Retinal Bipolar Cell Input Mechanisms in Giant Danio. I. Electroretinographic Analysis

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Wong, Kwoon Y., Alan R. Adolph, and John E. Dowling. Retinal bipolar cell input mechanisms in giant danio. I. Electroretinographic analysis. J Neurophysiol 93: 84–93, 2005. First published June 30, 2004; doi:10.1152/jn.00259.2004. Electroretinograms (ERGs) were recorded from the giant danio (Danio aequipinnatus) to study glutamate-mediated input mechanisms onto bipolar cells. Glutamate analogs were applied to determine which receptor types mediate synaptic transmission from rods and cones to ON and OFF bipolar cells. Picrotoxin, strychnine, and tetrodotoxin were used to isolate the effects of the glutamate analogs to the photoreceptor–bipolar cell synapse. Under photopic conditions, the group III metabotropic glutamate receptor (mGluR) antagonist (RS)-α-cyclopentyl-4-phosphonophenylglycine (CPPG) only slightly reduced the b-wave, whereas the excitatory amino acid transporter (EAAT) blocker α-L-threo-β-benzyl-oxyspartate (TBOA) removed most of it. Complete elimination of the b-wave required both antagonists. The α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA)/kainate receptor antagonist 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzof[l]quinonoxaline-7-sulfonamide (NBQX) blocked the d-wave. Under scotopic conditions, rod and cone inputs onto bipolar cells were studied by comparing the sensitivities of the b-wave to photopically matched green and red stimuli. The b-wave was >1 log unit more sensitive to the green than to the red stimulus under control conditions. In CPPG or l-AP4 (l-(-)-2-amino-4-phosphonobutyric acid, a group III mGluR agonist), the sensitivity of the b-wave to the green stimulus was dramatically reduced and the b-waves elicited by the 2 stimuli became nearly matched. The d-wave elicited by dim green stimuli, which presumably could be detected only by the rods, was eliminated by NBQX. In conclusion: 1) cone signals onto ON bipolar cells involve mainly EAATs but also mGlur (presumably mGlur6) to a lesser extent; 2) rods signal onto OFF bipolar cells by mainly mGlur6; 3) OFF bipolar cells receive signals from both photoreceptor types by AMPA/kainate receptors.

INTRODUCTION

The first synapse in the visual system is between the photoreceptors and bipolar cells. All photoreceptors hyperpolarize in response to light, whereas the bipolar cells come in 2 varieties: OFF bipolar cells hyperpolarize whereas the ON bipolar cells depolarize to light (Werblin and Dowling 1969). l-Glutamate transmits signals from the photoreceptors to both types of bipolar cells, and thus the polarities of the bipolar cell responses are determined by postsynaptic receptors (Ayoub et al. 1989; Copenhagen and Jahr 1989; Slaughter and Miller 1981, 1983). In many animals, the identities of these postsynaptic glutamate receptors are known. OFF bipolar cells hyperpolarize in response to light; they use a sign-preserving mechanism that enables them to depolarize to glutamate, which is released from photoreceptors in darkness. α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA)/kainate receptors are found postsynaptically in these cells in all species studied so far, such as cat (Sasaki and Kaneko 1996), rabbit (McGillem and Dacheux 2001), rat (Euler et al. 1996), and salamander (Hensley et al. 1993). Glutamate activates a nonselective cation channel in these receptors, leading to an inward flow of cations and consequently depolarization of the cell (Ozawa et al. 1998).

By contrast, ON bipolar cells depolarize in response to light, and thus they use sign-inverting mechanisms that cause them to hyperpolarize to glutamate. It is believed that in many animals, this postsynaptic mechanism depends on the group III metabotropic glutamate receptor mGlur6 (Nakajima et al. 1993; Thoreson and Witkovsky 1999). In response to glutamate, mGlur6 activates an intracellular biochemical pathway that ultimately closes a nonselective cation channel, thus hyperpolarizing the cell (Nawy and Jahr 1991; Shiells and Falk 1990). In teleosts, however, 2 glutamate input mechanisms on the ON bipolar cell exist. Saito et al. (1979) reported that ON bipolar cells in carp respond to light signals from rods with a conductance increase, but cones with a conductance decrease. Navy and Copenhagen (1987) subsequently reported that the group III mGlur agonist l-(-)-2-amino-4-phosphonobutyric acid (l-AP4, or APB) completely blocked rod-driven light responses of goldfish ON bipolar cells, but that after mGlur6 receptors were saturated by l-AP4, additional glutamate induced a conductance increase, presumably reflecting a cone-driven, non-mGlur6 mechanism. Grant and Dowling (1995) found the non-mGlur6 component (named “IGlu” in their study) to be associated with a chloride conductance that could be partially blocked by excitatory amino acid transporter (EAAT) antagonists. They proposed an EAAT could account for the sign-inverting action of glutamate on the ON bipolar cells. As it transports glutamate from the synaptic cleft into the cell, chloride is also enabled to passively enter the cell, resulting in hyperpolarization. In response to light, the EAAT becomes less activated by glutamate and less chloride enters the cell, resulting in depolarization.

Together, these studies indicate that mGlur6 is the primary mediator of rod signals. However, it remains unclear whether cone inputs are mediated partially or exclusively by EAATs. If EAATs are the main mechanism and mGlur6 plays a lesser role, the net change in glutamate-induced conductance could still be an increase, in agreement with the measurements made by Saito et al. (1979). Grant and Dowling (1995, 1996) could...
not rule out the possibility that the cone channel uses mGluR6 as well as EAATs because light-evoked responses were not analyzed in great detail in their studies. Another possibility is that a third mechanism is involved in mediating cone signals to on bipolar cells because the EAAT antagonists they used could only partially (≤50%) block the non-mGluR6 component.

To test these possibilities, we recorded electroretinograms (ERGs) from the giant danio. The ERG is a field potential consisting of 3 main components, a-, b-, and d-waves, arising mainly from photoreceptor, on bipolar cell, and off bipolar cell activities, respectively (Fig. 1, top; Chappell and Rosenstein 1996; Dowling 1987; Green et al. 1999; Sieving et al. 1994; Stockton and Slaughter 1989). Earlier ERG studies suggested that the photopic b-wave in fish is mediated in large part by an mGluR6 mechanism because L-AP4 abolished nearly all of the b-wave in goldfish (DeMarco and Powers 1989; DeMarco et al. 1991) and zebrafish (Ren and Li 2004; Saszik et al. 2002; Van Epps et al. 2001). These ERG findings seemingly contradict the intracellular studies summarized above. The experiments reported herein were performed to examine these issues.

The giant danio was chosen in this and the companion study because of its phylogenetic closeness to zebrafish (Danio rerio) (Meyer et al. 1993). Zebrafish are an important model for genetic studies of the visual system and thus an understanding of its retinal neurophysiology is of importance for the analysis of mutants. Unfortunately, zebrafish have small retinal neurons and consequently single-cell recording has been technically challenging. In contrast, giant danio has considerably larger retinal cells and is more amenable to electrophysiological experiments. A number of the ERG experiments described herein were repeated using larval zebrafish and similar results were obtained (Wong et al. 2004), indicating that retinal bipolar cells of giant danio and zebrafish are driven by similar synaptic mechanisms.

METHODS

Animals

All experiments were performed on adult giant danios (Segrest Farms, Gibsonton, FL; EkkWill Waterlife Resources, Gibsonton, FL; Central Mass Aquatics, Worcester, MA) between 1.5 and 3 in. in length. Animals were maintained according to Harvard University and National Institutes of Health guidelines.

Electroretinography

All procedures were approved by the Institutional Animal Care and Use Committee at Harvard University. An in situ eyecup preparation was used. Animals were anesthetized in 0.016% (g/mL) tricaine (ethyl 3-aminobenzoate methanesulfonate) and paralyzed with 2–10 μL 1% gallamine triethiodide dissolved in phosphate-buffered solution (PBS). They were placed on a wet sponge with one eye facing the light stimulus. The fish were kept oxygenated by passing fish water containing 0.0004% tricaine through their gills. The cornea and lens were removed, and the tip of the superfusion tubing put in the vitreous humor. Glass electrodes filled with PBS with tip diameters of 10–20 μm were placed in the vitreous humor. The ground wire was inserted into the wet sponge. For photopic experiments, the above procedures were performed under room light. For scotopic experiments, they were done in red light. After the red light had been turned off, the animals were allowed to dark-adapt for >35 min before data acquisition began.

A 2-channel optical bench with separate 100-W tungsten light sources for the stimulus and the background illumination was used. The unattenuated intensities for the stimulus and the background were 9.5 × 10^3 and 1.4 × 10^3 μW cm^-2 at the fish’s eye, respectively. Neutral-density and interference filters were put in the light paths to adjust the intensity and wavelength of the stimuli. The unattenuated intensities for the 520- and 640-nm stimuli were 91 and 160 μW cm^-2, respectively. Stimulus flash duration was controlled by an electromechanical shutter. Recording started after the retina had been superfused by the Ringer solution for ≥25 min to allow the retina to adjust to the new chemical environment. Voltage signals were low-cut–filtered at 0.1 Hz and high-cut–filtered at 100 Hz and amplified by a Dagan EX1 differential amplifier (Dagan, Minneapolis, MN). Data were acquired with PCLAMP software (Axon Instruments, Union City, CA), and were either single responses or averages of 2 to 12 responses depending on signal-to-noise ratios. The sampling rates ranged from 200.1 Hz to 1.01 kHz. The amplitudes of the b-waves were measured from the bottom of the a-wave to the peak of the b-wave, and those of the d-waves from the bottom of the small...
negative-going component at light off to the peak of the d-wave. All error estimates, including error bars in the figures, are SEs. Origin software (Microcal, Northampton, MA) was used to calculate \( P \) values, with the significance level set at 0.05.

**Results**

**Photopic ERG**

The group III mGluR agonist L-AP4 removed most but not all of the b-wave in normal Ringer’s. To isolate cone-driven responses, a full-field white background of an intensity of about 35 \( \mu \text{W cm}^{-2} \) was used; an intensity of 0.07 \( \mu \text{W cm}^{-2} \) is sufficient to saturate rod responses in the goldfish retina (Mackintosh et al. 1987). ERGs were elicited by a series of white stimulus flashes of increasing intensity, first in the presence of the control Ringer solution (Fig. 1, top left). In response to a prolonged flash (500–1,000 ms), the ERG response demonstrates a small, negative-going a-wave shortly after light on, followed by a positive-going b-wave, and a positive-going d-wave shortly after light off (Fig. 1, top left). In the experiments shown in Fig. 1, the perfusion was then switched to a solution containing the group III mGluR agonist L-AP4 (150–500 \( \mu \text{M} \)). After the effects of L-AP4 on the ERG had stabilized, the same stimulus flashes were presented again. In the presence of L-AP4, the amplitude of the b-wave was reduced by 71.2 \( \pm 3.1\% \) (SE) for the response to the –1 log unit stimulus and comparably at other intensities (Fig. 2, top). When L-AP4 was now added to the PST Ringer’s, the b-wave was only slightly reduced in amplitude, by 23.1 \( \pm 4.7\% \) at –1 log unit (Fig. 2). Thus when the photoreceptor-to-bipolar cell synapse is isolated in this manner, a majority of the b-wave survives the L-AP4 treatment, suggesting that mGluR6 is not the main mechanism that mediates cone-driven bipolar cell responses. All subsequent experiments were performed in the presence of PST.

As shown in Fig. 2, top, PST dramatically enhanced the a- and b-waves of the ERG. PST also broadened the d-wave somewhat and delayed the latency of its peak, but had little effect on its amplitude (Fig. 2, top). When L-AP4 was now added to the PST Ringer’s, the b-wave was only slightly reduced in amplitude, by 23.1 \( \pm 4.7\% \) at –1 log unit (Fig. 2). Thus when the photoreceptor-to-bipolar cell synapse is isolated in this manner, a majority of the b-wave survives the L-AP4 treatment, suggesting that mGluR6 is not the main mechanism that mediates cone-driven bipolar cell responses. All subsequent experiments were performed in the presence of PST.
COMPLETE ELIMINATION OF THE PHOTOPIC B-WAVE REQUIRES BOTH MGLUR6 AND EAAT ANTAGONISTS. The group III mGluR antagonist CPPG (2–3 mM) also only removed a minor portion (15.4 ± 3.7% at −1 log unit; *n* = 5) of the photopic b-wave when the retina was in PST Ringer (Fig. 3). On the other hand, the remaining b-wave was nearly completely abolished by the EAAT blocker TBOA (75–250 μM; Fig. 3). This suggests that EAATs are responsible for most of the synaptic transmission from cones to on bipolar cells. The small reduction of the b-wave by CPPG could mean that the transmission of cone signals to the on bipolars uses mGluR6 to a small extent, but it could also be attributable to CPPG’s having other effects, such as on presynaptic group III mGluRs on photoreceptors (Koulen et al. 1999). Therefore this experiment could not rule out the possibility that synaptic transmission from cones to on bipolar cells uses only EAATs.

To test these possibilities, the experiment was repeated with the order of drug application reversed. TBOA (50 μM) eliminated most but not all (85.5 ± 1.2% at −1 log unit; *n* = 5) of the b-wave, whereas the small remaining b-wave could subsequently be removed by CPPG (Fig. 4). Therefore a component of the photopic b-wave could be blocked by CPPG but not by TBOA, confirming that mGluR6 makes a small contribution to the b-wave. A similar result was obtained when a small stimulus spot (0.5–1 mm diameter) focused onto the center of the retina was used (*n* = 3; not shown), demonstrating that the CPPG-sensitive component did not arise from rod photoreceptors in the peripheral retina that could not be saturated by the bright background.

THE CPPG-SENSITIVE COMPONENT PERSISTED WHEN GAP JUNCTIONS WERE BLOCKED. Heterologous coupling exists between rods and cones in teleosts (Baldridge et al. 1998; Mangel et al. 1994; Wang and Mangel 1996), and therefore it is conceivable that even though the former could not directly respond to light in these photopic experiments, the cones might pass signals onto the rods by gap junctions, which subsequently activate mGluR6 receptors on the on bipolars. To test this possibility, the gap junction blocker carbenoxolone (CBX; 50 to 100 μM) was added to the control solution for about 30 min, which is much longer than the time required for CBX to get into the retinas.
distal retina in the turtle eyecup preparation (Pottek et al. 2003). As shown in Fig. 5, top, CBX reduced the b- and d-waves somewhat (see DISCUSSION). TBOA was then applied in addition to CBX. As in the experiments without CBX, TBOA removed most but not all of the b-wave (72.9 ± 2.6% at −1 log unit; n = 8). CPPG was again required to eliminate the residual b-wave (Fig. 5). Similar results were obtained when another gap junction blocker, 1-octanol (1 mM), was used (n = 3; not shown). This suggests that cones can directly drive ON bipolar cells by mGluR6 receptors.

THE PHOTOPIC D-WAVE WAS ELIMINATED BY BLOCKING AMPA/KAINATE RECEPTORS. Because CPPG had little effect on the photopic d-wave, whereas TBOA actually enhanced it (Figs. 3 and 4), the mechanisms generating the d-wave are distinct from those that give rise to the b-wave. The AMPA/kainate receptor antagonist NBQX (20 μM) almost completely removed the photopic d-wave (96.2 ± 3.7% at −1 log unit; n = 7), indicating that these glutamate receptors mediate the cones’ synaptic input to OFF bipolar cells (Fig. 6). In most cases, the b-wave was also slightly reduced (17.3 ± 4.8% at −1 log unit; n = 7; Fig. 6), and a possible explanation for this will be presented in the DISCUSSION.

Scotopic ERG

THE ROD-DRIVEN B-WAVE IS MORE SENSITIVE TO MGLUR6 BLOCKADE THAN THE CONE-DRIVEN B-WAVE. The experiments described so far were performed using a rod-saturating background illumination. To study rod-driven ON bipolar cell responses, ERGs were recorded under scotopic conditions in which both rods and cones are functional. To separate rod and cone contributions to the ERG, we took advantage of the fact that rods (λ_{max} = 502 nm; see Schwanzara 1967) are more sensitive than cones to green stimuli, whereas cones are more

![Figure 5](http://jn.physiology.org/FIG. 5. When gap junctions were blocked with 50–100 μM carbenoxolone (CBX), elimination of the photopic b-wave also required both TBOA and CPPG. Top: stimulus was a 1-s white flash. Bottom: summary of the results from 8 retinas, with the responses in PST omitted. Largest response obtained from each retina in PST + CBX was normalized to 1.

![Figure 6](http://jn.physiology.org/FIG. 6. 2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoline-7-sulfonamide (NBQX) (20 μM) eliminated the photopic d-wave. Top: stimulus was a 1-s white flash. Bottom: summary of the results from 7 retinas, for the d-wave (left) and the b-wave (right) amplitudes. In both cases, the largest response obtained from each retina in PST was normalized to 1.)
sensitive than rods to red stimuli (for the red cones, $\lambda_{\text{max}} = 560–570 \text{ nm}$; see Palacios et al. 1996; Robinson et al. 1993). A 640-nm (red) stimulus was used to activate the cones selectively, whereas a 520-nm (green) stimulus was used to activate the rods selectively. The intensities of these 2 stimuli were photopically matched, i.e., under photopic conditions both stimuli elicited b-waves with similar thresholds (Fig. 7, bottom right “control”).

Increasing intensities of these stimuli were first presented to retinas in the PST-containing control solution and then in the presence of 2–3 mM CPPG or 600 $\mu$M L-AP4. Criterion thresholds (20 $\mu$V) were determined under both conditions. In control Ringer, the threshold intensity for the b-wave elicited by the 520-nm stimulus was on average 1.29 + 0.14 log units (n = 9) lower than that for the 640-nm–elicited b-wave; that is, the rods are more sensitive than the cones to the 520-nm stimulus (Fig. 7, bottom left). CPPG and L-AP4 were then applied to 3 and 6 retinas, respectively. Because the effects of these drugs on the threshold intensities were similar, data from both types of experiments were pooled together to generate the plots shown in Fig. 7, bottom. Blocking mGluR6 with CPPG or L-AP4 raised the 520-nm threshold by 1.33 ± 0.18 log units and the 640-nm threshold by 0.33 ± 0.16 log units, on average. As a result, the b-waves induced by these photopically matched stimuli became nearly matched again (Fig. 7, top and bottom left). The remaining 0.30 ± 0.12 log difference was only weakly significant ($P = 0.039$). In other words, in CPPG or L-AP4, the b-wave that remained was mostly cone-driven, suggesting that rod-driven on bipolar cell responses had been almost completely eliminated by blocking mGluR6.

Because the threshold intensity for the 640-nm stimulus was raised by CPPG or L-AP4, the cone-driven b-wave probably also had an mGluR6 component. To confirm this, the experiment was repeated under photopic conditions, where only the cones had contributions to the ERG. CPPG or L-AP4 also raised the threshold intensity for the 640 nm-elicited b-wave, by a similar amount (0.335 ± 0.047 log units, n = 11; $P$ value of the elevation = $3.5 \times 10^{-5}$) (Fig. 7, bottom right). Therefore the elevation of the scotopic b-wave threshold for the 640-nm stimulus by CPPG or L-AP4 was a result of the action of these drugs on the cone input. This result reinforced the conclusion drawn from the photopic experiments shown in Figs. 3 and 4; that is, mGluR6 makes a small but significant contribution in the transmission of cone signals to the on bipolar cells.

The rod-driven d-wave could be removed by NBQX. To analyze rod-driven off bipolar cell responses, we studied the effect of NBQX (60 $\mu$M) on the scotopic d-wave elicited by 520-nm flashes ≤1 log unit above the absolute threshold. Under dark-adapted conditions, the d-wave in the teleost ERG is small and not easy to detect (e.g., Ren and Li 2004). For that reason, we used a longer flash (2 s) to induce a clearly discernible d-wave. This d-wave was nearly completely eliminated by the application of NBQX (90.7 ± 6.1%; n = 5), suggesting that the AMPA/kainate receptors are responsible for the transmission of rod signals onto the on OFF bipolars (Fig. 8, left). NBQX did not reduce the rod-driven b-wave (Fig. 8, left). However, it decreased the b-wave elicited by dim, 640-nm flashes (Fig. 8, right), similar to its effect on the white light-induced b-wave recorded under photopic conditions (see above and DISCUSSION).

**DISCUSSION**

The importance of isolating the synapse of interest

The electroretinogram is generated mainly by radially oriented retinal neurons, and therefore arises primarily from photoreceptors and bipolar cells (Pugh et al. 1998). Horizontal, amacrine, and ganglion cells are not believed to have substantial, direct contributions to the ERG. Based on these assumptions, in most ERG studies, glutamate analogs are applied alone to investigate the glutamatergic inputs on the bipolars. However, other neuronal types have actions on bipolar cells, and thus can have indirect influences on the ERG. For example, amacrine cells make inhibitory GABA and glycnergic syn-
apases onto bipolar cells (Suzuki et al. 1990; Tachibana and Kaneko 1987) and blocking or activating these synapses has effects on the ERG (Arnarsson and Eysteinsson 1997, 2000; Kapousta-Bruneau 2000; Popova et al. 1995). In addition, there are cells that release neuromodulators that can alter the excitability of bipolar cells, such as the dopaminergic neurons. Indeed, '*'-AP4 has been shown to suppress the release of dopamine (Boatright et al. 1994; Dong and McReynolds 1991), and dopamine modulates glutamate receptors and voltage-gated channels on bipolar cells (Fan and Yuzulla 2001; Maguire and Werblin 1994).

When an agonist or an antagonist is applied to a retina, the drugs act on all neurons with receptors for the drugs. '*'-AP4, for example, acts on all group III mGluR receptors in the retina. Although it was thought initially that the receptor for '*'-AP4 was found only on the ON bipolar cell dendrites (Slaught-er and Miller 1981), subsequent studies have shown that amacrine and ganglion cells (chick: Caramelo et al. 1999; rat: Akazawa et al. 1994; Tehrani et al. 2000; rabbit: Gafka et al. 1999; Linn and Gafka 1999), bipolar cell axon terminals (rat: Brandstätter et al. 1996; salamander: Awatramani and Slaught-er 2001; Higgs et al. 2002), and even photoreceptors (rat: Koulom and Brandstätter 2002), horizontal cells (goldfish: Takahashi and Copenhagen 1992; catfish: Gaflka et al. 1999; Linn and Gafka 1999), bipolar cell axon terminals (rat: Brandstätter et al. 1996; salamander: Awatramani and Slaught-er 2001; Higgs et al. 2002), and even photoreceptors (rat: Koulom et al. 1999; salamander: Higgs et al. 2002) possess group III mGluRs and thus can be affected by '*'-AP4. Therefore, the effect of '*'-AP4 on the ERG cannot be assumed to be completely attributable to its direct action on mGluR6 recep-tors on the ON bipolar cells.

The importance of these considerations is made clear in the present study. When '*'-AP4 was given alone, it eliminated a majority of the photopic b-wave (Fig. 1). When PST Ringer’s was added to isolate the photoreceptor-to-bipolar cell synapse, '*'-AP4 removed only a small portion of the b-wave (Fig. 2). A plausible explanation for this finding is that in addition to a small direct action on mGluR6 receptors on the ON bipolar cells, '*'-AP4 activated group III mGluRs on amacrine cells, which suppressed ON bipolar cells by inhibitory synapses. Together, these 2 effects of '*'-AP4 led to a dramatic reduction of the photopic b-wave (Fig. 1), a result observed in other teleosts (DeMarco et al. 1991; Van Epps et al. 2001), but inconsistent with findings based on intracellular analyses of cone-driven ON bipolar cell responses (see INTRODUCTION). The present study resolves this inconsistency by showing that polysynaptic effects of '*'-AP4 lead to a substantial overestimate of the role of mGluR6 in the generation of the photopic b-wave.

**on** bipolar cells respond to cones mainly through EAATs, and to rods mainly through mGluR6

Most experiments in this study were performed in the presence of PST Ringer’s, which served to isolate the photo-receptor-to-bipolar cell synapse. It is possible that '*'-AP4 and other drugs used in this study could still influence the ERG by acting on the photoreceptors, the horizontal cells, and/or the bipolar cell axon terminals. It would have been more nearly ideal if mGluR6-specific glutamate analogs had been used, but such compounds have yet to be developed.

PST substantially enhanced both the a- and b-waves (Fig. 2, top). Assuming that the a-wave arises from photoreceptor activity, this enhancement suggests that photoreceptors are normally under GABAergic and/or glycinergic inhibition, which was blocked by picrotoxin and strychnine, and/or under inhibition by neuromodulators, the release of which was blocked by TTX. Alternatively, the hyperpolarization of OFF bipolar cells at light onset could contribute to the a-wave, and a recent study has shown evidence for this in the primate ERG (Rangaswamy et al. 2003). Thus PST may remove inhibition onto OFF bipolar cells, which hyperpolarize more vigorously at light onset, leading to a larger a-wave. However, the application of NBQX, which blocks OFF bipolar cell light responses, only slightly reduced the a-wave in PST Ringer’s (Fig. 6, top), suggesting that the hyperpolarization of the OFF bipolars makes only a small contribution to the a-wave. Thus although the potentiation of the OFF bipolar cell response by PST may cause the a-wave to increase somewhat, the majority of the increase is probably attributable to a removal of inhibition onto the photoreceptors. GABA receptors and GABA-mediated responses have been found on photoreceptors in some animals, including carp and goldfish (Wu and Dowling 1980), primates (Vardi and Sterling 1994), salamander (Wu 1986), and turtle (Tachibana and Kaneko 1984).

By performing experiments in PST, we observed that the photoreceptor inputs generating the b-wave are mediated by a combination of mGluR6 and EAATs. Rod inputs are mediated mostly by mGluR6 (Fig. 7, bottom left), as predicted by previous intracellular studies. However, cones signal not only onto EAATs, but also to some extent on mGluR6 (Figs. 3 and 4). This does not contradict the earlier findings by Saito et al. (1979), who reported that cone-driven light responses triggered a conductance decrease. If the ON bipolar cells respond to cones mainly with EAATs, and mGluR6 is responsible for only a minor portion of the response, the resistance change associated with EAATs would still dominate the net change in resistance.

Gap junction blockers CBX and I-octanol were used to test the possibility that cones and rods share light-evoked responses by gap junctions. Thus cones could pass their light responses to rods, which activate mGluR6 receptors on postsynaptic dens-drites. However, in the presence of these blockers, elimination of the photopic b-wave still required the application of both TBOA and CPPG, suggesting the cones directly signal onto both types of glutamate receptors (Fig. 5). The caveat here is that there was no independent way of confirming that these gap
junction blockers completely uncoupled rods and cones. In other studies, CBX and 1-octanol have shown varying degrees of effectiveness in blocking different types of gap junctions (Rozental et al. 2001), and it is possible that in our experiments, they were not sufficiently potent at the gap junctions between rods and cones. Moreover, there is no guarantee that the concentrations of CBX and 1-octanol used in this study reached the distal retina. However, they slightly reduced the amplitudes of the b- and d-waves, which argues that at least some of them reached the distal retina. Such reduction might be attributable to these blockers’ suppressing the feedback of horizontal cells onto photoreceptors, leading to a reduction in the calcium-dependent release of glutamate from photoreceptors onto bipolar cells (Kamermans et al. 2001a,b).

An additional line of evidence that rod–cone coupling is not required for the cone-induced CPPG-sensitive ON bipolar cell response is provided by comparing the effect of CPPG or L-AP4 on the threshold of the b-wave elicited by the 640-nm stimulus under photopic and scotopic conditions. The coupling between rods and cones is not static but depends on whether the retina is light- or dark-adapted (Mangel et al. 1994; Wang and Mangel 1996; Yang and Wu 1989). Because L-AP4/CPPG raised the threshold for the 640-nm-induced b-wave by similar amounts under photopic and scotopic conditions (which presumably led to different degrees of rod–cone coupling), the mGluR6 component of the b-wave probably did not depend on the passage of signals from cones to rods (Fig. 7, bottom).

Using TBOA to study the ERG

This is the first study to use the recently developed EAAT blocker TBOA for analyzing ERG mechanisms (Shimamoto et al. 1998; Winkler et al. 1999). Earlier studies suggested that blocking EAATs with d-aspartate and threo-hydroxyaspartic acid (L-tHA) on the rat ERG. They found that when glutamate reuptake was suppressed in this manner, all postsynaptic responses were quickly removed, leaving only the photoreceptor response. High doses (2–4 mM) of these compounds were used because they are actual substrates for EAATs and block the reuptake of glutamate by competing with endogenous glutamate for EAATs (Bridges et al. 1999). When low (<1 mM) doses are used, these drugs have virtually no effect on the giant dianio ERG (not shown), presumably because glutamate is still the dominant molecular species transported through the EAATs.

In the present study, TBOA was applied at much lower concentrations (as low as 50 μM), which was possible because TBOA is a potent nontransportable EAAT blocker (Mitrovic et al. 2001; Shigeri et al. 2001). It acts on all 5 types of EAATs (Shigeri et al. 2001; Shimamoto et al. 2000) and thus can block all EAATs in the retina. However, rather than nondiscriminatively blocking all postreceptoral responses, it specifically reduced the photopic b-wave in the giant dianio ERG. The most likely reason is that among the 5 EAAT types, TBOA is the most potent at EAAT5 (Shigeri et al. 2001; Shimamoto et al. 2000), which is believed to be the EAAT on teleost ON bipolar cells (Thoreson and Witkovsky 1999). Thus at relatively low concentrations such as those used in this study, TBOA can block most EAAT5 activity while having less effect on other EAATs. However, when these low concentrations are applied for prolonged periods or when much higher concentrations (>500 μM) are used, TBOA eliminates both b- and d-waves (not shown); that is, it has the same effects as d-aspartate and L-tHA in the study by Winkler et al. Under these conditions, glutamate reuptake (e.g., into Müller cells by EAAT1) in most parts of the retina is severely compromised, resulting in widespread accumulation of glutamate that saturates postsynaptic receptors on both ON and OFF bipolar cells. For this reason, proper dosage and incubation time are critical. All of our data were obtained as soon as the effects of TBOA on the ERG had become relatively stable, which was typically within 15–25 min after TBOA was first added to the retinas.

Therefore the experiments described here could have benefited from using an EAAT5-specific blocker. L-tHA is a nontransportable blocker for EAAT5 (Bridges et al. 1999). However, as mentioned above, low doses of L-tHA have virtually no effect on the ERG because it is a transportable substrate for EAAT1–EAAT4 (Bridges et al. 1999). Most of the applied L-tHA would be rapidly removed by EAATs on Müller and other cells before reaching the synapse between photoreceptors and the ON bipolar cells. When high doses are used, as in the study by Winkler and colleagues, it blocks all types of EAATs and nondiscriminately reduces both b- and d-waves. Thus at present TBOA is probably the best EAAT blocker for studying EAAT5 on ON bipolar cells.

Besides reducing the photopic b-wave, TBOA also reduced the a-wave (Figs. 3 and 4). This is expected because EAATs have been found on photoreceptors in salmonid and rat retinas (Amara and Fontana 2002; Eliasof and Werblin 1993; Picaud et al. 1995a,b; Pow and Barnett 2000). An obvious concern is that TBOA’s action on photoreceptors is responsible for a reduced ON bipolar cell response. For example, TBOA might prevent the reuptake of glutamate by the photoreceptors, and the glutamate that accumulates in the synaptic cleft saturates the postsynaptic receptors on the ON bipolar cells. If this were the case, the AMPA/kainate receptors on the OFF bipolar cells should also be saturated, reducing their light-evoked responses and consequently the size of the d-wave. However, the d-wave not only survived the TBOA treatment but was actually enhanced by it (Fig. 4, top). Therefore most of the reduction of the photopic b-wave was probably a result of a direct action of TBOA on the ON bipolar cells.

AMPA/kainate receptors mediate the light response of OFF bipolar cells

AMPA/kainate receptor antagonists (CNQX, NBQX, kynurenic acid, etc.) were previously shown to block selectively the d-wave in various species including zebrafish (Wesolowska et al. 2002; Wong et al. 2000), frog (Szikra and Witkovsky 2001), monkey (Sieving et al. 1994), and skate (Chappell and Rosenstein 1996). In those studies, the antagonists were applied alone, and PST was not used to minimize polysynaptic actions of these drugs. In the present study, PST was first applied, followed by NBQX, and the d-wave was likewise blocked, under both scotopic and photopic conditions (Figs. 6 and 8). However, the photopic b-wave was also slightly reduced (Fig. 6). This may be attributable to the action of NBQX on the AMPA/kainate receptors on horizontal cells. Horizontal cells are activated by glutamate released from photoreceptors in the dark, which depolarizes the cells. In light, the horizontal cells hyperpolarize and feed back onto photore-
ceptrons to inhibit their light responses (Baylor et al. 1971; Mangel 1991; Smith and Sterling 1990). NBQX blocks the AMPA/kainate receptors on horizontal cells, thus hyperpolarizing the cells. This causes the horizontal cells to be constantly activating the feedback mechanism, thereby keeping the cones in an inhibited state. Therefore the b-wave is slightly reduced by NBQX. Likewise, in the scotopic state, NBQX had a tendency to reduce the b-wave elicited by dim 640-nm (cone-selective) flashes. However, NBQX had no effect on or even enhanced the scotopic b-wave induced by dim 520-nm (rod-selective) flashes (Fig. 8). This differential effect of NBQX may be evidence that horizontal cells make inhibitory feedback onto cones but not onto rods.

Most of the earlier ERG studies cited above reported that under photopic conditions, blocking AMPA/kainate receptors enhanced the b-wave, apparently contradicting the results found in this study. This apparent disparity can be explained by a blockade of amacrine cell light responses by these AMPA/kainate antagonists. With their light responses blocked, amacrine cells no longer exert negative feedback onto the bipolar cells. Freed of inhibitory inputs from amacrine cells, the ON bipolar cells respond to light stimuli more vigorously, resulting in a larger b-wave. In this study, the PST cocktail blocked most of this amacrine cell feedback; thus the effect of NBQX on the horizontal cell-mediated feedback onto cones is revealed. This again illustrates the importance of isolating the synapse of interest in pharmacological experiments.

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