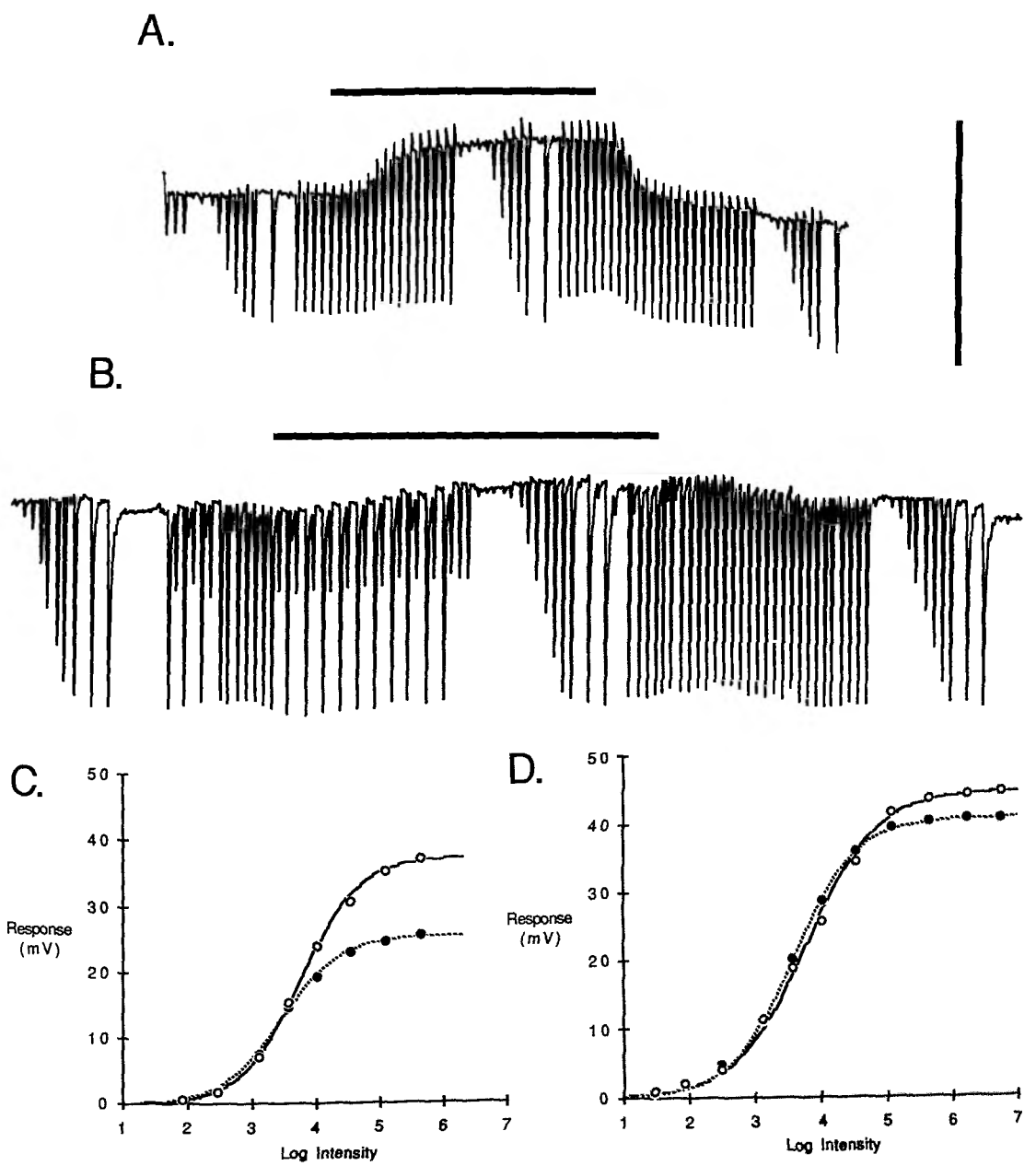

Fig. 3. The effect of lowering calcium concentration from 2 to 0.5 mM on two L-type horizontal cells. The period of superfusion is indicated by the horizontal bar above each record. A, the effect of 93 s of 0.5 mM calcium superfusion recorded in a cell characterized by small maximum response amplitude. B, the effect of 136 s of 0.5 mM calcium superfusion on a cell with large-amplitude maximum photoresponses. C, intensity-response curves of the cell shown in (A) measured in 2 mM (filled circles) and in 0.5 mM calcium (open circles). The curves, through the data points, describe a Naka-Rushton relationship, $V = V_{max} * I^n/(I^n + \sigma^n)$, with the following parameters: dashed curve, log $\sigma = 3.45$, $n = 0.95$; continuous curve, log $\sigma = 3.75$, $n = 0.95$. D, intensity-response curves of the experiment in (B) measured in 2 mM and 0.5 mM calcium (filled and open circles, respectively). The following parameters have been used to fit the curves (Naka-Rushton relationship) to the data points: dashed curve, log $\sigma = 3.6$, $n = 0.9$; continuous curve, log $\sigma = 3.85$, $n = 0.8$. Vertical calibration bar for A and B = 50 mV.

summarized in the plots of Fig. 4. The extent of depolarization (Fig. 4A) and the degree of augmentation of the saturating photoresponse (Fig. 4B), produced by the low-calcium solutions, were inversely related to the amplitude of the maximal photoresponse that could be evoked in the horizontal cells by bright flashes when the retina was superfused with 2 mM calcium solution. The correlation between response augmentation and the extent of the depolarization, which are the major electrophysiological parameters modulated by low calcium, can be appreciated in the parametric plot of Fig. 4C. Decreasing calcium below 2 mM would generally augment the amplitude of the maximum photoresponse that could be evoked by the cell; the degree of the augmentation was directly related to the extent of depolarization induced by the reduced calcium concentrations.

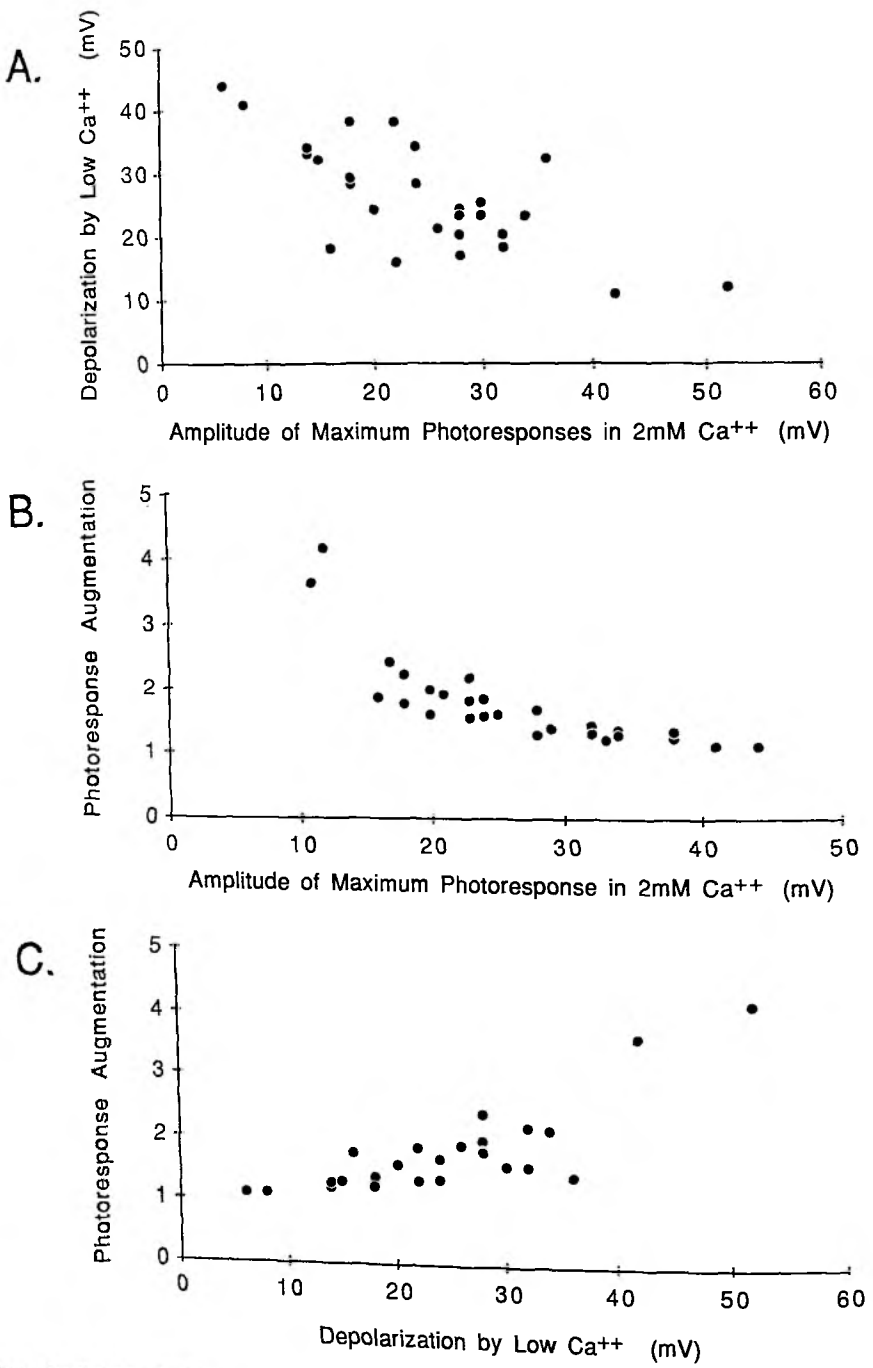

Fig. 4. Horizontal cell depolarization and response augmentation caused by lowering calcium from 2 mM to 0.1–0.5 mM. A, horizontal cell depolarization *versus* the amplitude of the maximum light-evoked response measured in 2 mM calcium. B, degree of augmentation of horizontal cell maximum response *versus* the amplitude of the maximum light-evoked response measured in 2 mM calcium. C, parametric plot from A and B; horizontal cell response augmentation *versus* horizontal cell depolarization evoked by reduced calcium.

In some of the cells studied, the dark resting potential was measured at the end of the experiment by withdrawing the microelectrode from the impaled cell. For these cells we could relate the amplitude of the maximum photoresponse to the absolute dark resting potential. Figure 5 shows this relationship for cells superfused with 2 mM calcium solution (filled circles) and during exposure to 0.1–0.5 mM calcium levels (open circles). The data in Fig. 5 were obtained from 34 different horizontal cells studied in different isolated retina preparations. Not all of the impaled cells were stable enough to follow a complete experiment of low-calcium superfusion. It can be appreciated from Fig. 5 that horizontal cells with large (more hyperpolarized) dark potentials are also characterized by small-amplitude maximum photoresponses. Exposure to low calcium tends to cluster the horizontal cells, regardless of initial properties, in a relatively narrow range of dark resting potential (−30 to −20

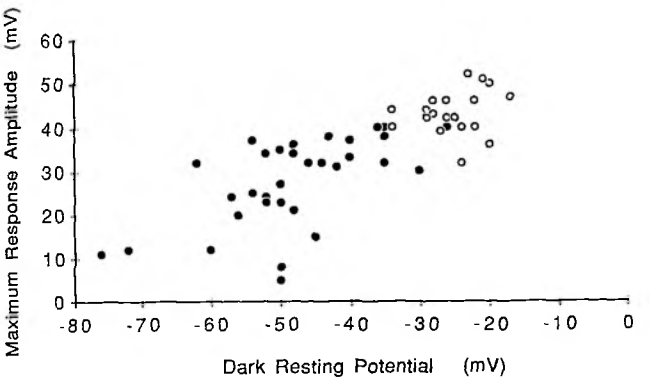

Fig. 5. The dependency of maximum photoresponse amplitude on dark resting potential for L-type horizontal cells in the isolated turtle retina. The data points were obtained from preparations superfused with 2 mM calcium solution (filled circles) or during applications of solutions containing 0.1–0.5 mM calcium (open circles).

mV). However, it is stressed again that large-amplitude photoresponses could often be recorded for prolonged periods of time in isolated retinas superfused with 2 mM calcium.

Effects of 0.1–0.5 μM calcium

When solutions containing concentrations of free calcium of 0.1 or 0.5 μM range were used, effects similar to those shown in Fig. 3 were seen; the horizontal cell depolarized and its responses to dim light stimuli decreased while those evoked by bright stimuli were augmented. However, this phenomenon was a transient one. As superfusion continued, the horizontal cell hyperpolarized back toward and usually exceeded the level recorded during 2 mM calcium superfusion. The photoresponses substantially decreased in amplitude and, in some cases, reversed in polarity (Rowe, 1987).

Figure 6 shows a typical experiment on an L-type horizontal cell which underwent three periods of exposures to a solution containing 0.1 μM of free calcium (2 mM EGTA + 0.75 mM calcium chloride). Four phases can be identified in the response of the horizontal cell to this solution as marked in the upper trace (first exposure to the 0.1 μM calcium solution). The first two phases occur during the superfusion with the low-calcium solution, while the last two are evident during the recovery period (superfusion with 2 mM calcium). In phase "1," the cell depolarized and the responses to bright light were augmented. In phase "2," hyperpolarization was seen and the photoresponses were reduced in amplitude and sometimes reversed in polarity. Phase "3" represents a fast depolarization associated with a modest growth of response amplitude after return to "normal" solution (2 mM calcium). During this phase, the response kinetics of the horizontal cell changed drastically; the "off" part slowed down considerably. In phase "4," the cell hyperpolarized, its sensitivity (amplitude of response to dim stimuli) increased while the maximum response amplitude decreased. At this stage of the experiment, the physiological condition of the retina was compromised compared to the pre-low calcium exposure as indicated by the substantially reduced photoresponses. Consecutive exposures to low calcium (0.1 μM) evoked similar effects on the horizontal cell but the physiological condition of the retina continued to deteriorate. Although

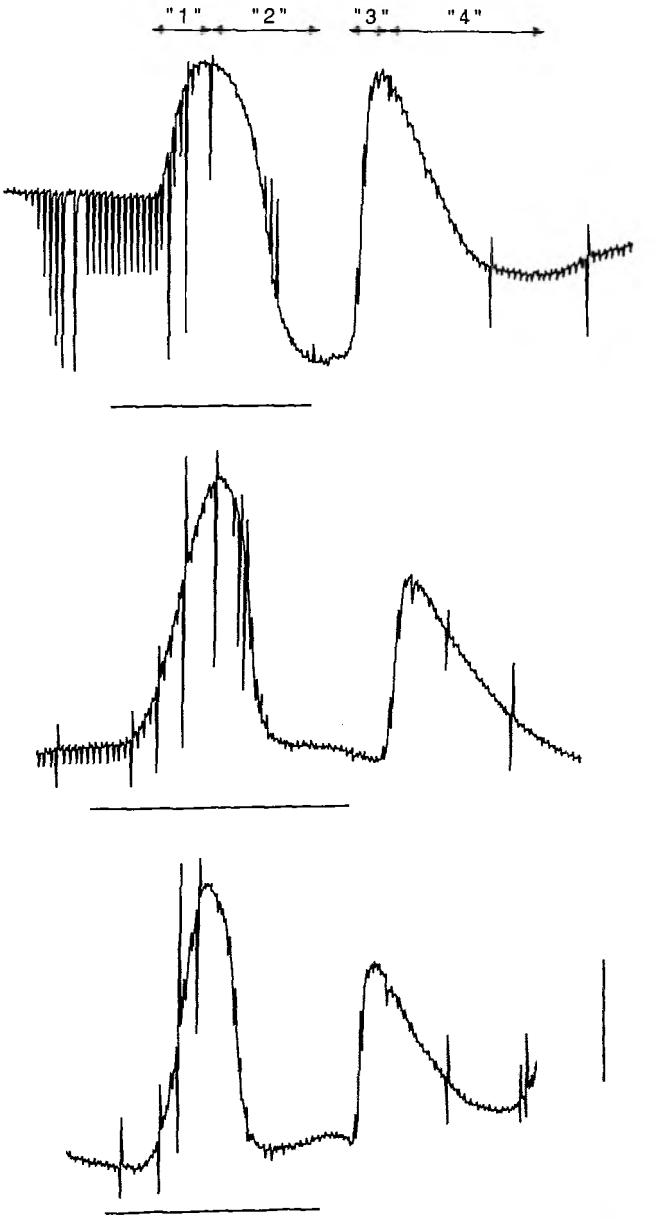

Fig. 6. The effects on an L-type horizontal cell in the isolated turtle retina of three consecutive exposures to a solution containing 0.1 μM calcium. The three traces were separated for clarity. The three traces do not represent a continuous record. The upper and middle traces are separated by a period of about 4 min of superfusion with 2 mM calcium solution in order to assure optimal recovery from the initial exposure to low calcium. The duration of superfusion with low calcium solution (98 s, 126 s, and 103 s, respectively) is indicated below each record by the horizontal line. The four phases of the effects of very low calcium solution are noted above the upper trace, representing the first exposure to low calcium solution. Calibration bar: vertical = 25 mV.

the reduced amplitude photoresponses illustrated in this figure could reflect a deterioration of the recording conditions, the large changes in the resting potential and the degree of maximum response augmentation (phase "1") produced by repeated exposures to very low calcium concentration argue against this possibility. Furthermore, every new cell impaled at this stage of the experiment was characterized by a very hyperpolarized dark

resting potential (greater than −50 mV) and small-amplitude photoresponses (10–15 mV). Thus, very low calcium levels seem to be toxic to the isolated turtle retina. This irreversible damage inflicted by the exposure to low-calcium solutions argues against the idea that "healthy" horizontal cells in an isolated retina preparation require low extracellular calcium concentrations.

Photoresponse kinetics

In addition to changes in resting potential and modulation of photoresponse amplitude discussed above, superfusion with low-calcium solutions also produced changes in the kinetics of the horizontal cell photoresponses. Figure 7 shows photoresponses, recorded from L-type horizontal cells in the non-superfused turtle eyecup (A) and in the isolated turtle retina, superfused with 2 mM calcium (B) and after lowering the calcium concentration to 0.1 mM (C). For these three cases, a series of photoresponses are shown to illustrate the entire dynamic range of the cells. The responses of the horizontal cell in the eyecup (A) were elicited by light stimuli of intensities ranging from 1.9–6.2 log effective quanta (633 nm)$s^{-1}\mu m^{-2}$, separated by about 0.5 log unit. The responses recorded in the isolated retina (B and C) were elicited by light stimuli covering an intensity range from 3.1–6.2 log effective quanta (633 nm)$s^{-1}\mu m^{-2}$ separated by about 0.5 log unit. In general, horizontal cells recorded in the eyecup are more sensitive to light than those studied in the isolated retina. This is demonstrated by the intensity of the light stimulus needed to elicit small criterion responses and by the slow kinetics of the "off" phase of the photoresponses evoked by bright light stimuli. Among the two sets of responses recorded from the isolated retina, the one obtained under low calcium (C) is closer in amplitude to that recorded in the eyecup. However, when response kinetics are

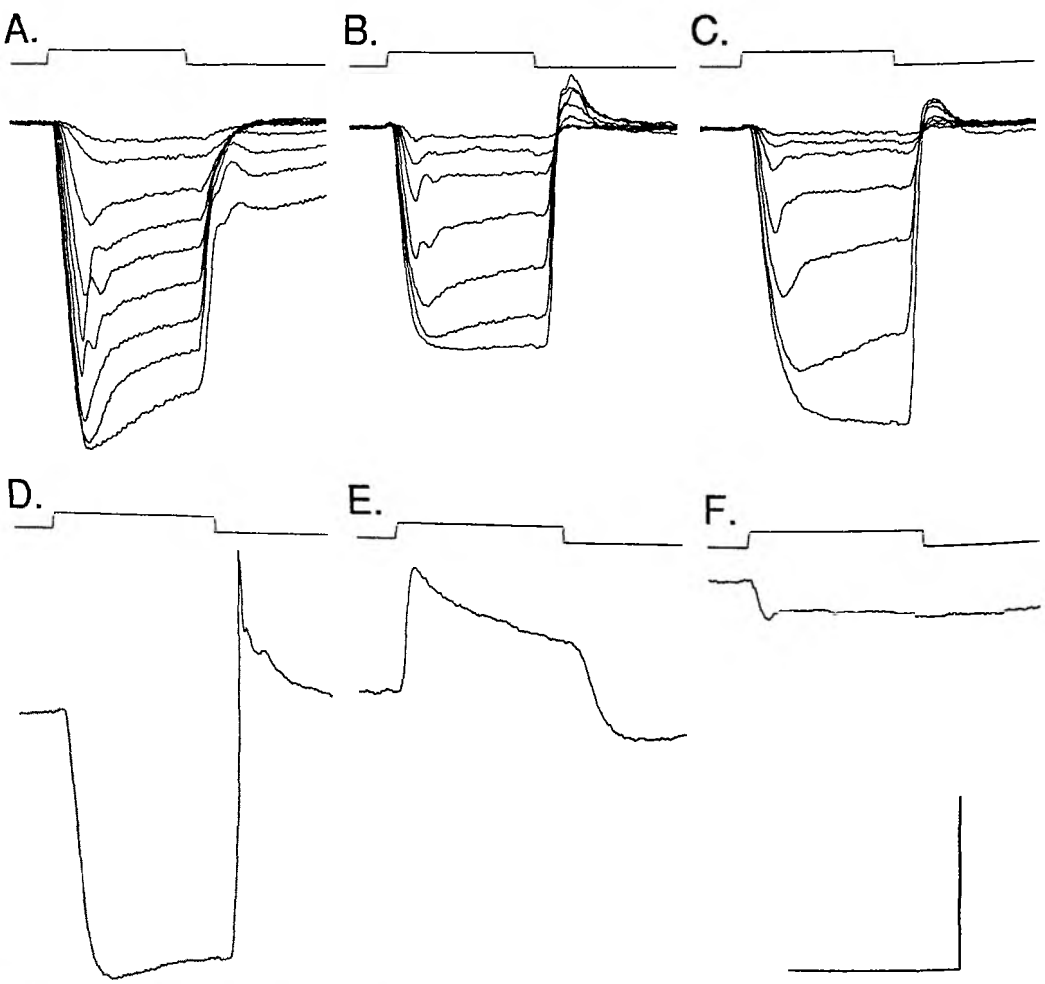

Fig. 7. Photoresponses elicited by light stimuli in different L-type horizontal cells under different conditions. The photoresponses in A were evoked by light stimuli intensities 1.9, 2.5, 3.1, 3.6, 4.0, 4.5, 5.1, 5.6, and 6.2 log effective quanta (633 nm)$s^{-1}\mu m^{-2}$ from an L-type horizontal cell in a non-superfused turtle eyecup. Photoresponses recorded in the isolated turtle retina were recorded during superfusion with 2 mM calcium solution (B) and after switching to 0.1 mM calcium solution (C). These two sets of responses were elicited by light stimuli of intensity 3.1, 3.6, 4.0, 4.5, 5.1, 5.6, and 6.2 log effective quanta (633 nm)$s^{-1}\mu m^{-2}$. The effects of exposure to 0.1 μM calcium solution on the photoresponses elicited by bright light stimuli of saturating intensity. In phase "1" a "spike" like event is observed at stimulus offset (D). During phase "2", depolarizing responses are often seen (E) and in phase "3" during the recovery period the photoresponse is drastically prolonged (F). Calibration bars: vertical = 25 mV; horizontal = 500 ms.

examined, the photoresponses recorded in the isolated retina superfused with 2 mM calcium (B) are more similar to those of the eyecup (A) especially when the "on" phase of the large-amplitude photoresponses is considered.

During superfusion with very low calcium concentration (0.1 μM), more complex changes in the horizontal cell photoresponse were noted. In these experiments, the time course of the low-calcium effect was too fast to allow recording the entire intensity-response series at each phase. Therefore, only photoresponses elicited by bright light stimuli are shown. In phase "1" of the effect of the second exposure to low calcium (Fig. 6, middle trace), a large transient "spike" is evident in the "off" part of the photoresponse (Fig. 7D). During phase "2" of the same experiment, a depolarizing response was elicited (Fig. 7E), similar to that previously seen in the isolated fish retina (Rowe, 1987) and in the turtle eyecup (Normann et al., 1988). During phase "3," which developed after superfusion was switched back to 2 mM calcium solution, a very prolonged photoresponse of small amplitude was recorded.

Discussion

Electrophysiological studies of the pharmacological properties of the neurons in the distal turtle retina have been hindered by the lack of a simple technique to isolate the retina from the pigment epithelium. The major impediment to retinal isolation is the strong adhesion between the pigment epithelium and the retina which causes most of the photoreceptor outer segments to be torn off of their inner segments during the separation procedure. In the fish, a period of 3–4 h of dark adaptation has been shown to weaken the interaction between the pigment epithelium and the retina (Rowe & Ruddock, 1982). Recently, it has been reported that several days of dark adaptation promotes the separation of the turtle retina from the pigment epithelium (Miyachi & Murakami, 1989). We report here that refrigeration of the eyecup at 10°C for a period of about 4–8 h appears to decrease the adhesion between the photoreceptor outer segments and the apical processes of the pigment epithelium. Once this interaction has been weakened, the pigment epithelium, choroid, and sclera can be peeled from the retina (when attached to a piece of Millipore filter) leaving a preparation which contains large areas with a full complement of photoreceptors with complete outer segments (Fig. 1). If the refrigeration is limited to the 4–8 h period, the retina retains its normal physiological function (Fig. 2). A postmortem modification of the adhesion of the retina to the pigment epithelium has been previously observed in the rabbit retina (Endo et al., 1988). However, in that preparation, maintenance in cold temperature seemed to oppose this weakening process.

Our experiments using low-calcium superfusion were directed at determining the calcium level which is the best estimate of the extracellular concentration in the turtle retina. The experiments demonstrated a complex dependency of horizontal cell physiology on the extracellular calcium concentration. We divided the effects of low calcium on horizontal cells into two broad classes depending upon the level to which calcium concentration had been reduced.

Effects of 0.1–0.5 mM calcium

Horizontal cells studied in 2 mM calcium concentrations manifested a large variability in the resting potential and in the amplitude of the photoresponse elicited by saturating stimuli. In agreement with previous reports on fish retinas (Rowe & Ruddock, 1982; Hankins et al., 1985; Rowe, 1987), lowering extracellular calcium level to 0.1–0.5 mM depolarized all of the L-type horizontal cells studied and augmented their maximal photoresponses (Figs. 3 and 4). The exposure to low calcium also reduced the physiological variability between different horizontal cells, recorded from different preparations, and tended to cluster their dark potentials and maximum amplitudes in a relatively narrow range. All cells exposed to low calcium were characterized by dark resting potentials of −20 to −30 mV and maximal response amplitudes of 40–50 mV (Fig. 5). In contrast to the augmentation described above, lowering calcium level to 0.1–0.5 mM reduced the horizontal cell sensitivity to light (Fig. 3C). Furthermore, the kinetics of the photoresponses differed significantly from those recorded in the conventional eyecup preparation (Fig. 7). Another difficulty with low calcium was a permanent reduction in the amplitude of the saturating photoresponses upon return to 2 mM calcium superfusion. Similar effects of low calcium on resting potential, sensitivity, and the amplitude of photoresponses to bright flashes have been observed in red cones in the turtle eyecup (Bertrand et al., 1978; Normann et al., 1988). Thus, it is likely that the effects described here on L-type horizontal cells are manifestations of events occurring at the cone level. Possible, although not exclusive, mechanisms underlying the low-calcium effects on cones are the opening of more light-sensitive sodium channels in the plasma membrane of the outer segment, modulation of the phototransduction mechanism (Yau & Nakatani, 1985; Nakatani & Yau, 1988), or direct effects of calcium on calcium channels or on calcium-dependent potassium and chloride channels (Maricq & Korenbrot, 1988).

Effects of 0.1–0.5 μM calcium

Very low levels of calcium were achieved by buffering with EGTA. These solutions produced a highly complex behavior of the horizontal cells which could be divided into four phases. The first two phases were observed during superfusion with very low calcium and the last two phases were associated with the return to 2 mM calcium superfusion (Fig. 6). Phase "1" was characterized by effects which were similar to those produced by superfusion with 0.1–0.5 mM calcium concentrations and consisted of a depolarization and response augmentation. These effects likely reflect similar events occurring in the cones. During phase "2," the horizontal cell hyperpolarized and its photoresponses gradually decreased in amplitude to almost a nonrecordable level. In some experiments the photoresponses reversed polarity (Fig. 7E) as has been previously demonstrated in the fish retina (Rowe, 1987) and the turtle eyecup (Normann et al., 1988). Phase "2" of the effect of very low calcium on horizontal cells is consistent with the reduced release of neurotransmitter from the photoreceptors (Cervetto & Piccolino, 1974). Switching to "normal" Ringer solution evokes phase "3": a large depolarization accompanied by an increase in response amplitude and a very marked prolongation of the horizontal cell photoresponses. Phase "3" probably reflects the sudden release of neurotransmitter due to the increase in the extracellular calcium concentration. Phase "4" represents the gradual recovery of the horizontal cells as "normal" superfusion proceeds. This phase is characterized by hyperpolarization, an increase in sensitivity and maximal photoresponse amplitude, and return to

more normal response kinetics. However, when compared to the pre-exposure situation, the horizontal cells showed permanent damage. Photoresponses did not recover to their initial levels even after prolonged superfusion with "normal" solution. Consecutive exposures to very low calcium solution produced quantitatively similar results but with each additional exposure, the physiological state of the retina was further compromised.

In conclusion, the retinal isolation procedure described in this paper is simple, yet produces large regions of the retina with photoreceptors that contain complete outer segments. Photoresponses of horizontal cells in the isolated retina preparation superfused with 2 mM calcium manifest kinetics and maximal amplitudes that are very similar to those recorded in eyecup preparations. Thus, horizontal cell physiology in the isolated turtle retina lends support to the notion that the extracellular calcium levels *in vivo* and in the eyecup preparation is in the millimolar range as has been measured in the carp (Kaila et al., 1984). Reduced calcium concentrations produces a variable degree of response augmentation but simultaneously changes the horizontal cell physiology in a manner that may lead to erroneous conclusions regarding the neurocircuitry of the retina. In fact, isolated turtle retina preparations that require reduced calcium superfusion before "normal" amplitude photoresponses can be recorded may have either suffered structural damage during the isolation procedure and contain a significant proportion of photoreceptors with damaged outer segments or have been compromised by prior exposures to very low calcium levels.

Acknowledgments

This research was supported by an NEI Grant EY 03748 to R.A. Normann. I. Perlman was a Research to Prevent Blindness International Research Scholar.

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