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Selective Transmission of Single Photon Responses by Saturation at the Rod-to-Rod Bipolar Synapse

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Summary

A threshold-like nonlinearity in signal transfer from mouse rod photoreceptors to rod bipolar cells dramatically improves the absolute sensitivity of the rod signals. The work described here reaches three conclusions about the mechanisms generating this nonlinearity. (1) The nonlinearity is caused primarily by saturation of the feedforward rod-to-rod bipolar synapse and not by feedback from horizontal or amacrine cells. This saturation renders the rod bipolar current insensitive to small changes in transmitter release from the rod. (2) Saturation occurs within the G protein cascade that couples receptors to channels in the rod bipolar dendrites, with little or no contribution from presynaptic mechanisms or saturation of the postsynaptic receptors. (3) Between 0.5 and 2 bipolar transduction channels are open in darkness at each synapse, compared to the approximately 30 channels open at the peak of the single photon response.

Introduction

Most of the operational range of rod vision is characterized by the sporadic arrival of photons at individual rod photoreceptors. At visual threshold only 1 in 10,000 rods absorbs a photon within the 0.2 s integration time of rod vision (reviewed by Walraven et al., 1990). To complicate matters, noise generated in all the rods threatens to obscure this sparse signal (Baylor et al., 1984; Van Rossum and Smith, 1998). Thus, downstream retinal neurons that receive input from multiple rods face the problem of convergence of sparse, noisy signals. This problem is by no means unique to rod vision. For example, just-detectable odors activate $<1\%$ of the olfactory receptors projecting to a single glomerulus (DeVries and Stuiver, 1961). Similarly, sensory signals are often sparsely represented in cortex (Young and Yamane, 1992; Vinje and Gallant, 2000), a representation that will carry over to subsequent computations. In each such instance, linearly combining inputs will inevitably mix signal and noise, compromising sensitivity. Indeed, the retina does not read out the rod array linearly, but instead applies a threshold-like nonlinearity to signals from each rod before they are combined (Field and Rieke, 2002). Here we investigate the mechanisms producing this nonlinear readout.

Near absolute threshold, rod signals traverse the mammalian retina through a specialized pathway (Dacheux and Raviola, 1986; Smith et al., 1986): rod → rod (ON) bipolar → AII amacrine → cone bipolar → ganglion cell. Rod bipolar cells receive converging input from 20–100 rods, making signal transfer from rods to rod bipolar cells the last opportunity to process single photon responses from individual rods. Baylor and colleagues (1984) suggested that thresholding the rod signals could reduce rod noise while retaining single photon responses. Van Rossum and Smith (1998) proposed that such a threshold could be provided by saturation of the postsynaptic machinery at the rod-to-rod bipolar synapse. This proposal, however, has not been tested experimentally.

Evidence for and against saturation at synapses in the central nervous system has been debated vigorously over the past decade (reviewed by Frerking and Wilson, 1996; Walmsley et al., 1998). Recent work indicates that some synapses are saturated by a single presynaptic action potential (Poncer et al., 1996; Foster, et al., 2002; Harrison and Jahr, 2003) while others are not (Frerking et al., 1995; Mainen et al., 1999; McAllister and Stevens, 2000; Yamashita, et al., 2003). Several mechanisms have been implicated in causing saturation. Signals at some synapses may be saturated presynaptically, as one action potential causes the fusion of no more than one vesicle (Edwards et al., 1976). At other synapses, release of multiple vesicles by a single action potential may elevate the transmitter concentration in the synaptic cleft sufficiently to saturate postsynaptic receptors (Wadiche and Jahr, 2001; Harrison and Jahr, 2003). One proposed function of saturation is to make a synapse reliable—that is to insure that the transmitted signal is insensitive to variations in the synaptic machinery (Foster et al., 2002).

While saturation may also play a functional role at the rod-to-rod bipolar synapse, this synapse differs in several ways from conventional synapses. First, the ribbon-type synapses rods make with rod bipolars (Sjöstrand, 1953) transmit small ($\sim$1mV), graded changes in voltage. Second, rods release transmitter continuously in the dark and slow release when hyperpolarized by light (Trifonov, 1968; Dowling and Ripps, 1973). Third, rod bipolar cells use metabotropic glutamate receptors to sense changes in transmitter release from the rods (Nakajima et al., 1993). Activity of these receptors leads to closure of nonselective cation channels (Nawy and Jahr, 1990; Shiells and Falk, 1990) through a poorly understood signaling cascade (Nawy, 1999). This G protein cascade provides another possible site of saturation at the rod-to-rod bipolar synapse.

Results

Nonlinear Synaptic Transmission between Rods and Rod Bipolar Cells

The light responses of mouse rod bipolar cells show a supralinear dependence on flash strength that arises in the transfer of signals from rods to rod bipolar cells (Field and Rieke, 2002). This nonlinearity could be an inherent property of the rod-to-rod bipolar synapse or could be produced by feedback from horizontal or amacrine cells.

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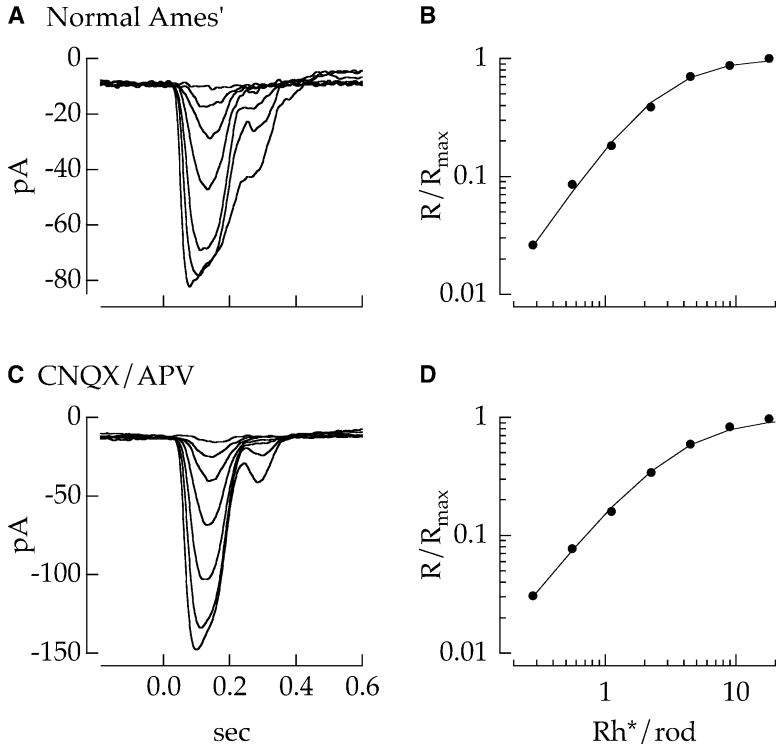

Figure 1. Nonlinearity Produced at the Rod-to-Rod Bipolar Synapse

Flash families and stimulus-response relations are shown for voltage-clamped rod bipolar cells superfused in normal Ames' (A and B) and Ames with 10 μM CNQX and 10 μM APV added (C and D). In each case the average responses to flashes producing between 0.28 and 18 Rh*/rod are superimposed. The Hill exponents for the fits to the stimulus-response relations were 1.60 in (B) and 1.38 in (D). Cells were voltage clamped at −60mV. A perforated-patch recording was used in (A) and a whole-cell recording in (C). Bandwidth was 0–30 Hz.

Figure 1A superimposes average responses of a voltage-clamped rod bipolar cell to flashes producing between 0.28 and 18 active rhodopsins per rod (Rh*/rod). The supralinear dependence on flash strength is particularly clear in the two smallest responses, which differ in amplitude by more than the 2-fold difference in the strength of the flashes generating them. We quantified the extent of this nonlinearity by fitting the cell's stimulus-response relation with the Hill equation (smooth curve in Figure 1B; see Experimental Procedures):

$$\frac{R}{R_{max}} = \frac{1}{1 + (\phi_{1/2}/\phi)^n}, \quad (1)$$

where R/R_{max} is the normalized response amplitude, $\phi_{1/2}$ is the half-maximal flash strength, and n, the Hill exponent, expresses the power of the relation between the response amplitude and flash strength ϕ. A Hill exponent of 1 indicates a linear relation at low flash strengths, and an exponent >1 indicates a supralinear relation. The component of the Hill exponent >1 quantifies the extent of nonlinearity. The Hill exponent for the fit to the stimulus-response relation in Figure 1B was 1.60; the average Hill exponent for voltage-clamped cells was 1.51 ± 0.04 (mean ± SEM, n = 30).

To test whether the nonlinearity in the rod bipolar light responses required amacrine or horizontal feedback, we suppressed activity of the ionotropic glutamate receptors expressed by these cells with CNQX and APV. CNQX and APV reduced the amplitude of the responses of horizontal (n = 6) and AII amacrine cells (n = 4) more than 10-fold (data not shown), indicating effective block of the ionotropic receptors expressed by these cells. Figures 1C and 1D show the flash family and stimulus-response relation for a rod bipolar cell when horizontal and amacrine responses were suppressed. The Hill exponent for the fit to the stimulus-response relation in this cell was 1.38; the average Hill exponent in the presence of CNQX and APV was 1.43 ± 0.06 (mean ± SEM, n = 11). Suppressing amacrine feedback by inhibiting GABA and glycine receptors on the rod bipolar synaptic terminal with picrotoxin and strychnine also produced little or no change in the nonlinearity (Field and Rieke, 2002). These experiments indicate that the nonlinearity is produced primarily at the feedforward rod-to-rod bipolar synapse with little contribution from horizontal or amacrine feedback.

Nonlinearity Generated within the Transduction Cascade

Nonlinear transfer of signals from rods to rod bipolar cells causes the bipolar current to be relatively insensitive to small changes in rod voltage, indicating a mechanism that is partially or completely saturated in darkness—i.e., the gain of the mechanism is low in darkness and increases with increasing rod hyperpolarization. Such a saturation could occur either pre- or postsynaptically. Presynaptically, the rate of glutamate release could be insensitive to small changes in rod voltage. This is unlikely because transmitter release in amphibian rods is more sensitive to small changes in rod voltage than large ones (Attwell et al., 1987; Belgum and Copenhagen, 1988; Witkovsky et al., 1997), opposite the effect required to explain the supralinearity of the rod bipolar responses. Postsynaptically, saturation of the glutamate receptors or of the cascade linking them to ion channels could cause the rod bipolar current to be insensitive to small changes in glutamate concentration. The experiments described below indicate that the nonlinearity

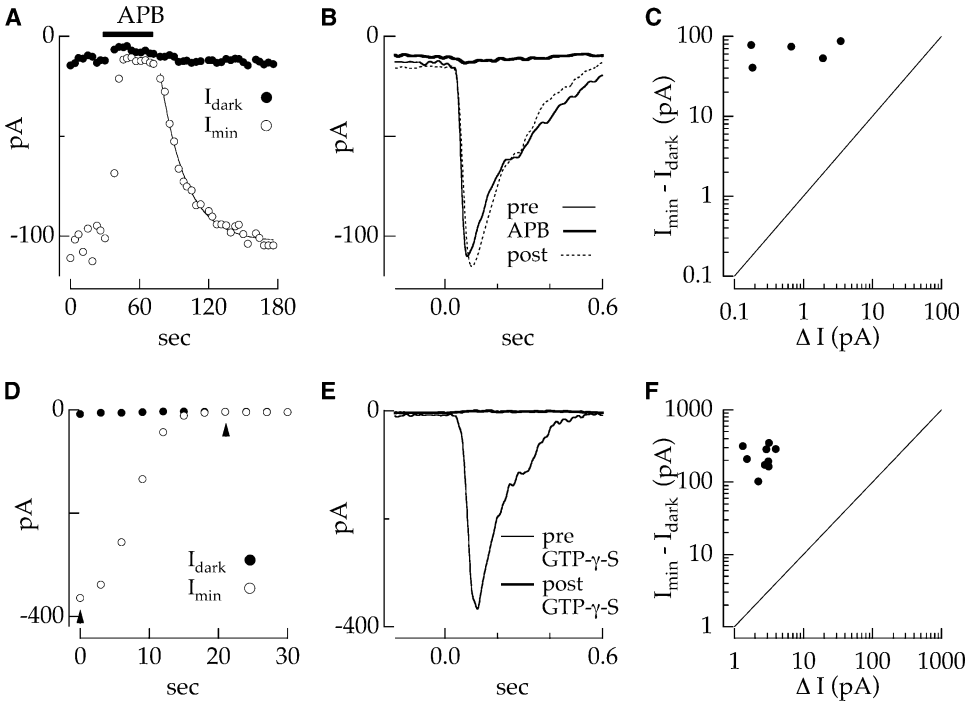

Figure 2. Current in Darkness Is Close to the Limit Set by Maximal Receptor and G Protein Activity

(A) Amplitude of the saturating light response (○) and the dark current (●) were monitored while the cell was superfused with a solution containing 8 μM APB during the period indicated. Flashes producing 36 Rh*/rod were delivered every 3 s. The smooth curve is an exponential fit (τ = 26 s) to the recovery of the light response upon return to Ames' solution.

(B) Average responses measured before, during, and after superfusion with APB are plotted.

(C) The amplitude of the saturating light response is plotted against the change in dark current produced by APB for five cells. The line of unity slope represents the expectation if the dark current and saturating response were equally sensitive to APB. Results are from perforated-patch recordings.

(D) Amplitude of the saturating light response (○) and the dark current (●) were monitored after initiating a whole-cell recording with a pipette solution containing 50 μM GTP-γ-S. Flashes producing 40 Rh*/rod were delivered every 3 s.

(E) Individual responses measured before and after decay of the light response are plotted; response times are indicated by arrows in (D).

(F) The amplitude of the saturating light response is plotted against the change in dark current for nine cells. The line of unity slope represents the expectation if the dark current and saturating response were equally sensitive to GTP-γ-S. Bandwidth was 0–30 Hz.

was generated by saturation within the rod bipolar transduction cascade.

The Rod Bipolar Transduction Cascade Operates Near Saturation in Darkness

Rod bipolar cells sense glutamate through mGluR6 (group III) metabotropic glutamate receptors, whose activity leads to a closure of nonselective cation channels (Nawy and Jahr, 1990; Shiells and Falk, 1990). The number of active (i.e., glutamate-bound) receptors and the strength with which their activity is coupled to ion channel activity determine the dependence of the rod bipolar current on the glutamate concentration.

Figures 2A–2C compare the current in darkness with the currents produced by minimal and maximal receptor activity. Minimal receptor activity was produced at the peak of a saturating light response. Maximal receptor activity was produced with a high concentration of APB, an agonist of mGluR6 receptors (Slaughter and Miller, 1981). Figure 2A shows the amplitudes of the dark current (the inward current in darkness at −60mV) and saturating light response before, during, and after superfusion with APB. Figure 2B shows saturating light responses measured in each period. APB strongly suppressed the light response but produced only a small change in dark current, 3 pA in this cell compared to the nearly 100 pA suppression of the light response. Figure 2C plots the change in amplitude of the saturating light response against the change in dark current produced by APB for five similar experiments. On average, APB produced a change in dark current 50 times smaller than the saturating light response. Thus the dark current was near the limit set by maximal receptor activity. This high level of dark receptor activity will cause the rod bipolar current to be more sensitive to decreases in glutamate than increases.

The insensitivity of the rod bipolar dark current to APB could be produced by saturation of the postsynaptic receptors or saturation of the machinery coupling receptor activity to channels. The first step in the bipolar transduction cascade is activation of a G protein. If the glutamate level in darkness is sufficient to saturate the mGluR6 receptors but not the transduction cascade, then there should be channels open in darkness that can be closed by increasing the G protein activity. If the receptor activity in darkness is sufficient to saturate the transduction cascade, then increasing the G protein activity should produce little or no change in the number of open channels and the dark current.

The experiment of Figures 2D–2F tested the effect of increasing G protein activity on the membrane current. The strategy was similar to that in Figures 2A–2C: We compared the dark current with two limits—the current produced at the peak of a saturating light response and the current remaining after the cell was dialyzed with GTP-γ-S. GTP-γ-S is a poorly hydrolyzed GTP analog that will increase the active lifetime of the G protein at least 100-fold (Lamb and Matthews, 1988). Figure 2D shows the amplitudes of the saturating light response and the dark current as GTP-γ-S diffused into the cell. The response to the saturating flash quickly decayed due to the additional G protein activity produced by GTP-γ-S. Figure 2E shows light responses measured immediately after establishing the recording and after the light response had decayed. When GTP-γ-S was omitted from the pipette solution, light responses maintained half of their maximal amplitude for at least 2 min (data not shown, $n = 16$). GTP-γ-S also decreased the inward current, apparent as a small shift in baseline in the superimposed responses in Figure 2E (see also Figure 7). In this cell GTP-γ-S reduced the inward dark current by <5 pA, a small fraction of the nearly 400 pA saturating light response. Figure 2F plots the amplitude of the saturating light response against the change in dark current for nine such experiments. The average change in dark current was 1.2% of the saturating light response, indicating that the current in darkness was near the limit set by the G protein activity. This will cause the rod bipolar current to be more sensitive to decreases in G protein activity (e.g., those produced by light) than increases.

Changing Number of Available Receptors Alters Nonlinearity

The GTP-γ-S experiments indicate that the rod bipolar transduction cascade operates near saturation in darkness. To test whether saturation of the transduction cascade contributes to the nonlinearity in the rod bipolar light responses, we decreased the number of receptors responding to changes in glutamate using the mGluR6 agonist APB. Because it is a high-affinity agonist, APB remains bound to the receptors for a period much longer than the duration of the light response (see Experimental Procedures). Thus APB should divide the receptor population into two groups: receptors bound to APB will be constitutively active and insensitive to glutamate throughout the light response, while the remaining receptors will retain their normal glutamate sensitivity (Figure 3A). If the nonlinearity in the rod bipolar light responses is caused by saturation of the receptors themselves, APB should decrease the amplitude of the light response but not alter the nonlinearity, since the APB-free receptors will still be subject to saturation. However, if the nonlinearity is generated downstream of the receptors, APB should exacerbate it by increasing receptor activity and hence saturation of the transduction cascade.

Figure 3B shows a response family measured from a rod bipolar cell superfused in Ames'. Figure 3C shows a family from the same cell with APB added to the perfusate. As the aim of these experiments was to test how a small change in receptor activity influenced the nonlinearity, we chose an APB concentration that produced a small but consistent decrease in the amplitude of the saturating light response, 3 pA in this cell (average decrease ~20% in 10 cells). APB produced a proportionally larger decrease in the amplitude of the smallest responses and increased the separation in their amplitudes. These effects are apparent in the stimulus-response relations (Figure 3D), where APB increased the slope at low flash strengths. The Hill exponent (see Equation 1) increased from 1.5 in Ames' to 1.7 in the presence of APB. The effect of APB on the shape of the stimulus-response relation was reversible and repeatable (Figure 3D, inset).

Figure 3E plots the Hill exponent in APB against that in Ames' for ten cells. In nine of ten cells APB produced a significant increase in the nonlinearity ($p < 0.05$). On average, the Hill exponent in APB was 1.16 ± 0.03 times that in Ames' (mean ± SEM, $n = 10$). This represents a 70% increase in the extent of the nonlinearity as measured by the supralinear part of the Hill exponent—i.e., the component of the Hill exponent greater than 1. The ability of APB to increase the nonlinearity, rather than simply scaling the responses as expected for receptor saturation, indicates that saturation within the transduction cascade accounts for at least part of the nonlinearity in the rod bipolar light responses.

Rod Bipolar Glutamate Receptors Are Not Strongly Saturated in Darkness

We tested for a contribution of presynaptic or receptor saturation to the nonlinearity in the rod bipolar light responses using the metabotropic glutamate receptor antagonist, LY341495. Like APB, LY341495 unbinds slowly from mGluR6 receptors. Thus LY341495 should divide the available receptors into two groups: some receptors will be inactivated by LY341495 throughout the light response, while the remainder will retain their normal glutamate sensitivity (Figure 4A; see Experimental Procedures). With fewer active receptors, saturation in the rod bipolar transduction cascade should be decreased or eliminated. If mechanisms upstream of the transduction cascade contribute substantially to the nonlinearity in the rod bipolar responses, LY341495 should be unable to restore linear or near-linear behavior. We used a high concentration of LY341495 to alleviate saturation in the transduction cascade as much as possible.

Figures 4B and 4C show flash families measured in Ames' and Ames' plus LY341495. LY341495 increased the inward dark current and decreased the amplitude of the saturating light response. More importantly, LY341495 reduced the nonlinearity in the rod bipolar light responses. Figure 4D plots stimulus-response relations in each condition. In this cell the responses to the dimmest flashes in LY341495 scaled linearly or near linearly with flash strength; the Hill exponent for the fit to the stimulus-response relation was 1.0, compared to 1.5 in Ames'. The change in Hill exponent was reversible and repeatable (Figure 4D, inset). LY341495 also decreased the inward current at the peak of the saturating response; this could be produced by a decreased transduction gain due to maintained Ca^{2+} influx (Shiells and Falk, 1999; Nawy, 2000) or an action of LY341495 as a partial agonist. Neither effect, however, alters interpretation of the decrease in Hill exponent in LY341495 as a relief of saturation in the rod bipolar transduction cascade.

Figure 4E plots the Hill exponent in LY341495 against that in Ames' for ten cells. LY341495 significantly re-

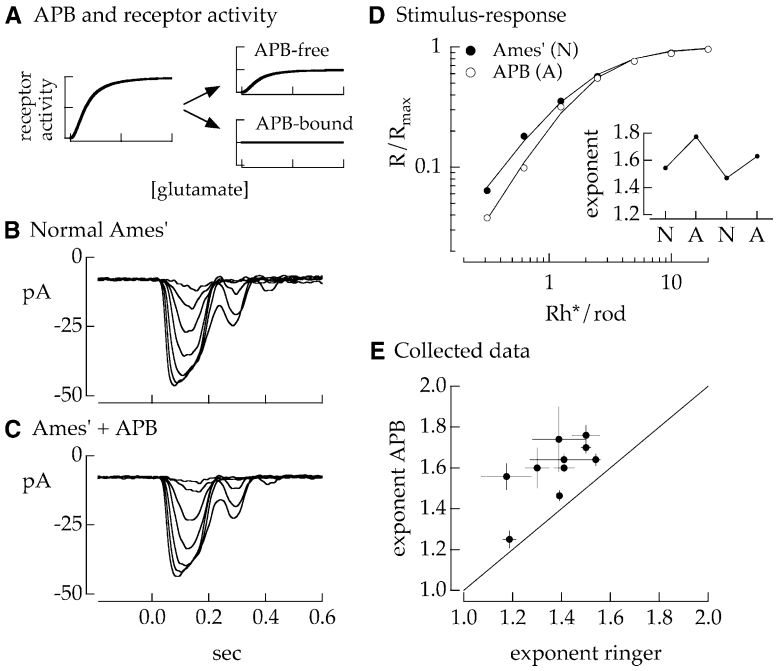

Figure 3. APB Increased the Nonlinearity in the Rod Bipolar Responses

(A) As a high-affinity agonist, APB will effectively divide the receptors into two groups. Receptors bound with APB will be active throughout the light response, while unbound receptors will retain their normal glutamate sensitivity.

(B) The flash family for a cell superfused with normal Ames' is shown.

(C) The flash family for the same cell superfused with Ames' containing 1.6 μM APB is shown.

(D) Stimulus-response relations are plotted for the families in (B) and (C). The smooth lines are Hill curves (Equation 1) with exponents of 1.51 (Ames') and 1.70 (Ames' + APB). The inset plots the Hill exponent for several changes in the superfusion solution (N = normal Ames', A = Ames' + APB).

(E) The Hill exponent (±SEM) measured in Ames' + APB is plotted against that measured in Ames' for ten cells. The line of unity slope plots the expectation if APB did not alter the nonlinearity in the rod bipolar light responses. Results are from perforated-patch recordings. Bandwidth was 0–30 Hz.

duced the nonlinearity in the light responses of each cell ($p < 0.05$) and eliminated the nonlinearity within the precision of our measurements in four cells. The average Hill exponent in LY341495 was 1.16 ± 0.05 (mean ± SEM) compared to 1.55 ± 0.07 in Ames'. The extent of nonlinearity was measured from the supralinear part of the Hill exponent—i.e., the part greater than one; LY341495 reduced the nonlinearity by more than a factor of three (0.55/0.16, ~3.4).

The experiments of Figure 4 place an upper bound on the contribution of presynaptic or receptor saturation to the nonlinearity in the rod bipolar responses. The remaining nonlinear behavior in the presence of LY341495 could be produced by mechanisms upstream of the rod bipolar transduction cascade or by failure of the drug to abolish completely the nonlinearity in the cascade (e.g., due to an action as a partial agonist). In either case, the large decrease in nonlinearity produced by LY341495 indicates that the rod bipolar transduction cascade is the primary source of nonlinearity in the bipolar light responses.

Background Light Decreases Nonlinearity

Saturation of the rod bipolar transduction mechanism should be relieved if the rate of glutamate release from the rods decreases. Background light will cause such

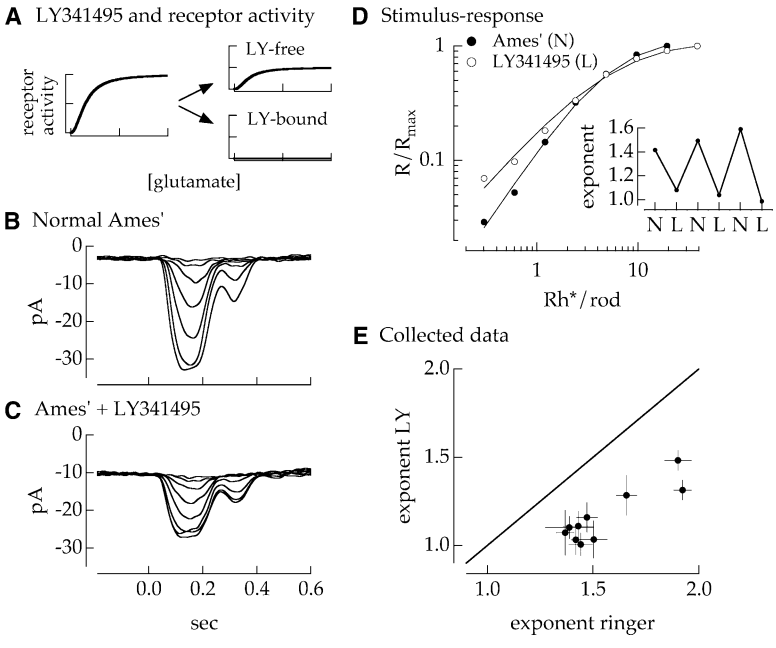

Figure 4. LY341495 Reduced Nonlinearity in the Rod Bipolar Responses

(A) As a high-affinity antagonist, LY341495 will effectively divide the receptors into two groups. Receptors bound with LY341495 will be inactive throughout the light response, while the unbound receptors will retain their normal glutamate sensitivity.

(B) The flash family for a cell superfused with normal Ames' is shown.

(C) The flash family for the same cell superfused with Ames' containing 2 μM LY341495 is shown.

(D) Stimulus-response relations are plotted for the families in (B) and (C). The smooth lines are Hill curves (Equation 1) with exponents of 1.50 (Ames') and 1.03 (Ames' + LY341495). The inset plots the Hill exponent for several changes in the superfusion solution (N = normal Ames', L = Ames' + LY341495).

(E) The Hill exponent (±SEM) measured in Ames' + LY341495 is plotted against that measured in Ames' for ten cells. The line of unity slope plots the expectation if LY341495 did not alter the nonlinearity in the rod bipolar light responses. Results are from perforated-patch recordings. Bandwidth was 0–30 Hz.

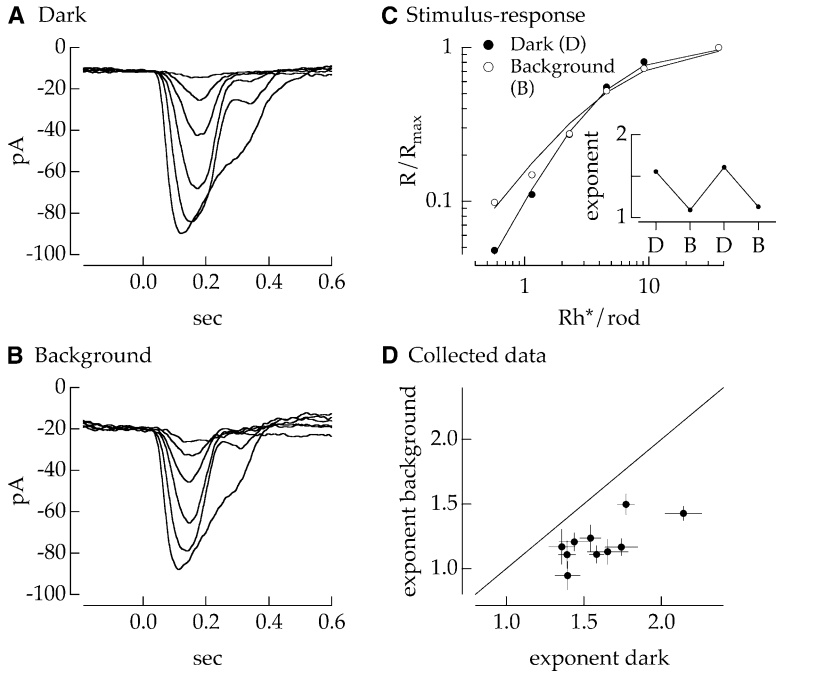

Figure 5. Background Light Decreased the Nonlinearity in the Rod Bipolar Light Responses

(A) The flash family for a cell in the absence of background light is shown.

(B) The flash family for the same cell in the presence of a background light producing 17 Rh*/rod/s is shown.

(C) Stimulus-response relations are plotted for the families in (A) and (B). The smooth lines are Hill curves (see Experimental Procedures) with exponents of 1.58 (dark) and 1.11 (background). The inset plots the Hill exponent for several presentations of background light (D = dark, B = background).

(D) The Hill exponent (±SEM) measured in the presence of background light is plotted against that measured in darkness for ten cells. The line of unity slope plots the expectation if the background did not alter the nonlinearity in the rod bipolar light responses. Results are from perforated-patch recordings. Bandwidth was 0–30 Hz.

a reduction in glutamate by hyperpolarizing rod photoreceptors.

Figures 5A and 5B show flash families from a rod bipolar cell in darkness and in the presence of background light. The background, which produced 17 Rh*/rod/s, caused a 10 pA increase in inward current. Backgrounds of this intensity cause the rod photoreceptor responses to adapt; however, unlike the effect of adaptation in the rods, the background increased the amplitude of the rod bipolar responses to dim flashes. Two effects are present when the stimulus-response relations with and without the background are compared (Figure 5C): a small rightward shift (more apparent in some cells), likely due to adaptation in the rods or at the rod-to-rod bipolar synapse, and a decrease in slope at low flash strengths due to a relief of the nonlinearity. Background light changed the Hill exponent for the fit to the stimulus-response relation from 1.5 to 1.1, a change that was reversible and repeatable (Figure 5C, inset). Figure 5D collects results from ten cells, plotting the Hill exponent in the presence of the background against that in darkness. Background light significantly reduced the nonlinearity in the light responses of 9 of 10 cells ($p < 0.05$).

Shiells and Falk (2002) found a similar effect of background light on the responses of dogfish bipolar cells. They also found that dialyzing the bipolar with 20 μM cGMP removed the nonlinearity, suggesting that cGMP and weak backgrounds operated through a common mechanism. We failed to observe an effect of cGMP on the nonlinearity in the rod bipolar responses; the Hill exponent for fits to the stimulus-response relations for ten cells dialyzed with 1 mM cGMP was 1.50 ± 0.04 (mean ± SEM), virtually identical to that when cGMP was excluded (1.51 ± 0.04, n = 30). This indicates that cGMP does not play an important role in controlling the nonlinearity in the responses of mouse rod bipolar cells.

Few Transduction Channels Are Open in Darkness

The small changes in current produced by saturating APB (Figures 2A–2C) and by GTP-γ-S (Figures 2D–2F) suggested that few transduction channels in the rod bipolar dendrites were open in darkness. We estimated the number of open channels from an estimate of the single channel current and the magnitude of the fluctuations in the rod bipolar dark current.

Two approaches to estimating the single channel current provided similar results. First, we applied nonstationary noise analysis to the falling phase of the saturating light response (Figure 6A). We divided each of several saturating responses into 10 ms bins starting 150 ms after the flash and measured the mean and variance of the current in each bin. The variance measured in darkness was subtracted from each measurement during the response. The resulting difference variance scaled approximately linearly with the mean current, as shown in Figure 6B. This relation between the variance and mean is usually parabolic, with maximal variance occurring at a channel open probability of 0.5 and minimal variance occurring when either no or all channels are open (Verveen and Derksen, 1969). The near-linear scaling indicates that the open probability throughout the response was less than 0.5. Assuming the variance was produced by independent channel gating, the slope of the best-fit line through the data, 0.28 pA in this cell, estimates the single channel current.

Figure 6C collects data from 11 similar experiments; the estimated single channel current from the collected data is 0.27 pA (99% confidence interval 0.26–0.28 pA). This estimate is nearly three times smaller than that obtained from nonstationary noise analysis of glutamate-sensitive currents of isolated cat rod bipolars (de la Villa et al., 1995); the reasons for this discrepancy are not clear.

The mean and variance of the rod bipolar current in

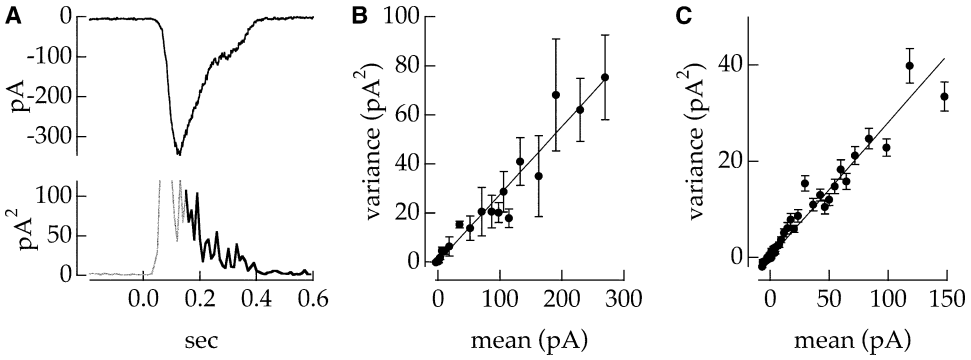

Figure 6. Estimate of Single Channel Current from Fluctuations in Saturating Light Response

(A) Single response to a flash producing 40 Rh*/rod is plotted in the top panel. Each such response was divided into 10 ms bins starting 150 ms after the flash, and the mean and variance of the current in each bin were calculated. The variance is plotted in the bottom panel.

(B) The variance is plotted against the mean for three responses like that in (A). Error bars are SEM. The straight line fit to the data has a slope of 0.28 pA, the estimated single channel current for this cell.

(C) The variance is plotted against the mean for 11 cells analyzed as in (B). The straight line has a slope of 0.27 pA. Results are from whole-cell recordings. Bandwidth was 0–3000 Hz.

darkness provided a second estimate of the single channel current. Figure 7A shows a section of current record in darkness. Figure 7B shows a section of record from the same cell after GTP-γ-S diffused into the dendrites and eliminated the light response. In addition to a small decrease in inward current (see also Figures 2D–2F), GTP-γ-S reduced the current fluctuations. Two components of noise apparent in darkness were absent or attenuated in GTP-γ-S: occasional 10–20 pA discrete inward currents and rapid, smaller baseline fluctuations. Figure 7C shows histograms of the current amplitudes measured before and after GTP-γ-S. The discrete inward events create the long tail in the pre-GTP-γ-S histogram, and the rapid baseline fluctuations create the central peak. We estimated the mean and variance of the baseline fluctuations by fitting a Gaussian to the symmetric region of the histogram (currents > -6 pA in this cell; smooth lines in Figure 7C) and comparing the mean and variance of the fits before and after GTP-γ-S. Assuming that the baseline fluctuations represent independent gating of transduction channels, the ratio of the variance increase to the increase in mean inward current estimates the single channel current. In this cell, the variance-to-mean ratio indicated a single channel current of 0.52 pA, compared to the estimate of 0.32 pA obtained from the variance during recovery of the saturating light response. In six cells the variance-to-mean ratio indicated a single channel current of 0.35 ± 0.05 pA (mean ±

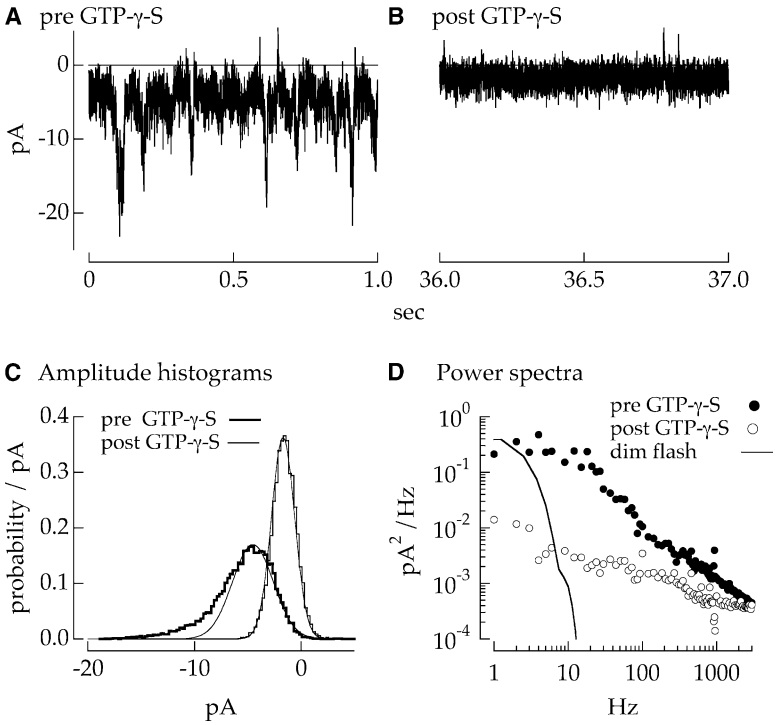

Figure 7. Properties of Dark Noise in Rod Bipolar Current

(A) A section of current recorded in darkness is plotted.

(B) A section of current recorded after GTP-γ-S had diffused into the cell and eliminated the light response is plotted. GTP-γ-S decreased the current fluctuations and produced a small decrease in inward current.

(C) Histograms of current values from recordings before and after dialyzing with GTP-γ-S, including records in (A) and (B). Smooth curves are Gaussian fits. The fit to the dark histogram was calculated using only currents > -6 pA to eliminate the asymmetric tail for larger inward currents.

(D) Power spectra of the current fluctuations before and after GTP-γ-S dialysis. The solid line is the power spectrum of the average dim flash response from 30 rod bipolar cells. Results are from whole-cell recordings. Bandwidth was 0–3000 Hz.

SEM). This estimate is similar to that obtained for the nonstationary noise analysis from the same cells (0.28 ± 0.03 pA), consistent with the idea that the baseline current fluctuations are generated by independent or nearly independent channel openings.

The two analyses described above indicate that the single channel current was near 0.3 pA. The total baseline current variance divided by the square of the single channel current provided an estimate of the number of channels open in darkness. In the cell of Figure 7, this ratio indicated 25 open channels. On average, the baseline current variance corresponded to 35 ± 6 open channels (mean ± SEM, n = 6). This is an upper bound because it assumes that all the baseline current variance can be attributed to transduction channels, while some of the variance certainly comes from other sources (e.g., instrumental noise).

A second estimate of the number of open transduction channels was obtained by assuming that GTP-γ-S closed all the channels. In this case the number of channels open in darkness is given by the change in mean current produced by GTP-γ-S divided by the single channel current. For the nine cells in Figure 2F, this ratio corresponds to 10 ± 1 (mean ± SEM) channels open in darkness. This is a lower bound because it assumes all channels are closed by strong G protein activation. Because mouse rod bipolar cells receive input from about 20 rods (Tsukamoto et al., 2001), we conclude that between 0.5 and 2 channels are open in darkness at each synapse. For comparison, the single photon response in the rod bipolar cell has a peak amplitude of approximately 10 pA (Field and Rieke, 2002), representing the opening of about 30 channels.

Synaptic Gain for Rod Signal and Noise

Previous work argued that the nonlinearity at the rod-to-rod bipolar synapse plays an important functional role by selectively transmitting signals from those rods receiving photons while eliminating noise from the remaining rods (Field and Rieke, 2002). The properties of the noise in the rod bipolar currents provide additional evidence for this conclusion.

Figure 7D shows the power spectra of the rod bipolar dark noise and the noise remaining after dialyzing the cell with GTP-γ-S. The spectrum in GTP-γ-S provides an upper bound to instrumental noise. Noise in the outer segment currents of rod photoreceptors has a similar frequency composition as the rod light response (Baylor et al., 1984). Thus variations in bipolar current that are inherited from the rods should have a similar frequency composition as the bipolar light response, which is dominated by temporal frequencies <5 Hz (smooth curve in Figure 7D). The rod bipolar dark noise, however, exceeds instrumental noise up to temporal frequencies well beyond those dominated by the light response. Thus the temporal properties of the rod bipolar dark noise suggest that it is generated at the synapse or in the bipolar transduction cascade rather than inherited from the rod photocurrent.

Fluctuations in the rod bipolar current at temporal frequencies between 0 and 5 Hz threaten to obscure the rod single photon responses. The standard deviation of the rod bipolar current in this frequency range was 1.2 ± 0.1 pA (mean ± SEM, n = 6). Assuming this noise is generated independently at each of the 20 rod-to-rod bipolar synapses, the contribution of each synapse is less than 0.3 pA (1.2 pA/$\sqrt{20}$ synapses). This means that the signal-to-noise ratio of the ~10 pA single photon response is about 30 at an individual synapse. For comparison, the signal-to-noise ratio for a single photon response in the rod itself is less than 4 (Field and Rieke, 2002).

Discussion

A thresholding nonlinearity in rod-to-rod bipolar signal transfer separates the rod single photon responses from dark noise, providing a near-optimal readout of the rod signals at visual threshold (Field and Rieke, 2002). We have investigated the mechanisms responsible for this nonlinearity. We find that it is produced by saturation within the G protein cascade linking glutamate receptors and ion channels in the rod bipolar dendrites. We also find that <2 transduction channels are open in darkness at each rod-to-rod bipolar synapse. The small number of open channels reduces noise intrinsic to the rod bipolar cell and may account for saturation of the transduction cascade.

Mechanisms Producing Nonlinearity at the Rod-to-Rod Bipolar Synapse

The nonlinearity at the rod-to-rod bipolar synapse causes the bipolar current to be insensitive to small changes in rod voltage, such as those produced by rod dark noise. Two observations indicate that the nonlinearity is generated within the rod bipolar signaling cascade. First, the rod bipolar dark current is near the minimum level set by G protein activity, making the bipolar current more sensitive to decreases in glutamate than increases. Second, manipulating the mGluR6 receptor activity altered the nonlinearity on the rod bipolar light responses. Thus, increasing receptor activity with the agonist APB increased the nonlinearity, while decreasing receptor activity with the antagonist LY341495 decreased the nonlinearity. Because APB and LY341495 unbind from mGluR6 receptors slowly, their ability to alter the nonlinearity indicates that it is generated downstream from the receptors—between the receptors and transduction channels and/or at the channels themselves.

Another constraint on the operation of the rod bipolar transduction cascade comes from the small size of the fluctuations in the bipolar current. From the magnitude of the current fluctuations and an estimate of the single channel current, we conclude that 0.5–2 channels are open in darkness at each rod synaptic contact. For comparison, approximately 30 channels are open at the peak of the single photon response.

Figure 8 illustrates two models for the rod bipolar transduction cascade that can account for the above results. In the first model, the nonlinearity in the rod bipolar responses is produced by saturation at the transduction channels themselves. Changes in rod voltage are linearly related to the signal S^* controlling channel gating. S^* could either be created when the receptors are active and act to close channels (as in Figure 8A)

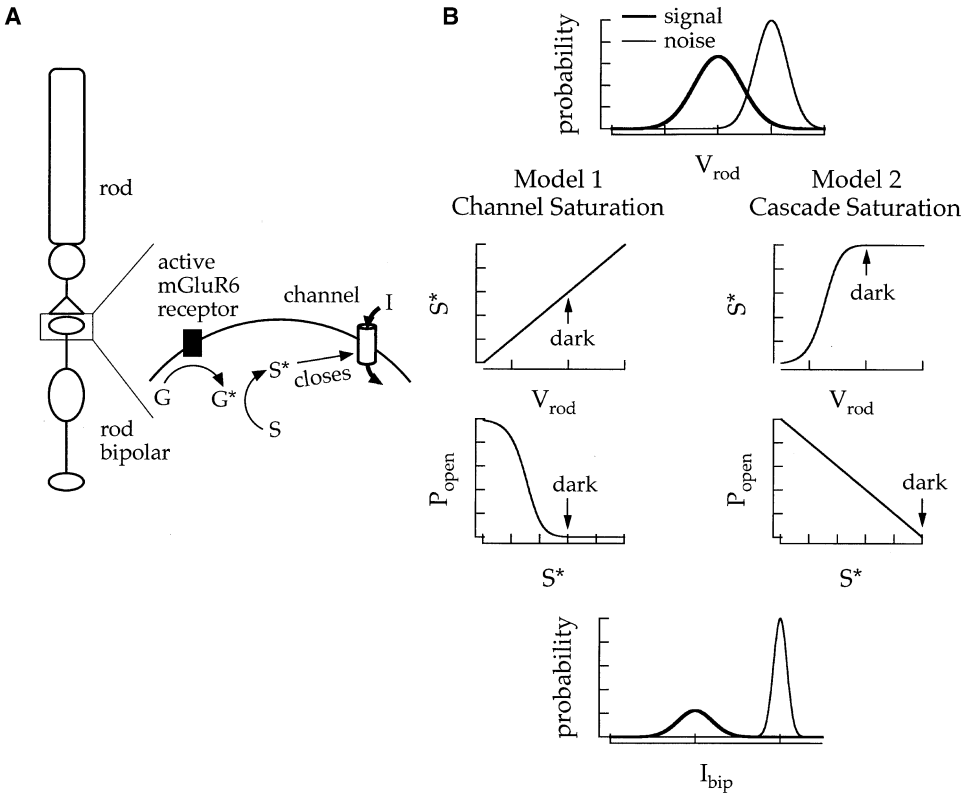

Figure 8. Models for Operation of Rod Bipolar Signal Transduction

(A) A schematic of the transduction cascade is shown. Activation of mGluR6 receptors leads to closure of nonselective cation channels. The steps between the receptors and ion channels are not well understood. We include the one known component—the G protein (*G*)—and a gating signal (*S*). Though this is certainly an oversimplification, it is sufficient to account for our results.

(B) Two models are illustrated that could account for the experimental observation that there is greater separation in single photon responses and dark noise in rod bipolar cells (bottom histogram) compared to the rod photoreceptor (top histogram). In Model 1, signal and noise are separated by rectification at the rod bipolar transduction channels. The gating signal *S** depends linearly on the rod voltage, but the amplitude of the gating signal in darkness produces a near-zero channel open probability P_{open}. In Model 2, signal and noise are separated by saturation of the gating signal. Thus activity of the mGluR6 receptors in darkness produced a near-maximal activity *S**. To explain the small number of channels open in darkness, the dark level of the gating signal must also coincide with a near-zero P_{open}.

or could be destroyed by receptor activity and act to open channels. The mean level of the gating signal causes the open probability P_{open} of the transduction channels to be near zero. More importantly, the open probability is insensitive to small changes in the gating signal about its mean value because of the nonlinear relation between *S** and P_{open}. Thus small changes in rod voltage, even if faithfully replicated in the level of the gating signal, do not produce substantial changes in the number of open channels—i.e., the cascade is partially saturated. Large changes in rod voltage, however, free the channels from saturation.

In the second model, the nonlinearity is produced by saturation of a component of the transduction cascade other than receptors or channels. Saturation produces a nonlinear relation between rod voltage and the gating signal, causing the activity of the gating signal to be less sensitive to small changes in rod voltage than large changes. Saturation could occur at the gating signal itself or at an upstream component of the transduction cascade. In general such saturation does not place a constraint on the number of channels open in the dark—i.e., channels could remain open even when the transduction cascade is saturated. To account for the observation that only a few channels are open in the dark, saturation of the cascade must coincide with a low channel open probability.

The two models illustrated in Figure 8 are not mutually exclusive. However, our results, particularly the large decrease in the nonlinearity of the rod bipolar light responses with LY341495, indicate that mechanisms other than those illustrated in Figure 8 do not contribute substantially.

Constraints on G Protein Cascades Produced by Speed, Amplification, and Noise

Signaling cascades in sensory receptors face numerous constraints, including amplification, speed of signaling, and noise. These are not independent; for example, ongoing activity in a transduction cascade can increase signaling speed at a cost in noise (Rieke and Baylor, 1996; Detwiler et al., 2000). Saturation at or near the output of the cascade can reduce this noise if the signals of interest are highly amplified (Lowe and Gold, 1995).

This strategy appears to be at work in the rod bipolar cell and other sensory neurons.

Amplification permits a single active receptor to activate many copies of downstream components in the transduction cascade. In rod phototransduction a single active rhodopsin leads to activation of 100–1000 transducin and phosphodiesterase molecules (Vuong et al., 1984; Leskov et al., 2000) and the hydrolysis of $\sim 10^6$ cGMP molecules (Yee and Liebman, 1978). Recovery of the response requires not only shutoff of the receptor, but also the return of these downstream components to their original state—e.g., the restoration of cGMP to its dark level. The rate of cGMP turnover in the dark is an important determinant of how quickly the rod current recovers following a flash (Hodgkin and Nunn, 1988). Indeed the speeding of the rod light response in the presence of background light is produced largely by an increased cGMP turnover rate (Gray-Keller and Detwiler, 1996; Nikonov et al., 2000). Turnover of cGMP in darkness, however, generates continuous noise in the rod currents (Baylor et al., 1980; Rieke and Baylor, 1996). It is this noise that threatens to swamp the rod single photon responses.

The rod bipolar transduction cascade, unlike that in the rods, operates in saturation in darkness, making the output of the cascade insensitive to small changes in receptor activity. Saturation of the rod bipolar transduction mechanism serves to separate the rod single photon responses from noise generated in phototransduction and in transmitter release. In addition to reducing rod noise, the rod bipolar transduction process itself generates little noise. Noise due to stochastic channel gating is minimal because few channels are open in the dark. Furthermore, saturation will render the rod bipolar currents insensitive to noise introduced by ongoing activity of components upstream of the site of saturation. Such ongoing activity, by analogy with the phototransduction cascade, is likely required to explain the speed of signaling in the rod bipolar cell.

Other signaling cascades also operate with few open channels and thus may use a similar strategy to maintain speed and low noise. Fly photoreceptors provide one example. Single photons elicit clearly identifiable responses in these cells in large part because few or no channels are open in the dark and the fluctuations in dark current are minimal (Borsellino and Fuortes, 1968). Single photons are thought to activate at most a few of the cell's 30,000 microvilli (Henderson et al., 2000), unlike vertebrate rods where the single photon response encompasses at least 3%–5% of the outer segment length (Baylor et al., 1979). Thus low dark noise is critical for single photon detection because noise generated in the inactive microvilli could easily overwhelm the response. Occasional discrete events about one-fifth the amplitude of the single photon response are the smallest discernable source of dark noise; these events are likely generated by spontaneous G protein activation and are subject to downstream amplification, including positive feedback (Hardie et al., 2002). Smaller fluctuations due to ongoing activity of the transduction machinery downstream of the G protein do not appear to reach the threshold for positive feedback and hence fail to produce a detectable change in current. These cells have remarkably fast light responses ($\sim$30 ms in duration), requiring that the concentrations of second messengers within the transduction cascade are rapidly restored to their dark values following photon absorption.

In each of the above instances, fast signaling requires a high level of ongoing activity in the transduction cascade. This ongoing activity also produces fluctuations that could compromise sensitivity. Nonlinear processing can enhance the signal-to-noise by masking these fluctuations.

Specialization of the Rod-to-Rod Bipolar Synapse

It is widely appreciated that rod photoreceptors are specialized for detecting single photons (Baylor et al., 1979). A growing literature has shown that these specializations are not restricted to the rod outer segment, but are also reflected in the transfer of signals to rod bipolar cells. The depolarized membrane potential rods maintain in darkness positions the rod output synapse in a regime where transmitter release is sensitive to small voltage changes, such as those produced by the absorption of a photon (Attwell et al., 1987; Belgum and Copenhagen, 1988; Witkovsky et al., 1997). Furthermore, a high vesicle release rate guards against random lapses in release that could be mistaken for responses to light (Falk and Fatt, 1972; Rao et al., 1994; Rao-Mirotznik et al., 1998). Finally, linear filtering properties of the rod-to-bipolar synapse are matched to the rod signal and noise (Ashmore and Falk, 1980; Bialek and Owen, 1990; Armstrong-Gold and Rieke, 2003). The present work adds to this picture by demonstrating that saturation within the rod bipolar transduction cascade produces a threshold-like nonlinearity that improves the fidelity of rod signals by causing the rod bipolar cells to selectively retain responses from rods absorbing photons while rejecting noise from the remaining rods.

Experimental Procedures

Slicing and Recording Procedures

Mice (C57BL/6) were dark adapted overnight and sacrificed according to protocols approved by the Administrative Panel on Laboratory Animal Care at the University of Washington. Retinas were isolated and stored in a light-tight container at 37°C in Ames' solution (Sigma, St. Louis, MO) equilibrated with 5% CO_2/95% O_2. Slices were prepared as described previously (Field and Rieke, 2002; Armstrong-Gold and Rieke, 2003) and superfused with Ames' solution heated to 35°C–37°C. The dissection, slicing, and recording were all carried out under infrared light (>950 nm) to maintain the dark-adapted state of the retina. Unless specified, all chemicals were purchased from Sigma.

Light responses of rod bipolar cells were recorded with whole-cell or perforated-patch recordings. Pipettes for whole-cell recordings were filled with 125 mM K-Aspartate, 10 mM KCl, 10 mM HEPES, 5 mM NMG-HEDTA, 0.5 mM $CaCl_2$, 1 mM ATP-Mg, 0.2 mM GTP-Mg; pH was adjusted to 7.2 with NMG-OH. For perforated-patch recordings, 0.5 mg/ml amphotericin B (solubilized formulation) was added to this solution. Light responses measured under whole-cell conditions typically lasted 2–4 min. Light responses measured under perforated-patch conditions typically lasted 10–20 min. Thus, perforated-patch recordings were used for all experiments requiring solution changes and/or long-term stability.

Light stimuli were delivered from an LED with a peak output wavelength of 470 nm. Calibrated photon densities (in photons/μm^2) at the preparation were converted to photoisomerizations per rod (Rh*/rod), assuming a rod collecting area of 0.5 μm^2 (Field and Rieke, 2002).

Stimulus-Response Relations

The amplitude of the rod bipolar response at a given flash strength was estimated by fitting the entire response with a scaled template generated from the average response across flash strengths. The amplitudes were taken as the maximal inward deflection of the template compared to the baseline prior to the flash and were normalized by the amplitude of the response to the brightest flash. Plots of response amplitudes against flash strength were fit with the Hill Equation (Equation 1). Similar fits were obtained when the amplitudes were instead estimated from the maximum inward current deflection; the template-scaling procedure, however, provided more consistent estimates of the amplitudes of responses to the noisier dim flashes.

High-Affinity Pharmacological Agents

The experiments of Figures 4 and 5 rely on APB (2-amino-4-phosphonobutyrate) and LY341495 (2S-2-amino-2-(1S,2S-2-carboxycyclopropyl-1-yl)-3-(xanth-9-yl) propanoic acid; Tocris Cookson, Ellisville, MO) unbinding from mGluR6 receptors slowly compared to the 200–400 ms duration of the light response. Although the unbinding rate constants have not been measured directly, the evidence described below indicates that they are indeed slow.

LY341495 binds to expressed mGluR6 receptors with an affinity near 30 nM (Wright et al., 2000). The unbinding rate constant is given by $k_{off} = k_{on} K$, where K is the affinity and k_{on} is the binding rate constant. The binding of glutamate or related compounds to a variety of glutamate receptors has been studied (Clements and Westbrook, 1991; Bowie et al., 1998; Clements et al., 1998), with rate constants that vary from 1 to 50 $\mu M^{-1}\ s^{-1}$. These values predict unbinding rate constants for LY341495 between 0.03 s^{-1} and 1.5 s^{-1}. Binding rate constants for LY341495 to group II metabotropic glutamate receptors fall at the lower end of this range (Johnson et al., 1999). Thus even with the fastest observed binding rate constants, LY341495 should stay bound to the receptors for a period at least twice that of the light response.

Direct evidence that the unbinding rate was slow under the conditions of our experiments came from monitoring the recovery of the rod bipolar responses following perfusion with APB or LY341495. We used the much lower affinity antagonist CPPG [(R,S)-a-cyclopropyl-4-phosphonophenylglycine, Tocris Cookson, Ellisville, MO] to obtain an upper bound on the time required to clear compounds from the synaptic cleft. The increase in inward current produced by CPPG recovered with a time constant of 10 $\pm$ 2 s (mean $\pm$ SEM, n = 7; data not shown) after removing CPPG from the superfusion solution. The increase in inward current produced by LY341495 and the decrease in saturating light response produced by APB recovered with time constants of 25 $\pm$ 4 s (mean $\pm$ SEM, n = 9; data not shown) and 31 $\pm$ 8 s (n = 5; e.g., Figure 2A). The slower recovery following exposure to LY341495 or APB compared to CPPG indicates that these drugs unbind slowly from mGluR6 receptors.

CPPG also provided another test of how decreasing receptor activity affected the nonlinearity in the rod bipolar light responses. The average Hill exponent in the presence of 80 μM CPPG was 0.84 $\pm$ 0.05 times that in Ames' alone (mean $\pm$ SEM, n = 7; data not shown). This is consistent with a relief of postsynaptic saturation, but because CPPG is not likely a strong antagonist it does not distinguish receptor and cascade saturation.

The APB and LY341495 experiments additionally rely on the assumption that these agents act primarily on postsynaptic receptors. Several studies have reported metabotropic glutamate receptors on photoreceptors (Koulen et al., 1999; Higgs and Lukasiewicz, 2002). Activation of these presynaptic receptors lowers the rate of transmitter release from photoreceptors. Thus presynaptic actions of APB and LY341495 would have effects on the rod bipolar current opposite those observed. This indicates that the primary actions of APB and LY341495 were indeed postsynaptic rather than presynaptic.

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