Light-Evoked Responses of Bipolar Cells in a Mammalian Retina

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Euler, Thomas and Richard H. Masland. Light-evoked responses of bipolar cells in a mammalian retina. J. Neurophysiol. 83: 1817-1829, 2000. We recorded light-evoked responses from rod and cone bipolar cells using patch-clamp techniques in a slice preparation of the rat retina. Rod bipolar cells responded to light with a sustained depolarization (ON response) followed at light offset by a slight hyperpolarization. ON and OFF cone bipolar cells were encountered, both with diverse temporal properties. The responses of rod bipolar cells were composed primarily of two components, a nonspecific cation current and a chloride current. The chloride current was reduced greatly in axotomized cells and could be suppressed by coapplication of the GABA_A antagonist bicuculline and the GABA_C antagonist (1,2,5,6-tetrahydropyridine-4-yl)methylphosphinic acid. This suggests that it largely reflects feedback from GABAergic amacrine cells. The response latency of intact rod bipolar cells was shorter than that of the axotomized cells, and the sensitivity curve covered more than twice the dynamic range. Application of the GABA receptor antagonists partially mimicked the effects of axotomy. These findings suggest that functional properties of the axon terminal system—notably synaptic feedback from amacrine cells—play an important role in defining the response properties of mammalian bipolar cells.

INTRODUCTION

Most signal processing by the mammalian retina takes place within the inner plexiform layer, involving interactions among bipolar, amacrine, and ganglion cell processes. At the center of these interactions is the bipolar cell’s axon terminal system, a highly specialized structure capable of releasing glutamate continuously at its ribbon synapses (Heidelberg et al. 1994; Rouze and Schwartz 1998; von Gersdorff and Matthews 1994). A bipolar cell receives graded photoreceptor signals and translates these into a modulation of its release of glutamate. This output signal is shaped through several mechanisms. First, the dendritic glutamate receptors receiving the photoreceptor input may be modified via intracellular pathways (reviewed in Hatt 1999); the second-messenger pathways that depolarizing (ON) bipolar cells use for signal transduction offer many such possibilities (Shiells and Falk 1995; Walters et al. 1998). Second, bipolar cells express several types of voltage-gated calcium and potassium channels. Certain K+ channel subunits (Klumpp et al. 1995) and L-type Ca2+ channels (De la Villa et al. 1998; Hartveit 1999; Protti and Llano 1998; Satoh et al. 1998) seem to be located preferentially on the bipolar axon terminal, supporting its role in shaping the time course of the output. Finally, bipolar cells possess high densities of ionotropic GABA and glycine receptors on their axon and terminals (reviewed in Wässle et al. 1998). There, amacrine cells synapse and deliver reciprocal and nonreciprocal feedback inhibition (Burkhardt 1972; Chun et al. 1993; Dong and Werblin 1998; Dowling and Boycott 1966; Pan and Lipton 1995; Toyoda and Fujimoto 1984).

Bipolar cells come in different morphological types (Cajal 1893; Cohen and Sterling 1990; Famiglietti 1981). It has been suggested that these types represent parallel signaling pathways through the retina, carrying different temporal, spatial, and chromatic aspects of the visual stimulus. In the retinas of cold-blooded animals, especially the dogfish and tiger salamander, the responses of bipolar cells to light have been studied extensively, leading to much important information on the polarity and the time course of their responses, the currents involved, and their receptive field organization (Ashmore and Falk 1980; Kaneko and Shimazaki 1976; Lasansky 1992; Saito and Kuijiraoka 1982; Saito et al. 1979, 1981, 1985; Werblin 1974). However, the degree to which information from cold-blooded animals can be translated to mammals (and vice versa) is not entirely clear. Although the cell’s fundamental biophysical properties are certainly similar, their wiring is not necessarily the same. In the mammalian retina, the cells are harder to record from than in cold-blooded vertebrates, but a systematic anatomic classification exists: one type of rod bipolar cell (RBC) and seven to nine types of presumed cone bipolar cells (CBCs) have been described (reviewed in Euler and Wässle 1995). In principle, one now can begin to map the various kinds of physiological responses onto the morphological types, but in mammals the responses to light have been little studied.

In this study, we addressed the mechanisms that shape the light-evoked responses of bipolar cells. We focused on the role of the bipolar cell’s axon terminal system by comparing the functional properties of intact and axotomized rod bipolar cells. In addition, we studied the effects of GABA receptor antagonists on the response behavior of these cells. The results, together with those of others, suggest that events at the bipolar axon terminals—amacrine cell feedback as well as axonal membrane conductances—importantly control the dynamic properties of a bipolar cell’s response.

METHODS

Animals

Three- to 7-wk-old albino rats were used. No systematic age-dependent differences in their responses to light were observed. Although the retinas of hypopigmented rats are susceptible to light-induced damage, the majority of studies on the rat retina have been done in albinos. In the young animals we used, light-induced retinal damage is probably negligible (Weisse et al. 1990). Because physiological studies show no significant differences in the sensitivity to light between these animals and pigmented strains (Green et al. 1991; Herreros et al. 1992), we used albino rats for comparability.
Light stimulus

Below the recording chamber, a conventional yellow light-emitting diode (LED; emission maximum at 585 nm) diffusely illuminated the microscope field. The LED was controlled either directly by an analogue output channel of the Digidata or by an external stimulator (S88, Grass Instrument, West Warwick, RI), which then also triggered the recording.

The stimulus duration was varied between 1 ms and 2 s. The brightness of the LED was calibrated using a photodiode (S1133–12; Hamamatsu Photonics, Japan) and a photometer (LS-100; Minolta, Japan). The stimulus intensity could be varied from 0.02 to 100 cd/m², covering the range from high scotopic into the photopic regime. Although the recording chamber was shielded, a very dim background illumination (not visible to the dark-adapted eye of the experimenter) may have been present during the recordings, precluding the observation of single photon responses.

The recorded bipolar cells generally were located near the upper surface of the slice, so that the stimulating light—entering the slice perpendicular to the outer segments of the photoreceptors—had to pass through tissue before it reached the photoreceptors connected to the examined cell. On its way, the light was attenuated, mainly by the intervening outer segments, in an amount depending on the thickness of the slice. This attenuation was probably similar in all experiments, though difficult to estimate. Therefore we give all intensities as luminance at the bottom of the recording chamber (in cd/m²).

Analysis of the responses of RBCs

Bipolar cells are thought to be electrotonically compact (Mennerick et al. 1997; Protti and Llano 1998), thus the potential recorded at the cell’s soma must be very similar to the potential at the axon terminals. The output of bipolar cells—the release of glutamate from their terminals—is modulated by the membrane potential of the cell (Tachibana et al. 1993; von Gersdorff and Matthews 1994), therefore the voltage response is presumably an adequate representation of the cell’s output signal. For this reason, we analyzed and compared properties of the voltage responses.

Several parameters were used to characterize the light responses of RBCs (illustrated in Fig. 3A). First, the maximum amplitudes of the two phases of the typical voltage response—the depolarizing phase at light onset and the succeeding hyperpolarization at light offset—were measured ($V_{\text{dep}}, V_{\text{hyp}}$ in mV). For the sensitivity curves, the response amplitudes of both phases were plotted over the logarithm of the stimulus luminance ($L$; Figs. 3C and 7) or the normalized luminance ($UL/L_{\text{ref}}$, Fig. 4). When comparing the curves of several cells in a diagram, the response amplitudes were normalized. The values for $V_{\text{dep}}$ were fitted using the Hill function: $V/V_{\text{max}} = L^h/(L^h + L^h_{50})$ (see also intensity-response function: Normann and Werblin 1974), with the maximal amplitude ($V_{\text{max}}$), the luminance ($L$; in cd/m²), the slope of the curve ($h$; the Hill coefficient), and the luminance evoking a half-maximal response ($L_{50}$). From this fit, the luminance evoking a 90%-response ($L_{90}$) also was determined. The width of the dynamic range ($w$; in log units) was estimated using following equation: $w = 2 \log L_{90} - \log L_{50}$.

Four timing parameters were measured (in ms) from the start of the stimulus ($t = 0$): $\tau_{\text{dep}}$ is the latency of the maximal amplitude of the depolarization $V_{\text{dep}}$; $\tau_{\text{hyp}}$ is the time that the rising depolarization response reached its maximal slope. The latter was determined by differentiating the rising phase and fitting a Gaussian function to find its peak. At $t_{\text{c}}$, the voltage response crossed the baseline, marking the start of the hyperpolarizing response phase, and at $V_{\text{c}}$ the hyperpolarizing phase reached its maximal amplitude, $V_{\text{hyp}}$.

The Student’s $t$-test (significance level $\leq 0.05$) was used to determine whether or not a parameter was significantly different between two groups of cells.

Tissue preparation

The animals were dark adapted for ≥2 h before the experiment. To reduce bleaching of the photoreceptors, the preparation was performed under dim red illumination. The rats were anesthetized deeply with 10 mg/kg xylazine (Bayer AG, Leverkusen, Germany) and 50 mg/kg ketamine (Fort Dodge Labs, IA). After anaesthesia, the animals were killed with an overdose of xylazine/ketamine, in accordance with institutional guidelines.

The retina was isolated in a petri dish perfused with Ames medium (Sigma Aldrich, St. Louis, MO), bubbled with 95% O₂/5% CO₂, and cooled to ≈20°C. Slices of ~150 μm were cut by hand using a scalpel blade (Boos et al. 1993). By adjusting the angle of the blade, vertical or slightly "wedged" slices were produced, increasing the frequency of intact or axotomized bipolar cells, respectively. The slices were transferred into a recording chamber and held in place with a nylon grid. The remaining tissue was maintained intact for ≥5 h at room temperature in bubbled Ames medium before being used for more slices.

Patch-clamp recordings

The recording chamber was placed on the fixed stage of an upright microscope (Axioskop, Zeiss, Germany) and perfused with warmed, bubbled Ames solution (35–37°C). A water-immersion objective was used (Acroplan ×63/0.9w Ph3, Zeiss). The microscope was equipped with differential interference contrast (DIC) optics and epifluorescence illumination for visualizing the Lucifer yellow (Sigma Aldrich)-filled cells at the end of the recordings.

Patch electrodes (8–12 MΩ) were pulled from borosilicate glass (1B150F-4; World Precision Instruments, Sarasota, FL) on a vertical electrode puller (Narishige, Japan). The recordings were performed with a PC-501A patch-clamp amplifier (Warner Instruments, Hamden, CT). As the reference electrode, an Ag/AgCl pellet in contact with the solution in the recording chamber was used. The recorded signals were low-pass filtered at 10 kHz or 500 Hz and digitized (4 or 5 times over-sampling) using AxoScope or Clampex (Axon Instruments, Foster City, CA) in conjunction with a Digidata 1200A digitizer board (Axon Instruments). The data analysis was performed with Origin (Microical Software, Northampton, MA).

Both whole cell and perforated-patch recordings were performed (Hamill et al. 1981; Horn and Marty 1988). For the perforated-patch configuration, nystatin was used (0.1–0.2 mg/ml intracellular solution). After a successful perforated-patch recording, the cell membrane was ruptured to fill the cell with Lucifer yellow. In some whole cell experiments, nystatin was added at a lower concentration (~0.01 μg/ml) to facilitate rupturing of the cell membrane under the electrode without breaking the seal. Perforated-patch recordings tended to be more stable than whole cell recordings. Apart from this, no differences relevant to our results were observed between the two recording modes.

Seal resistances >1 to 2 GΩ were commonly obtained. Series resistances ($R_S$) were estimated from the peak amplitudes of the capacitative currents or automatically determined by Clampex7. Because capacitative currents are capped by the limited sampling frequency of the system, $R_S$ was overestimated. For the cells that were analyzed in more detail, $R_S$ ranged from 35 to 85 MΩ (whole cell; for perforated-patch mode: 65–125 MΩ). Recordings of cells with higher $R_S$ were not used to determine the polarity of their light responses (ON or OFF). The built-in $R_S$ compensation of the amplifier was not activated routinely because it often caused oscillations that damaged the seal. Corrections for voltage errors related to $R_S$ were not made.

The experiments were performed in a dark room. The slices were visualized using dim red illumination. Immediately after sealing onto the targeted cell and while establishing the whole cell configuration, the illumination was turned off. Before the first light stimulus was applied, the slice was kept in darkness for ≥5 min to allow the photoreceptors to recover.
Solutions

The following intracellular solution was used (in mM): 10 NaCl, 1 CaCl$_2$, 1 MgCl$_2$, 1.1 EGTA, 4 KCl, 126 K$_2$-gluconate, 10 HEPES, 1 Mg$^{2+}$-ATP(H$_2$O)$_2$, and 1 Na$^+$,GTP(H$_2$O)$_2$ (pH 7.2). All chemicals were purchased from Sigma Aldrich. The solution also contained 0.025% Lucifer yellow (potassium salt) to visualize the recorded cells after the experiment. The chloride reversal potential (in Ames) was $-48.8$ mV; the (mean) reversal potential for nonspecific cation currents was $+0.2$ mV (assuming similar permeability for Na$^+$ and K$^+$). This difference in reversal potential enabled us to distinguish excitatory cation currents from inhibitory chloride currents. For the combination intracellular solution-Ames, a liquid junction potential of 18 mV was measured, close to the estimated value of 15.7 mV, and was used to correct all measured potentials. Antagonists were dissolved in Ames and applied directly with the bath. The solution in the recording chamber was completely exchanged within 3 min.

Identification of the bipolar cells

Because Lucifer yellow was included in the intracellular solution, we were able to classify the recorded cells according to their morphology (Euler and Hässle 1995; Hartveit 1997). RBCs can be identified easily from the stereotypical branching pattern of their axon terminals, by the size and the location of their somata, and especially by the characteristic appearance of their dendrites; their narrow and more tightly clustered dendritic tree enabled us easily to distinguish axotomized RBCs from other axotomized bipolar cells (Fig. 1).

In most mammals, only the RBC, one of the 8–10 types of bipolar cells, is known to exclusively contact rods (Cajal 1893). It is assumed that the remaining types of bipolar cells contact only cones, and they are hence referred to as cone bipolar cells. However, in many mammals, including rats, detailed knowledge of the wiring between photoreceptors and bipolar cell types is still lacking and recent evidence suggests that some “cone bipolar cells” in the mouse retina may connect also to rods (Brandstätter et al. 1998a; Soucy et al. 1998). Nonetheless, for simplicity’s sake, we refer to these “nonrod bipolar cells” as cone bipolar cells (CBCs).

RESULTS

Thirty-two RBCs and 35 CBCs were studied. Twelve of the RBCs lost their axon terminals during the preparation. These axotomized RBCs seemed to be as healthy as their intact counterparts; they could be studied for similar periods of time and showed no abnormal rundown of their light responses. Twenty-nine RBCs, including all axotomized cells, were ON cells. The remaining three RBCs hyperpolarized at light onset. Twelve of the CBCs were OFF cells; 23 CBCs were ON cells.

Resting potentials were measured in darkness, immediately after the recording had stabilized. The resting potential of intact RBCs was $-45.4 \pm 10.6$ mV ($n = 15$), that of axotomized RBCs was $-39.0 \pm 7.4$ mV ($n = 11$). The resting potentials were $-38.5 \pm 13.6$ mV ($n = 12$) and $-35.9 \pm 9.8$ mV ($n = 9$) for ON and OFF CBCs, respectively. Axotomized RBCs being slightly more depolarized than normal (although this difference was not statistically significant), could indicate that they leaked ions and thus were damaged. For two reasons, we think this was not the case. The cell’s axons were cut off during the dissection, generally $\geq 1$ h before the recordings. By then, damaged cells most likely have died off. The axotomized RBCs displayed stable responses to light during the whole experiment, indicating that the intracellular milieu in these cells was widely undisturbed. The likely explanation for the drop in resting potential is instead the lack of inhibitory axonal input from amacrine cells or the loss of axonal conductances, as studied further in the following text.

Rod bipolar cells—Light-induced voltage responses

A typical voltage response of an intact RBC is shown in Fig. 2. At light onset, the cell displayed a strong depolarization (ON response), followed by a smaller hyperpolarizing phase after light offset.

With increasing light intensity, the amplitude of the depolarization ($V_{\text{dep}}$) increased (Fig. 2A, bottom 4 traces). At higher intensities, $V_{\text{dep}}$ saturated, but the width of the depolarizing phase continued to increase (Fig. 2A, upper 6 traces). Note that the hyperpolarization also increased with increasing stimulus intensity. The maximal $V_{\text{dep}}$ ranged from 3.9 to 26 mV (15.1 ± 7.1 mV, $n = 24$) and no differences were observed between intact and axotomized cells. The maximal amplitude of the hyperpolarizing phase ($V_{\text{hyp}}$) ranged from $-1.0$ to $-11.5$ mV ($-4.5 \pm 3.1$ mV, $n = 9$) in intact RBCs and from $-0.6$ to $-5.0$ mV ($-2.2 \pm 1.2$ mV, $n = 11$) in axotomized cells. This difference, though small, was statistically significant suggesting that at least part of the hyperpolarization is caused by functional properties of the axon.

Stimuli of varying duration showed the responses of RBCs to have a large sustained component (Fig. 2C). To quantify this, the percentage of the amplitude remaining at the offset of saturating stimuli was determined: for 0.5 s stimuli this percentage ranged from 47 to 93% (75 ± 15, $n = 8$); for 2 s stimuli, it ranged from 62 to 89% (75 ± 12, $n = 5$). Note also that the longer the duration of the stimulus, the longer the time required for the cell to repolarize (Fig. 2C).

In the nonsaturating intensity range, the latency of the de-
polarizing response decreased with increasing luminance as Fig. 2B clearly shows (traces from A, marked by arrows). For saturating stimuli of increasing duration, the latency of the depolarizing phase remained constant (Fig. 2D; arrows in C). To characterize the light-induced voltage responses of RBCs in more detail, we separately analyzed three aspects: the response latency, the relationship between the response phases, and the sensitivity of the cells. As an example, Fig. 3 shows the results from an intact RBC.

**Rod bipolar cells—Latency**

Latencies were measured from the onset of the light stimulus to the time points marked by the symbols next to the voltage trace in Fig. 3A. The changes in the response latencies over the intensity range examined are revealed in Fig. 3B. First, we first determined the latency of the maximum depolarization ($t_{V_{dep}}$; upward triangles in Fig. 3B). For both intact and axotomized cells, $t_{V_{dep}}$ was relatively constant; in a few cells, a slight decrease in $t_{V_{dep}}$ was observed for increasing intensities. However, this trend was small. Therefore we calculated the mean $t_{V_{dep}}$ which was 176 ± 23 ms ($n = 10$) in intact cells and 216 ± 29 ms ($n = 10$) in axotomized cells. This difference was statistically significant and suggests that intact cells have shorter latencies.

To overcome the difficulties of pinpointing the exact latency of the maximum response, we also determined the latency of the maximal slope ($t_{MaxSlope}$; circles in Fig. 3B). In both intact and axotomized cells, $t_{MaxSlope}$ decreased significantly with increasing intensity until it reached a minimum for the highest intensities. However, intact cells had consistently shorter latencies. For intact cells, $t_{MaxSlope}$ was 132 ± 25 ms ($n = 10$) at low light levels and 73 ± 9 ms ($n = 10$) at saturating illumination, while the corresponding values for axotomized RBCs were 160 ± 26 ms ($n = 11$) and 106 ± 21 ms ($n = 11$). In both cases, the difference in $t_{MaxSlope}$ was statistically significant. Thus the depolarizing phase of RBC responses speeded up with increasing stimulus intensity. Furthermore, intact RBCs responded faster, on average, than axotomized cells, suggesting that the axon terminal system is involved in shaping the time course of the depolarizing phase.

Increasing the intensity of the stimulus also affected the latency of the hyperpolarizing portion of the RBC response. Both the latency of the hyperpolarization ($t_{V_{hyp}}$; downward triangles in Fig. 3B) and the time when the trace crossed the baseline ($t_{0}$; squares in Fig. 3B) increased with increasing...
stimulus intensity. The fit appears linear in the double-logarithmic plot, indicating that it can be fitted with a power function of the form $f(x) = a \log(x/b)$. This change in latency reflects the lengthening of the depolarizing phase.

**Rod bipolar cells—Relationship between the response phases**

We next wanted to explore the relationship, if any, between the size of the depolarizing and hyperpolarizing phases of the light response. Finding a proportional relationship between the two response phases could indicate that the depolarization directly causes the hyperpolarization, for example by a very simple form of reciprocal feedback.

The ratio of the amplitudes of the depolarizing and hyperpolarizing phase was studied to see whether it remained constant over the examined range of intensities. For each cell, we took the ratio of $V_{\text{hyp}}/V_{\text{dep}}$ at different intensities and calculated the mean. The ratio of $V_{\text{hyp}}/V_{\text{dep}}$ was quite different between axotomized and intact RBCs. We found a mean ratio of $0.29 \pm 0.13$ ($n = 8$) for intact cells, and a significantly smaller average ratio of $0.17 \pm 0.06$ ($n = 9$) for axotomized cells. The standard deviation (SD) of $V_{\text{hyp}}/V_{\text{dep}}$ was used as an estimate of the constancy of the ratio. The averaged SD was $0.11$ ($n = 8$) for intact and $0.08$ ($n = 9$) for axotomized cells. The variability of this ratio suggests that the relationship, if any, between the two response phases in both intact and axotomized RBCs is not straightforward. This means that the hyperpolarizing phase probably results from a more complex interaction than reciprocal feedback alone.

**Rod bipolar cells—Sensitivity**

Figure 3C shows the sensitivity curve for an RBC. The peak amplitudes, $V_{\text{dep}}$ (upward triangles) and $V_{\text{hyp}}$ (downward triangles), of the response are plotted over the stimulus intensity. The data points for $V_{\text{dep}}$ can be well fitted with the Hill function (see METHODS), describing the light intensity range within which the cell responds with graded potentials—the dynamic range of the cell. The data points for $V_{\text{hyp}}$ were simply fitted by linear regression.

We noted that the intact RBCs behaved differently than axotomized RBCs in terms of dynamic range and curve profile. To explore this quantitatively, we compared the normalized sensitivity curves of intact and axotomized RBCs. The curves for four cells of each group are shown: Fig. 4A contains the data for the intact RBCs; Fig. 4B shows the same plot but for the axotomized RBCs. The response amplitude of each cell was plotted as a function of the stimulus luminance on normalized axis. The amplitude was normalized to the maximal amplitude ($V_{\text{max}}$) and the luminance was normalized to the $L_{50}$ value, the luminance needed to evoke a half-maximal response. Solid lines represent the fits for the data points. For comparison, the fit from the other group of cells is added to the plots (dashed lines).

The comparison of the fits reveals a surprising difference: The curve for the intact RBCs is shallower and extends over a wider range of intensities than the curve for their axotomized counterparts. This is expressed in the slope of the curve ($h$; the Hill coefficient), which was $1.07 \pm 0.19$ ($n = 7$) for intact RBCs and $2.39 \pm 0.84$ ($n = 9$) for axotomized cells. The dynamic range of intact cells ($1.84 \pm 0.32$ log units; $n = 7$) was more than twice as wide as that of axotomized RBCs ($0.87 \pm 0.23$ log units; $n = 9$). The $L_{50}$ value ranged from 0.15 to 0.58 cd/m² (average = 0.34; $n = 7$) for intact RBCs and from 0.26 to 9.75 cd/m² (average = 2.01; $n = 9$) for axotomized RBCs. The relatively high variability of the $L_{50}$ value in both cell groups may reflect variations in number and sensitivity of rods connected to the recorded cell, but probably also depends on the synaptic integrity of the slice and the different attenuation of the stimulus by the tissue (see METHODS). The results suggest that the axon terminal system is involved in widening the dynamic range and lowering the threshold of RBC voltage responses.
fits from similar data for four axotomized RBCs (solid lines). The comparison with the lines show the fits for four axotomized RBCs (from bols) were fitted by linear regression (solid line). For comparison, the dashed function (solid curve). The data points for the hyperpolarization (open sym-

The data points of the depolarization (shaded symbols) were fitted with the Hill function (solid curve). The data points for the hyperpolarization (open symbols) were fitted by linear regression (solid line). For comparison, the dashed lines show the fits for four axotomized RBCs (solid lines). The comparison with the fits from A (dashed lines) shows that the sensitivity curves for the two cell groups clearly differ. C: separate fits for each intact (from A; thin lines) and axotomized (from B; thick lines) cell are plotted, further illustrating the difference between the two groups. The error bars represent a 95% confidence interval around the fitted curves from A and B. For the fitting parameters see Table 1 and METHODS.

For both groups of RBCs, \( V_{\text{hyp}} \) increased with increasing light intensity. This increase was smaller in axotomized cells, further supporting the hypothesis that the hyperpolarization partially reflects an axonal contribution.

To illustrate the significance of the difference between the two cell groups, the separate fits for the 8 cells are plotted in Fig. 4C (axotomized: thick lines; intact: thin lines). The error bars designate the width of a 95% confidence interval around the fits shown in panels A and B.

**Rod bipolar cells—Light-induced current responses**

To characterize the currents involved in the light responses of RBCs, we recorded in voltage clamp at different holding potentials \( (V_H) \). We used a low intracellular chloride concentration, setting the Cl\(^-\) reversal potential \( (E_{\text{Cl}}) \) to about \(-50 mV\), to distinguish inhibitory chloride currents and excitatory cation currents. By clamping the cells to \( V_H = -3 mV \ (E_{\text{Cation}}) \) and to \( V_H = -48 mV \ (E_{\text{Cl}}) \), respectively, we could isolate these two components of the light response.

A current family recorded from an intact RBC is shown in Fig. 5A. For negative holding potentials, the stimulus evoked a strong initial inward current, followed by a weak outward current rebound. For positive holding potentials, the polarity of the currents was reversed. At both the reversal potentials of cations \( (E_{\text{Cation}}; \text{marked a}) \) and of chloride \( (E_{\text{Cl}}; \text{marked b}) \), currents were observed. The outward current at \( E_{\text{Cation}} \) was probably a chloride current, while the inward current at \( E_{\text{Cl}} \) was very likely a nonspecific cation current. The two different currents are combined in the responses: when the cell was held at an intermediate \( V_H \) (e.g., \(-18 mV\)), one can see the two components, the outward and the inward current, contributing to the response.

Figure 5C1 shows the initiation of the responses at higher magnification. The inward currents developed smoothly. The outward currents often began with large, apparently discrete events and had a noisy characteristic. Nonetheless, both inward and outward currents started with approximately the same latency. Similar events were also seen in darkness (Fig. 5C2). Since these events reversed around \(-50 mV \ (E_{\text{Cl}}) \) both in the light response and in the dark they probably reflect postsynaptic inhibitory input, carried by chloride, and thus are IPSCs.

A similar current family for an axotomized RBC is shown in Fig. 5B. In contrast to the intact RBC, no outward current was measured when the cell was held at \( V_H = -3 mV \ (E_{\text{Cation}}; \text{marked a}) \). However, at \( E_{\text{Cl}} \) (marked b) the response was very similar to that of the intact RBC. This effect of axotomy is also clearly seen when comparing the response of the two cells at \( V_H = -18 mV \ (E_{\text{Cl}}) \) both in the light response and in the dark they probably reflect postsynaptic inhibitory input, carried by chloride, and thus are IPSCs.

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We attempted to separate the different current components by determining the net charge flowing through the membrane (area under the current trace) within the time limits marked by the shaded boxes underlying the traces (Fig. 5, A and B). This charge was normalized and plotted over the holding potential (charge-voltage relation; Fig. 5, D–F). For intact RBCs, the reversal potentials of the first (black squares) and the second (black circles) phases were between \( E_{\text{Cl}} \) and \( E_{\text{Cation}} \), suggesting a mixture of chloride and cation currents and indicating that the separation of the components could not be achieved. However, since the first phase reversed at a more negative potential...
than the second phase (−30 mV compared with −20 mV), chloride seemed to be more prominent in the initial phase of the response.

In axotomized RBCs, the first phase of the response was nearly absent (open squares). The second phase was pronounced and reversed around 0 mV. This “shift” in reversal potential further supports the idea that the chloride current is largely reduced in axotomized cells. The reversal potential of the second phase (circles) is an indicator of the composition of the currents: it was $-15.7 \pm 10.4$ (n = 6) in intact RBCs and $-2.3 \pm 4.7$ (n = 7) in axotomized cells (statistically significant).

The current rebound at light offset partially remained in axotomized cells and it probably caused the smaller hyperpolarization observed in those cells. Its charge-voltage relationship (triangles in Fig. 5F) is nonlinear, making it difficult to pinpoint the reversal potential between −20 and 0 mV. However, the negative slope suggests a reduction in current flow. This effect could be due to the kinetic properties of the APB receptors, the subsequent second messenger cascade or intrinsic membrane properties.

**Rod bipolar cells—GABA receptors shape the light-induced responses**

The fact that GABAergic amacrine cells provide significant synaptic input to RBC terminals (Fletcher et al. 1998; Kim et al. 1998; Sterling and Lampson 1986; Strettoi et al. 1990) suggests that the observed chloride current reflected inhibitory input mediated by ionotropic GABA receptors (GABARs). We tested this hypothesis by co-applying the GABA$_A$R antagonist bicuculline (100 μM; Feigenspan et al. 1993) and the GABA$_C$R antagonist TPMPA (50 μM; Ragozzino et al. 1996) to intact and axotomized RBCs.

Figure 6 shows the charge-voltage relations for two RBCs. The total area under the responses—corresponding to the first two shaded boxes in Fig. 5, A and B—was plotted. Under control conditions, the curve for the intact RBC (Fig. 6A, open circles) reversed between $E_{Cl}$ and $E_{Cation}$, reflecting a mixture of cation and chloride currents ($-33.8 \pm 17.0$ mV; n = 3). The co-application of the GABAR antagonists decreased the charge and shifted the reversal potential close to $E_{Cation}$ (shaded circles), indicating that the chloride component was strongly
Cone bipolar cells

While RBC responses were consistent across cells, CBCs displayed a wide variety of responses (Fig. 8). We recorded more ON than OFF CBCs, probably because we targeted cells in the outer third of the inner nuclear layer, where the somata of RBCs and CBCs stratifying in sublamina b are located (Euler and Wässle 1995). All ON responding bipolar cells—CBCs and RBCs—had their axon terminals in the inner part of the inner plexiform layer, while all OFF responding CBCs stratified in its outer portion; bipolar cells obey the ON/OFF subdivision of the inner plexiform layer (Euler et al. 1996; Famiglietti and Kolb 1976; Hartveit 1997; Kaneko 1979; Nelson et al. 1978; Werblin and Dowling 1969). Although we found no exception to this rule, we cannot exclude that exceptions exist given that we recorded from only 35 CBCs.

Figure 8, A and B, shows light-induced voltage responses of two representative ON CBCs. Some cells displayed sustained responses: they depolarized for the duration of the stimulus (Fig. 8A). Other ON CBCs responded with a brief depolarization at the onset of the stimulus, independent of the stimulus duration (transient response; Fig. 8B). Interestingly, axotomized CBCs tended to be more sustained than intact cells (data not shown), although this was not examined quantitatively.

Figure 8, C and D, shows two examples of light-induced responses for OFF CBCs. They also differed in the time course of their responses, showing more sustained (Fig. 8C) or more transient responses (Fig. 8D), although these characteristics were not as pronounced as in ON CBCs. We generally used light-on stimuli to reduce the effect of photoreceptor bleaching.
light-off stimuli might reveal transient and sustained OFF CBCs more clearly.

Most of the CBCs displayed biphasic voltage responses. For the brightest LED stimulus, ON CBCs displayed an initial depolarization at light onset ranging from 1.8 to 10.8 mV, and a hyperpolarization at light offset between $-0.9$ and $-8.4$ mV. For OFF CBCs, the initial hyperpolarization ranged from $-16.0$ to $-1.6$ mV, while the depolarization at light offset was 0.6 to 2.1 mV. However, since the intensity of the light stimulus was limited to the low photopic range (see METHODS), the brightest of our stimuli did not evoke the maximal responses. For several cells, we confirmed this by briefly switching on the fluorescent illumination of the microscope. This produced larger light responses than any of our LED stimuli.

Responses to light of CBCs were smaller and noisier than those of RBCs (compare traces in Fig. 2 with Fig. 8). In many CBC recordings, low frequency noise, probably a summation of discrete events, was observed. Larger single excitatory (EPSPs) and inhibitory events (IPSPs) can be clearly resolved, but were not further analyzed. The fact that the CBC responses were small can probably be explained by the relatively low density of cones in the rat retina, as well as by technical limitations: The probability of having enough intact cones connected to the recorded bipolar cell is low in a slice, especially for bipolar cells close to the surface of the slice.

**DISCUSSION**

By comparing the light-evoked responses of intact RBCs with those of axotomized or pharmacologically treated ones, we found evidence that the bipolar axon terminal—especially the GABAergic inhibition it receives—plays an important role in determining the cell’s dynamic response properties. The responses of various types of cone bipolar cells, whose most distinguishing characteristics are the shape and the stratification level of their axon terminals, differed not only in polarity but also in time course. Taken together, these findings support the view that these cell types represent parallel signaling pathways to the inner retina and that the axon terminals may be an important source of their functional specificity.

**Responses of rod bipolar cells to light**

The great majority of RBCs responded with a robust, sustained depolarization at light onset (ON response), followed by a smaller hyperpolarization at light-off. Only a few previous studies have described light responses of RBCs in other mammals. The depolarizing responses from rabbit RBCs in whole-mounts (Dacheux and Raviola 1986) resembled those we recorded in slices from the rat. Despite some sagging back after an initial peak, the cells stayed depolarized during the whole stimulus presentation. A hyperpolarizing phase was not noted...
for rabbit RBCs, but a small dip at light offset is visible in some of the published traces. As for rabbit RBCs, we observed a widening of the depolarizing phase and longer repolarization periods for postsaturating stimulus intensities. This could reflect nonlinearities in the rod responses (Dacheux and Raviola 1982; Kraft and Schnapf 1998; Schneeweis and Schnapf 1995).

Using voltage clamp recordings and separate reversal potentials for cation and chloride currents, we identified two components in the response of RBCs: a nonspecific cation current and a chloride current. Both currents were induced by light. Similar components have been reported in depolarizing bipolar cells in the salamander retina (Lasansky 1992). The cation current caused the depolarization at light-on and is most likely attributed to the APB-receptor/cGMP-gated channel mechanism (Nawy and Jahr 1991; Shiells and Falk 1990). Chloride currents in bipolar cells have been shown to originate from ligand-gated inhibitory receptors (postsynaptic currents, IPSCs) or Ca$^{2+}$-activated chloride channels (Hartveit 1999; Okada et al. 1995). Since the recorded RBC was voltage-clamped during the light stimulus, Ca$^{2+}$-activated chloride currents may have been continuously activated but were not modulated by the cell’s light response. Hence, the measured light-evoked chloride current instead reflected receptor-mediated inhibitory input, probably from amacrine cells at the RBC axon terminals. Two lines of evidence support this assumption. Under control conditions, the integrated current response of intact RBCs reversed at a value between the reversal potentials for chloride and cations, indicating a mixture of the two currents. During the co-application of GABA$_{A/C}$ receptor antagonists, the overall current response decreased and its reversal potential shifted toward that for cation currents, consistent with the suppression of a chloride current. Thus the chloride current is mediated by ionotropic GABA receptors, presumably located on the bipolar cell itself. Furthermore, axotomized RBCs lacked most if not all of this chloride current, which is consistent with the fact that RBCs receive their major inhibitory input at their axon terminals (Hartveit 1999; Karschin and Wässle 1990; Suzuki et al. 1990).

Our findings fit well into a picture in which GABAergic amacrine cells provide the bulk of inhibitory synaptic input to RBCs (Fletcher et al. 1998; Kim et al. 1998; Sterling and Lampson 1986; Strettoi et al. 1990). Anatomical studies have established that in the inner plexiform layer, RBCs make dyad synapses onto both glycinergic AII and GABAergic A17 amacrine cells (Famiglietti and Kolb 1975; Kolb and Nelson 1983; Nelson et al. 1976) and depolarize when glutamate receptors are probably not involved. Second, AII amacrine cells are ON cells (Bloomfield et al. 1997; Kolb and Nelson 1983; Nelson et al. 1976) and depolarize when glutamate is applied (Boos et al. 1993). Since they receive most of their bipolar cell input from RBCs (Famiglietti and Kolb 1975), RBCs should be ON cells as well. Third, the dendritic input of RBCs can be compromised—by damage to the fragile dendritic tips or the rod distal to the recorded cell—even when the dendritic tree appears visually to be intact (Euler et al. 1996; Hartveit 1996). Hence, while axotomized RBCs clearly had lost the majority of chloride currents, the hyperpolarizing cells behaved as though they had their dendrites cutoff and thus lost their cation currents (the chloride current would originate from amacrine cells driven by neighboring bipolar cells).

Hyperpolarizing rod bipolar cells

We found three RBCs that responded by hyperpolarizing at light onset (not shown). For several reasons we believe that these RBCs obtained reduced input from photoreceptors while still receiving inhibitory input at their axon terminals. First, current families indicated that the hyperpolarizing response of these RBCs was composed predominately of chloride currents. Thus these cells are not OFF cells in the sense that ionotropic glutamate receptors are probably not involved. Second, all amacrine cells are ON cells (Bloomfield et al. 1997; Kolb and Nelson 1983; Nelson et al. 1976) and depolarize when glutamate is applied (Boos et al. 1993). Since they receive most of their bipolar cell input from RBCs (Famiglietti and Kolb 1975), RBCs should be ON cells as well. Third, the dendritic input of RBCs can be compromised—by damage to the fragile dendritic tips or the rods distal to the recorded cell—even when the dendritic tree appears visually to be intact (Euler et al. 1996; Hartveit 1996). Hence, while axotomized RBCs clearly had lost the majority of chloride currents, the hyperpolarizing cells behaved as though they had their dendrites cutoff and thus lost their cation currents (the chloride current would originate from amacrine cells driven by neighboring bipolar cells).

Responses of cone bipolar cells to light

The responses of CBCs varied in polarity and time course. Four major types of responses were observed: transient ON, transient OFF, and sustained ON, OFF. Given the difficulties of patching specific CBCs, we have not recorded enough examples of each morphological type to firmly establish the physiological behavior of each type. Nonetheless, it is clear that different types of bipolar cells in mammals, as in cold-blooded vertebrates, carry different signals.

In the salamander retina, inhibitory feedback at the axon terminals of bipolar cells has been shown to make amacrine and ganglion cells respond more transiently, presumably by shaping the bipolar cell’s output (Dong and Werblin 1998).
Furthermore, it has been suggested that the temporal properties of bipolar cells results, at least partially, from the differential expression of inhibitory receptors (Łukasiewicz and Shields 1998; Pan and Lipton 1995). Supporting this hypothesis, recent studies (Euler and Wässle 1998; Łukasiewicz and Wong 1997; Shields et al. 1998) have found differences in the contribution of GABA_A and GABA_C receptors to the GABA responses of different types of bipolar cells. Because of their different kinetic properties, GABA receptors can tune the output of bipolar cells: GABA_A relays fast, transient inhibition, while GABA_C provides delayed, sustained inhibition (Pan and Lipton 1995). Furthermore, given the variety of GABA receptor subunits that have been localized to mammalian bipolar cells (Enz et al. 1996; Greferath et al. 1995; Grüner and Hughes 1993), each cell type could well express a unique combination of GABA receptors with specific subunit compositions, as recently demonstrated for RBCs (Fletcher et al. 1998; Koulén et al. 1998). Thus the axon terminals, and their associated amacrine cells, are probably important in generating the diversity of dynamic temporal properties of CBCs.

The role of the axon terminal system

The comparison of intact and axotomized RBCs enabled us to evaluate the role of the axon terminals in shaping the light response of RBCs. We quantitatively examined the response parameters listed in Table 1. Three major differences were found: intact RBCs 1) had generally a significantly shorter response latency ($\tau_{dep} / \tau_{MaxSlope}$), 2) displayed a larger hyperpolarizing phase ($V_{hyp}$), and 3) had wider sensitivity curves. In other words, functional properties of the axon terminals seem to speed up the responses of RBCs and extend their dynamic range. We believe that this is achieved through two mechanisms: inhibitory feedback from amacrine cells and axonally concentrated voltage-gated channels.

It has been proposed, that feedback from amacrine cells could extend the dynamic range in which a bipolar cell responds with graduated signals (Tachibana and Kaneko 1988).

Here, we demonstrated that by suppressing the GABA_A/C-mediated inhibitory input of intact RBCs, their shallow sensitivity curve could be transformed into a much steeper one. The transformed curve covered a smaller dynamic range and matched closely the sensitivity curves of axotomized RBCs. Thus inhibitory feedback from GABAergic amacrine cells at the bipolar axon terminal is an important factor in modifying the bipolar cell’s dynamic range.

Inhibitory feedback does not explain the longer latencies and smaller after-hyperpolarization observed in axotomized RBCs. During drug application, intact cells did not become significantly slower or displayed a reduced after-hyperpolarization. Both effects may be linked with the loss of voltage- and/or ion-gated conductances predominantly located in the axon. Voltage-gated channels play an important role in shaping the responses of bipolar cells (Mao et al. 1998; Mennerick et al. 1997). In addition to controlling transmitter release, calcium channels are thought to amplify the cell’s responses and decrease their latency (Zenisek and Matthews 1998). Potassium channels may accelerate the response by attenuating the gain (Mao et al. 1998). In rat bipolar cells, several types of both calcium and potassium channels have been described (Karschin and Wässle 1990). For contributing to the differences between intact and axotomized RBCs L-type Ca$^{2+}$ channels are good candidates. These channels are located in the axon terminals (Hartveit 1999; Pan and Lipton 1995; Protti and Llano 1998) and rapidly activate at membrane potentials between $-50$ and $-40$ mV, close to the resting potential of RBCs. Hence, Ca$^{2+}$ currents could be elicited by small depolarizations, amplifying the responses and decreasing the latency in dim light. This would result in “pushing” up the left side of the sensitivity curve. And indeed, we have found that axotomized RBCs, which lack L-type Ca$^{2+}$ channels (Hartveit 1999; Kaneko et al. 1989; Protti and Llano 1998), have longer latencies and need higher stimulus intensities to respond.

The sensitivity curve for the intact RBCs resembles the non co-operative Michaelis-Menten function (slope $h = 1$), and is similar to the sensitivity curve found in rods and cones (Norrmann and Werblin 1974; Schneeweis and Schnapf 1995). The

### Table 1. Summary of the characteristics of the light-induced voltage responses in intact and axotomized RBCs

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Intact RBCs</th>
<th>Axotomized RBCs</th>
<th>Statistically Different ($P \leq 0.05$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting potential, mV</td>
<td>$-45.4 \pm 10.6$ (15)</td>
<td>$-39.0 \pm 7.4$ (11)</td>
<td>No</td>
</tr>
<tr>
<td>$V_{dep}$, mV</td>
<td>$14.3 \pm 7.2$ (13)</td>
<td>$16.1 \pm 7.1$ (11)</td>
<td>No</td>
</tr>
<tr>
<td>$V_{hyp}$, mV</td>
<td>$-4.5 \pm 3.1$ (9)</td>
<td>$-2.2 \pm 1.2$ (11)</td>
<td>Yes</td>
</tr>
<tr>
<td>$V_{hyp}/V_{dep}$</td>
<td>$0.29 \pm 0.13$ (8)</td>
<td>$0.17 \pm 0.06$ (9)</td>
<td>Yes</td>
</tr>
<tr>
<td>$\tau_{dep}$, ms</td>
<td>$176 \pm 23$ (10)</td>
<td>$216 \pm 29$ (10)</td>
<td>Yes</td>
</tr>
<tr>
<td>$\tau_{MaxSlope}$, ms</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low intensity, ms</td>
<td>$132 \pm 25$ (10)</td>
<td>$160 \pm 26$ (11)</td>
<td>Yes</td>
</tr>
<tr>
<td>Saturating intensity, ms</td>
<td>$73 \pm 9$ (10)</td>
<td>$106 \pm 21$ (11)</td>
<td>Yes</td>
</tr>
<tr>
<td>Sensitivity curves</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$L_{cri}$, cd/m²</td>
<td>$0.15 \ldots 0.58$</td>
<td>$0.26 \ldots 9.75$</td>
<td>No</td>
</tr>
<tr>
<td>average $= 0.34$ (7)</td>
<td>average $= 2.01$ (9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$L_{cri}$, cd/m²</td>
<td>$1.14 \ldots 5.67$</td>
<td>$0.77 \ldots 16.55$</td>
<td>No</td>
</tr>
<tr>
<td>average $= 3.18$ (7)</td>
<td>average $= 4.45$ (9)</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>$h$</td>
<td>$1.07 \pm 0.19$ (7)</td>
<td>$2.39 \pm 0.84$ (9)</td>
<td>Yes</td>
</tr>
<tr>
<td>$w$, log units</td>
<td>$1.84 \pm 0.32$ (7)</td>
<td>$0.87 \pm 0.23$ (9)</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Summary of the characteristics of the light-induced voltage responses in intact and axotomized RBCs. $n$ is given in parentheses. $V_{dep}$, maximal amplitude of the depolarizing phase; $V_{hyp}$, maximal amplitude of the hyperpolarizing phase; $V_{hyp}/V_{dep}$, ratio of the amplitudes; $\tau_{dep}$, latency of the depolarizing phase; $\tau_{MaxSlope}$, latency of the maximal slope (at the onset of the response); $L_{cri}$, luminance needed to evoke 50 or 90% of the maximal amplitude of the depolarizing phase (range and average). $h$, slope of the fit using the Hill equation (= Hill coefficient); $w$, width of the dynamic range. For further explanations, see RESULTS and METHODS.
sensitivity function of axotomized RBCs, however, is best fitted using the Hill equation with $h > 1$ (positive co-operativity). In general, increasing the slope of the curve improves the cell’s ability to threshold its input and respond in a more binary fashion (Rao et al. 1994; Smith et al. 1986; Sterling 1998). One could speculate that interactions at the axon may modulate the thresholding transfer function of the input stage (equivalent to the axotomized RBC) to switch the cell between a ‘binary’ detection mode at scotopic conditions (steep curve) and an ‘analogue’ mode at mesopic conditions (shallow curve). The shallow sensitivity curves we found in intact RBCs support this speculation, since we recorded in the high scotopic to mesopic range.

Support for an important role of the axon in modulating the bipolar cell’s response comes also from recent evidence that shows bipolar cells to be quite homogenous in their direct response to photoreceptor input. All on bipolar cells use the same type of metabotropic glutamate receptor, mGluR6, to relay photoreceptors signals (Brandstätter et al. 1999b; Nomura et al. 1994; Vardi and Morigiwa 1997), suggesting that all on bipolar cells have similar dendritic responses. Recently, DeVries and Schwartz (1999) recorded the response of OFF cone bipolar cells to direct depolarization of single cones. Under their conditions, with feedback onto the bipolar cells eliminated pharmacologically, all cells responded similarly—even though the on and off bipolar cells studied clearly represented several morphological types. Thus the glutamate receptors directly involved in relaying the photoreceptor signals seem not to contribute much to the diversity of bipolar cell responses.

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