Molecular Mechanism of Spontaneous Pigment Activation in Retinal Cones

Alapakkam P. Sampath and Denis A. Baylor
Department of Neurobiology, Stanford University School of Medicine, Stanford, California 94305 USA

ABSTRACT Spontaneous current and voltage fluctuations (dark noise) in the photoreceptor cells of the retina limit the ability of the visual system to detect dim light. We recorded the dark current noise of individual salamander L cones. Previous work showed that the dark noise in these cells arises from thermal activation of the visual pigment. From the temperature dependence of the rate of occurrence of elementary noise events, we found an Arrhenius activation energy E_a of 25 ± 7 kcal/mol (mean ± SD). This E_a is similar to that reported for the thermal isomerization of 11-*cis* retinal in solution, suggesting that the cone pigment noise results from isomerization of the retinal chromophore. E_a for the cone noise is similar to that previously reported for the "photon-like" noise of rods, but the preexponential factor is five orders of magnitude higher. To test the hypothesis that thermal isomerization can only occur in molecules whose Schiff base linkage is unprotonated, we changed the pH of the solution bathing the cone outer segment. This had little effect on the rate of occurrence of elementary noise events. The rate was also unchanged when the cone was exposed to Ringer solution made up from heavy water, whose solvent isotope effect should reduce the probability, that the Schiff base nitrogen is naked.

INTRODUCTION

In the vertebrate visual system, random noise events that could be mistaken for light-evoked signals are rare. What noise there is seems to arise primarily within the retinal photoreceptor cells, the rods, and cones. The low dark noise of rods allows the scotopic system to reliably detect a flash eliciting less than 10 effectively absorbed photons (Hecht et al., 1942). Electrical recordings from rods revealed two components of the noise: a continuously present low amplitude component and a discrete component consisting of randomly occurring events resembling the response to a single absorbed photon (Baylor et al., 1980; Schwartz, 1977). The discrete events result from thermal activation of rhodopsin (Baylor et al., 1980), and the rate at which they occur sets a lower limit on visual sensitivity (Aho et al., 1988). The continuous component of rod noise results from the spontaneous activation of cGMP phosphodiesterase molecules (Rieke and Baylor, 1996).

Psychophysical experiments suggest that cones are noisier than rods (Barlow, 1958), and indeed the membrane current of macaque L and M cones had a fivefold greater dark noise variance than that of macaque rods (Baylor et al., 1984; Schnapf et al., 1990). The cone dark noise had the same spectral composition as noise generated by dim background light and was consistent with thermal activation of cone pigment molecules at a rate of roughly 10^3 s^{-1}.

Recently Rieke and Baylor (2000) reported that the dark noise of L and S cones of the salamander retina arises from different sources. Most of the current noise in L cones was apparently generated by thermal activation of the visual pigment. Thus, the noise disappeared after the visual pigment was bleached even though the dark current had recovered to near the prebleach level. Furthermore, the power spectrum of the dark noise had the same shape as the spectrum of the dim flash response and the spectrum of the noise generated by dim steady light, suggesting that the noise consisted of a superposition of random events with the average shape of the single photon response, occurring at a rate of 10^3 s^{-1}. Finally, internally dialyzing an L cone outer segment in darkness with a solution lacking GTP (guanosine triphosphate) caused an increase in the current dependent on cGMP (3′,5′cyclic guanosine monophosphate). This GTP dependence indicated the presence of cGMP phosphodiesterase activity due to spontaneous activation of the visual pigment. In contrast, the dark current noise of S cones appeared to arise from molecular species downstream to the pigment, the pigment itself being stable.

The aim of this work is to examine the molecular mechanism of thermal activation of the L-cone pigment. Initially we confirmed the observation (Rieke and Baylor, 2000) that the power spectrum of the dark noise of L cones resembles that of the dim flash response, as expected if the noise is produced by spontaneous activation of visual pigment molecules. The amplitude scaling of the two spectra enabled us to estimate the rate of occurrence of noise events at different temperatures and thus to characterize the energetics of the thermal activation process. Comparison of the derived thermodynamic parameters for the L-cone pigment with those obtained previously for rhodopsin (Baylor et al., 1980) provided insight into the basis for the large difference in the rates of thermal activation for the two types of pigment. Finally, we tested the hypothesis that pigment molecules can only undergo thermal activation when the Schiff base linkage between the chromophore and protein is unproto-

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Address reprint requests to D. A. Baylor, M.D., Department of Neurobiology, Stanford University School of Medicine, Fairchild, D-237, 299 Campus Drive West, Stanford, CA 94305. Tel.: 650-723-6510; Fax: 650-725-3958; E-mail: dbaylor@stanford.edu.

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nated (Barlow et al., 1993; see also Birge and Barlow, 1995). Given that the pKa of the Schiff base is 16 or greater for vertebrate rhodopsin (Steinberg et al., 1993) and much lower for cone pigments (Liang et al., 1994), this idea provided a possible explanation for the large difference in the rate constants for thermal isomerization in rods and L cones. We tested the hypothesis by observing the noise while changing the pH of the solution bathing the cone outer segment or substituting heavy water Ringer solution for normal Ringer.

MATERIALS AND METHODS

Preparation and solutions

Larval tiger salamanders (*Ambystoma tigrinum*) were obtained from Charles Sullivan Amphibians (Nashville, TN) and maintained at 9°C to 14°C on a 12/12 h light/dark cycle. Before an experiment the salamander was dark-adapted for at least 3 h but typically overnight. In accordance with protocols approved by the Animal Research Committee of Stanford University (Protocol #3596), the animal was rapidly decapitated in darkness, and the brain and spinal cord were pithed. Under infrared illumination the eyes were removed and hemisected, and each retina was peeled from the retinal pigment epithelium with the eyecup immersed in Ringer solution. The isolated retinae were then stored in darkness at 4°C. Before making recordings, a small piece of retina was mechanically dissociated with forceps or needles in a droplet of Ringer solution on a silane-coated glass slide, and the droplet was subsequently placed in the recording chamber on the stage of an inverted microscope. After allowing the cells to settle to the chamber floor for 10 to 15 min, the chamber was continuously perfused with Ringer solution at a rate of 0.5 mL/min. In some experiments the cones were incubated during this settling period in 12.5 μM BAPTA-AM (Molecular Probes, Eugene, OR) to increase the amplitude and duration of the elementary events underlying the pigment noise (Matthews et al., 1985). The experiments were performed exclusively on L cones, which were identified from their characteristic relative sensitivities to flashes of 600- and 460-nm light (Makino and Dodd, 1996).

The standard pH 7.6 Ringer solution had the following composition: 110 mM NaCl, 2.5 mM KCl, 1.0 mM $CaCl_2$, 1.6 mM $MgCl_2$, 10 mM HEPES, 0.02 mM EDTA, 10 mM glucose; the solution also contained 0.1 mg/mL bovine serum albumin and basal medium Eagle vitamins and amino acids. In pH 8.8 Ringer solution, the pH buffer HEPES was replaced with TAPS, which has a higher pKa. Heavy water Ringer solution was adjusted to an apparent pH of 7.2 to compensate for the voltage offset in the pH electrode (Root and MacKinnon, 1994) giving an effective pH identical to that in pH 7.6 Ringer solution. Unless otherwise stated, all remaining reagents were purchased from Sigma (St. Louis, MO).

Electrical recording and light stimuli

The membrane current from single cones was recorded with the suction electrode technique described by Baylor et al. (1979). The recorded currents were amplified with an Axopatch-1A amplifier (Axon Instruments, Foster City, CA), digitized with a 16-bit A/D converter (model ITC-16, Instrutech Corp., Port Washington, NY), and stored on a Macintosh G3 computer.

Unpolarized light stimuli were delivered from a double beam optical bench (Baylor and Hodgkin, 1973). Interference filters (Oriel Corporation, Stratford, CT) with nominal half bandwidths of 10 nm were used to control the wavelength, and the intensity was adjusted with a series of calibrated neutral density filters (Bausche and Lomb, Inconel).

Changes in extracellular solution bathing the outer segment

In some experiments we observed the dark noise of a cone while changing the proton concentration in the solution bathing the outer segment; the aim was to test whether the protonation state of the Schiff base linkage between the 11-*cis* retinal chromophore and the opsin protein influences the rate of occurrence of thermal noise events. Changes in external pH will effectively change the protonation state of the pigment's Schiff base linkage only if 1) protons in the extracellular solution have diffusional access to the chromophore-binding pocket and 2) access of protons from the intracellular solution to the pocket is negligible, so that the proton concentration in the pocket is not controlled by an unchanging internal proton concentration. The following considerations suggest that these conditions hold. The binding pocket is accessible to ions and small molecules in the external solution, as indicated by the fact that the chromophore in cone pigments is attacked by hydroxylamine (Wald et al., 1954; Fasick et al., 1999; Liang et al., 1994; Ma et al., 2001), as well as the fact that external anion substitutions shift the spectral absorption of L cones (Kleinschmidt and Harosi, 1992). Although the accessibility of the pocket to the intracellular solution has not been measured, it is almost certainly small. For instance, if the accessibility from the inside were comparable with that from the outside, each of the 10^8 pigment molecules in the outer segment would function as a leak conductance in parallel with the light-regulated channels and would attenuate the light-evoked signals. In striped bass cones Miller and Korenbrot (1993) were unable to measure any significant leakage conductance in the outer segment. Furthermore, at early times the flash response of cones rises nearly as fast as that of rods (Pugh and Lamb, 1993), in which the leakage conductance in parallel with the light-sensitive conductance is effectively zero (Baylor and Nunn, 1986).

When the solution bathing the outer segment was to be changed, the light-sensitive current was usually measured with the cone inner segment inside the suction electrode and the outer segment outside; the outer segment was positioned in a stream of flowing solution whose composition could be changed by switching to another stream with a piezoelectric translator (Burleigh Instruments, Fishers, NY). In other experiments the solution around the outer segment was altered by changing the bath solution. Current noise was typically measured 2 min after the extracellular pH was changed. When the light-sensitive current was recorded with the outer segment in the suction electrode, allowing a larger fraction of the current to be recorded, the solution surrounding the outer segment was changed with a back-to-back syringe arrangement (Baylor and Nunn, 1986). Two fused silica tubes (ID = 75 μm, OD = 150 μm; Polymicro Technologies, Phoenix, AZ) were placed very close to the tip of the suction electrode, and the desired solution was pushed out of one tube while simultaneously retracting the old solution with the other. Solutions changes made this way usually lasted more than 5 min as indicated by the shape of the flash response of the cone.

Temperature control

In temperature change experiments the Ringer solution traversed several coils of fine polyethylene tubing on a Peltier device before entering the recording chamber. The temperature in the chamber was measured with a miniature thermocouple (Model TMTSS020U6; Omega Engineering, Stamford, CT) placed within 2 mm of the tip of the suction electrode. Measured temperatures at various positions within the chamber varied by less than 1°C at the flow rates used (1–2 mL/min).

Noise acquisition and analysis

One-sided power spectral densities were calculated using the Fast Fourier Transform of the Igor program (Wavemetrics, Lake Oswego, OR) from data collected using an acquisition program written by Dr. Fred Rieke

(University of Washington). Power spectra were typically determined from 20 to 50 sweeps of the dark membrane current, each 4.25 s in length. The current was digitized at 200 Hz after low-pass filtering at 30 Hz with an eight-pole Bessel filter. In all spectra a horizontal line has been drawn to indicate the instrumental Johnson noise level, which was calculated from the measured electrode resistance (ρ) and absolute temperature (T) using the Nyquist Equation:

$$S(f) = \frac{4kT}{\rho},$$

in which $S(f)$ is the power spectral density as a function of frequency and k is Boltzman's constant (1.381×10^{-23} J/K). After subtracting the Johnson noise, the cellular dark noise variance was obtained from the integral of the power spectrum over the bandwidth 0 to 10 Hz.

The small size of the elementary noise event in L cones precluded simple counting of events as in rods (Baylor et al., 1980). Therefore, absolute rates of occurrence of thermal events in normal Ringer were estimated from the scaling between the power spectrum of the dark noise and the power spectrum of the elementary noise event. The elementary event, $r(t)$, was estimated from the averaged linear dim flash response, $f(t)$, using the relation:

$$r(t) = \frac{f(t)}{i_F A_C},$$

in which i_F is the strength of the flash, and A_c is the effective collecting area determined from the size of the cone outer segment, assuming a pigment concentration of 3.2 mM (Harosi, 1982). The size of the outer segment and the pigment concentration were also used to calculate N, the number of pigment molecules in the outer segment. The power spectrum of the elementary event was then calculated and scaled to match the power spectrum of the dark noise by a least squares fitting algorithm. This fitting used points in the frequency range 0 to $1/\tau_{int}$, where τ_{int} is the integration time (Baylor and Hodgkin, 1973) of the dim flash response. Restricting the fit to these points minimized the contribution of high frequency noise arising from channel flicker or spontaneous phosphodiesterase or guanylyl cyclase activity. The scaling factor for the spectrum of the elementary event, divided by the 4.25-s sampling interval gave the absolute event rate in that cone.

The event rate in a solution of new pH or in heavy water Ringer solution was estimated by applying Campbell's Theorem (Rice, 1944):

$$\sigma^2 = \upsilon \int r^2(t)dt.$$

In this expression, $r(t)$ is the elementary event, ν its frequency of occurrence, and σ^2 the noise variance. We assume that the change in external proton concentration does not alter the quantum efficiency of excitation for the cone pigment. The event rate ν_2 in a new condition can then be estimated from that in standard Ringer ν_1, the variances, and derived elementary noise events by:

$$\upsilon_2 = \upsilon_1 \frac{\sigma_2^2 \int r_1^2(t)dt}{\sigma_1^2 \int r_2^2(t)dt}.$$

Reductions in cellular dark noise became more difficult to characterize as the noise approached the Johnson level.

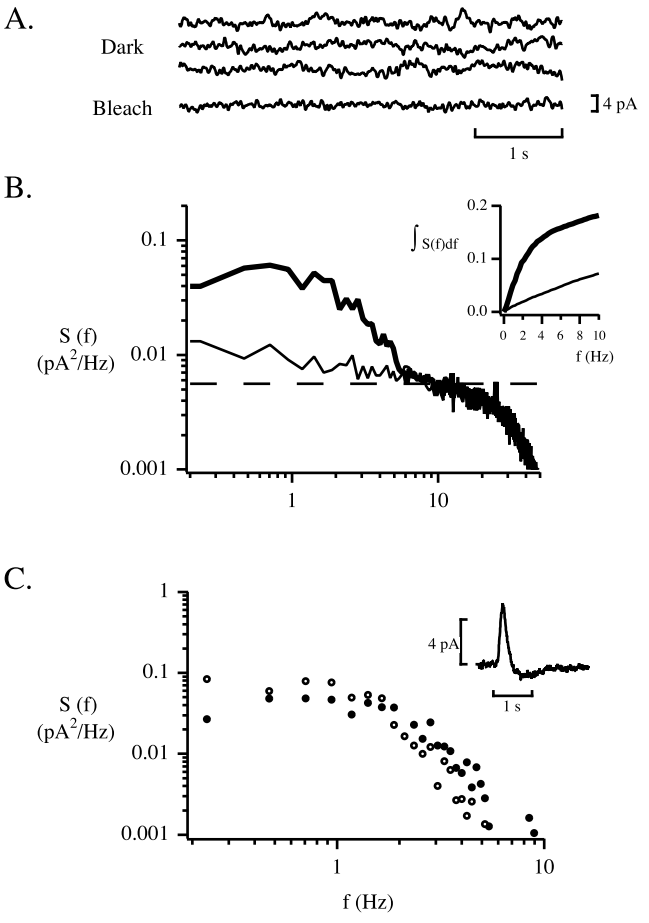

FIGURE 1 Dark noise from the visual pigment of an L cone. (*A*) Membrane current in darkness and after bleaching >99% of the visual pigment, having allowed the circulating current to recover. Bleaching reduced the fluctuations in the membrane current observed in darkness. (*B*) Power spectrum of the dark noise (*thick line*) and the noise after bleaching (*thin line*) at 23°C. The dashed horizontal line is the Johnson noise level, calculated from the Nyquist equation (see Materials and Methods). At frequencies higher than 10 Hz the spectrum falls below the Johnson level because of the low-pass filtering (see Materials and Methods). The inset is the integral of the power spectrum to 10 Hz, as used to quantify the total variance in darkness and after bleaching. (*C*) Difference spectrum (*solid circle*) of the dark noise determined as the noise in darkness minus the noise after bleaching. Superimposed on the difference spectrum is the power spectrum of the elementary response (*open circle*; determined by dividing the dim flash response (*inset*) by the average number of photoisomerizations per flash, which in this cell was 370). The power spectrum of the elementary response was multiplied by a scaling factor of 9490 to best fit the dark noise spectrum. The scaling factor divided by the 4.25-s sampling interval gives a rate of occurrence of thermal events of 2200 s^{-1} for the 3.4×10^8 pigment molecules in this cone outer segment, corresponding to a molecular rate constant for thermal activation, a, of 6.6×10^{-6} s^{-1}.

RESULTS

Origin of dark noise in L cones

We studied the dark noise of salamander L cones by recording the outer segment membrane current with the suction electrode method. Fig. 1 *A* shows recordings made from an L cone in darkness and after bleaching >99% of the visual pigment; the postbleach recording was obtained after the circulating current had recovered to near the amplitude in darkness. The current fluctuations in darkness were re-

duced after the pigment was bleached. The noise is quantified in the power spectra shown in Fig. 1 *B*, which plot the pre- and postbleach spectra, as well as the Johnson noise level for the measured electrode resistance. The difference spectrum (dark-bleach), which isolates the component of noise dependent on the presence of the unbleached pigment, is shown in Fig. 1 *C*. Superimposed on the difference spectrum is the scaled power spectrum of the single photon response of the same cone (see Fig. 1, legend), determined by dividing the dim flash response (Fig. 1 *C*, inset) by the average number of photoisomerizations per flash. The two spectra have similar shapes, consistent with the notion that the dark noise consists of a superposition of randomly occurring events with the same average shape as the single photon response. In this cone the absolute rate of occurrence of thermal events, ν, as estimated from the scaling factor between the two spectra was 2200 s^{-1}. The molecular rate constant, a, was then calculated as:

$$a = \frac{\nu}{N},$$

in which N is the number of pigment molecules in the outer segment. In this cell N was estimated as 3.4×10^8 (see Materials and Methods), giving $a = 6.6 \times 10^{-6}$ s^{-1}. The average value of a at room temperature for 12 L cones was 5.7×10^{-6} s^{-1}, as shown in Table 1. The corresponding value for rhodopsin at comparable temperature was 1×10^{-11} s^{-1} (Baylor et al., 1980), six orders of magnitude smaller. These results are consistent with the idea that the dark noise in L cones is produced by thermal activation of the cone pigment (Rieke and Baylor, 2000), which is much less stable than rhodopsin.

The L-cone pigment's large value for a could result from either a lower activation energy or from more frequent attempts to surmount the energy barrier. To distinguish between these possibilities, we examined the temperature dependence of the rate of occurrence of thermal events. Fig. 2 illustrates the dark noise and dim flash response of an L cone at several temperatures. Raising the temperature increased the amplitude of the light-suppressible current (Fig. 2, legend) but reduced the size of the dim flash response (Fig. 2, inset). A similar reduction in sensitivity at elevated temperatures was also seen in rod photoreceptors (Lamb, 1984). We assume that the amplitude reduction results from the change in the size of the single photon response with the quantum efficiency of response production remaining constant. Evidence that the latter condition should hold is provided by the fact that the quantum efficiency of bleaching of visual pigment is temperature independent (Dartnall et al., 1938). The rate of occurrence of thermal activation events at each temperature was estimated as before, by scaling the power spectrum of the dim flash response to best fit the power spectrum of the dark noise. Raising the temperature from 18°C to 29°C increased the thermal activation rate for the L cone in Fig. 2 by greater than fourfold. Results from 12 cells are presented in Table 1.

TABLE 1 Temperature dependence of dark noise in L cones

Cell	T(°C)	Event rate (s^{-1})	E_a (kcal/mol)	Pigment molecules	a (s^{-1})
1	15.2	200	31.3	1.5E+08	3.9E−06
	19.5	580			
	26.3	1600			
2	16.6	340	16.6	1.6E+08	2.6E−06
	20.9	400			
	27.4	940			
3	17.7	530	24.8	2.1E+08	5.1E−06
	22.3	1100			
	28.7	2500			
4	17.4	850	23.1	2.1E+08	4.0E−06
	21.8	1600			
5	16.7	590	15.9	2.3E+08	4.4E−06
	21.1	980			
	27.6	1600			
6	19.5	2000	20.2	2.5E+08	7.8E−06
	23.7	3500			
7	15.5	200	23.5	1.4E+08	3.4E−06
	20.0	490			
	27.2	1000			
8	16.5	770	33.4	2.1E+08	9.2E−06
	21.1	1900			
9	17.5	310	29.5	1.5E+08	5.7E−06
	21.9	830			
	26.7	1500			
10	17.5	1800	32.9	4.0E+08	1.0E−05
	22.1	4100			
	27.3	12000			
11	17.6	540	35.8	2.3E+08	5.0E−06
	22.1	1200			
	27.2	3900			
12	17.8	2500	17.1	3.3E+08	7.7E−06
	22.2	3900			
Mean			25.3	2.2E+08	5.7E−06
SD			7.1	7.8E+07	2.4E−06
SE			2.0	2.2E+07	7.0E−07

According to the Arrhenius equation the temperature dependence of the reaction rate is given by

$$a = Ae^{-E_a/RT}$$

in which a is the molecular rate constant, A the Arrhenius preexponential factor, R the gas constant (1.98×10^{-3} kcal/mol K), and T the absolute temperature. The activation energy (E_a) for the thermal activation process was estimated by plotting the natural log of the event rate versus $(T)^{-1}$; the slope of this plot is $-E_a/R$. For the cone of Fig. 3, E_a was 24.8 kcal/mol, and from 12 cones E_a was 25.3 ± 7.1 kcal/mol (mean ± SD; Table 1). This activation energy corresponds to a Q_{10} of 4.7 for the rate of occurrence of thermal activation events. Within experimental error, this value for E_a is the same as that derived for thermal activation of rhodopsin (22 kcal/mol; Baylor et al., 1980), suggesting a common mechanism of production of thermal noise events. The 10^6-fold difference in the molecular rate

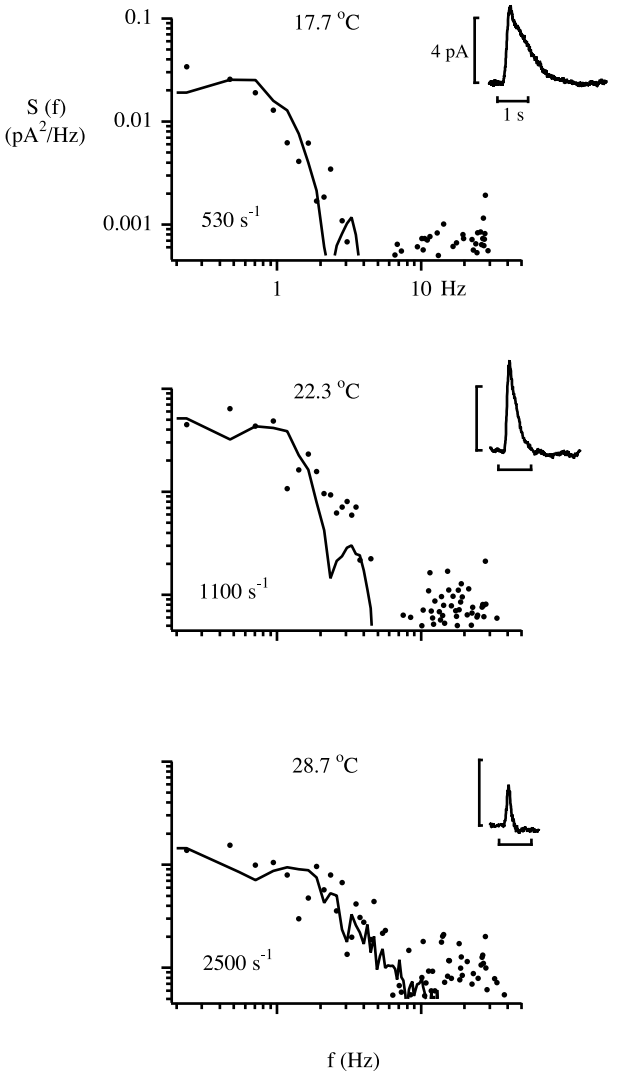

FIGURE 2 Temperature dependence of pigment noise in an L cone. Power spectra of membrane current fluctuations in darkness at different temperatures. Inset in each panel is the average response to a flash delivering 270 photons μm^{-2}. The dark current was 24.2 pA at 17.7°C, 36.3 pA at 22.3°C, and 35.6 pA at 28.7°C. The difference spectrum of the dark noise at each temperature, obtained as in Fig. 1 *C*, is shown as solid circles. Superimposed (*black line*) is the power spectrum of the average elementary event, obtained from the dim flash response divided by the number of photoisomerizations. The power spectrum of the average elementary event has been multiplied by a scaling factor to best fit the difference spectrum in the bandwidth 0 to $1/\tau_{int}$ of the dim flash response. The scaling factor divided by the 4.25-s sampling interval gives the estimated rates of thermal activation for the pigment molecules in the outer segment of this cone. These rates are shown at the lower left of each spectrum.

constants for thermal activation of the L-cone pigment and rhodopsin apparently arises from a higher preexponential factor, A, for the L-cone pigment. For the L-cone pigment the value of A, calculated from the molecular rate constant at room temperature (21°C) and the average value of E_a, was 4.5×10^{13} s^{-1}, compared with a value for rhodopsin of approximately 6×10^{6} s^{-1} (Baylor et al., 1980).

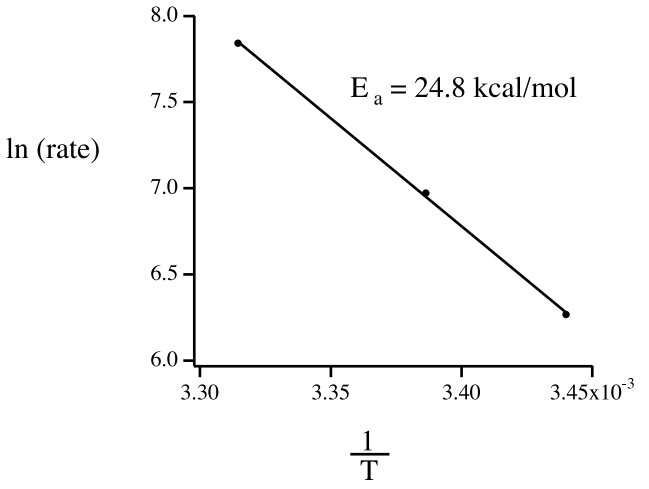

FIGURE 3 Natural log of the event rate as a function of the reciprocal absolute temperature. Results from the L cone of Fig. 2. From the slope of the relation, E_a was estimated as 24.8 kcal/mol. Results from 12 cells are summarized in Table 1.

The Arrhenius activation energy and the molecular rate constant for thermal activation were used to calculate the Gibbs free energy of activation, $\Delta G^{\ddagger}$, enthalpy of activation, $\Delta H^{\ddagger}$, and entropy of activation, $\Delta S^{\ddagger}$, using the relations (Moore, 1964; Baylor et al., 1980),

$$\Delta G^{\ddagger} = -RT \ln \frac{ha}{kT},$$

$$\Delta H^{\ddagger} = E_a - RT,$$

$$\Delta S^{\ddagger} = \frac{\Delta H^{\ddagger} - \Delta G^{\ddagger}}{T},$$

in which a is the molecular rate constant for thermal activation, h is Planck's constant, k is Boltzmann's constant, and T is the absolute temperature. The average results from 12 L cones (mean ± SD) were: $\Delta G^{\ddagger} = 24.2 \pm 0.3$ kcal/mol, $\Delta H^{\ddagger} = 24.8 \pm 7.1$ kcal/mol, and $\Delta S^{\ddagger} = 0.002 \pm 0.024$ kcal/mol K. The sizable variations in the value of $\Delta S^{\ddagger}$ arise from variations in the measured value of E_a, which is in the argument of an exponential.

Effects of pH changes on dark noise

Baylor et al. (1980) reported that the low thermal activation rate of rhodopsin can be explained by two factors. First, the pigment can only undergo thermal activation if its energy exceeds an E_a of 22 kcal/mol. Second, the Arrhenius preexponential factor is more than five orders of magnitude lower than the theoretical limit of kT/h, suggesting that only a small fraction of the rhodopsin molecules can undergo thermal activation at any instant. Barlow et al. (1993) provided a possible explanation for the small value for the

preexponential factor. Supported by evidence obtained in Limulus photoreceptors, they proposed that thermal activation could occur only in the small fraction of pigment molecules whose Schiff base linkage is unprotonated. The general applicability of this idea is not known.

We tested Barlow et al.'s (1993) hypothesis in the L cones. Whereas it may be argued that the large preexponential factor, *A*, for the L cones itself precludes any special structural requirement for thermal activation, it should be pointed out that the derived value of *A* is somewhat uncertain, and the true value may be much lower (see Table 1). The first test of the hypothesis involved changing the extracellular pH around the outer segment of a cone while observing the effect on the pigment noise. The usefulness of this manipulation depends on the assumption that the Schiff base linkage is accessible to the extracellular medium and inaccessible to the intracellular medium (see Materials and Methods). If this assumption holds, changing the external pH from 7.6 to 8.8 should increase the number of molecules with an unprotonated Schiff base by a factor of 16, because the pKa of the Schiff base is presumably much larger than 8 (Liang et al., 1994). On the model of Barlow et al. (1993), this should increase the rate of occurrence of thermal events by a factor of 16. Fig. 4 presents results from such an experiment. The membrane current recordings (Fig. 4 *A*) show that increasing the external pH caused little change in the dark noise. As usual, the noise largely disappeared after the pigment was bleached and the circulating current recovered. The power spectra of the dark noise (Fig. 4 *B*) reveal an increase in low frequency noise at pH 8.8. The noise variance in the bandwidth 0 to 10 Hz (Fig. 4 *B*, inset) was approximately twofold larger than that at pH 7.6. This elevation in low frequency noise was quantitatively accounted for by the increase in the integral of the square of the dim flash response (Fig. 4 *C*, inset; see Materials and Methods), which was larger and longer lasting than that determined at pH 7.6. Therefore the noise increase at pH 8.8 was entirely explained by an increase in amplitude and duration of the elementary noise event. There was no evidence for a change in the event rate. Similar results were obtained in seven L cones. There was a 2.0 ± 0.5-fold increase in dark noise variance upon changing from pH 7.6 to 8.8 (mean ± SE). The expected change in dark noise variance, calculated from the change in the elementary response, was 2.2 ± 0.8-fold.

Fig. 5 shows results from an experiment in which we lowered the extracellular pH from 7.6 to 7.0, which should reduce the number of unprotonated Schiff base linkages by a factor of 4. Power spectra of the dark noise at pH 7.6 and 7.0 are shown in Fig. 5 *B*. After subtracting the noise variance after pigment bleaching, the dark noise variance at pH 7.0 was 1.7-fold less than that at pH 7.6 (Fig. 5 *C*). From the integral of the squares of the elementary response, computed from the dim flash response (Fig. 5 *A*, inset), a 4.6-fold reduction in noise variance would be expected if the rate of occurrence of noise events had remained constant. The smaller reduction in the observed variance may reflect a 2.7-fold increase in the thermal event rate at

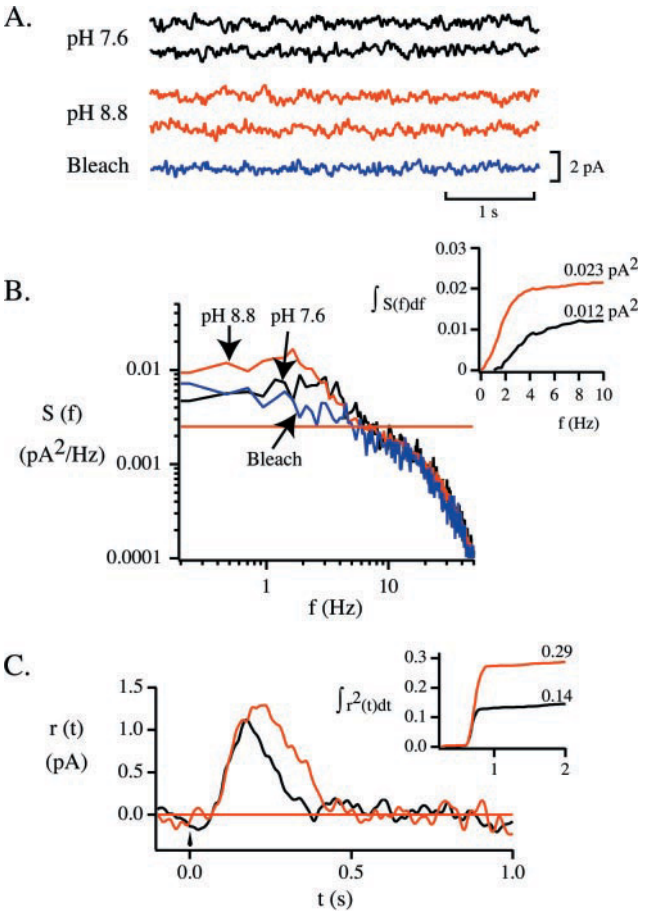

FIGURE 4 Pigment noise at pH 7.6 and 8.8. (*A*) Membrane current in darkness at pH 7.6 (*black*), 8.8 (*red*), and after a >99% bleach and recovery of the current to the steady level (*blue*). This cell was preloaded with BAPTA-AM (see Materials and Methods). (*B*) Power spectra of the dark noise. (*Inset*) Integrals of the power spectra with the noise variance after bleaching subtracted from the noise variance in each solution. (*C*) Responses to a dim flash of 160 photons μm^{-2} at pH 7.6 (*black*) and 8.8 (*red*). The dark current at pH 7.6 was 11.7 pA, and at pH 8.8 it was 8.5 pA. (*Inset*) Integrals of the squares of the dim flash responses, as used to compute the expected change in the noise variance resulting from a change in the elementary event using Campbell's Theorem (see Materials and Methods).

pH 7.0, or inaccuracy in determining the variance when the noise became small. In six cells tested, however, the observed variance was fourfold higher on average than that predicted from the change in the elementary event, suggesting that lowered pH may indeed have increased the thermal event rate. This change is in the direction opposite to that predicted by the Barlow et al. (1993) model. Perhaps protonation of a residue with a pKa less than 7 may increase the rate of occurrence of thermal events. Results from six cells gave an average reduction of 1.9 ± 0.3-fold (mean ± SE) in noise variance upon changing from pH 7.6 to 7.0. Based on the change in the elementary response, a 7.5 ± 2.7-fold reduction in dark variance was predicted.

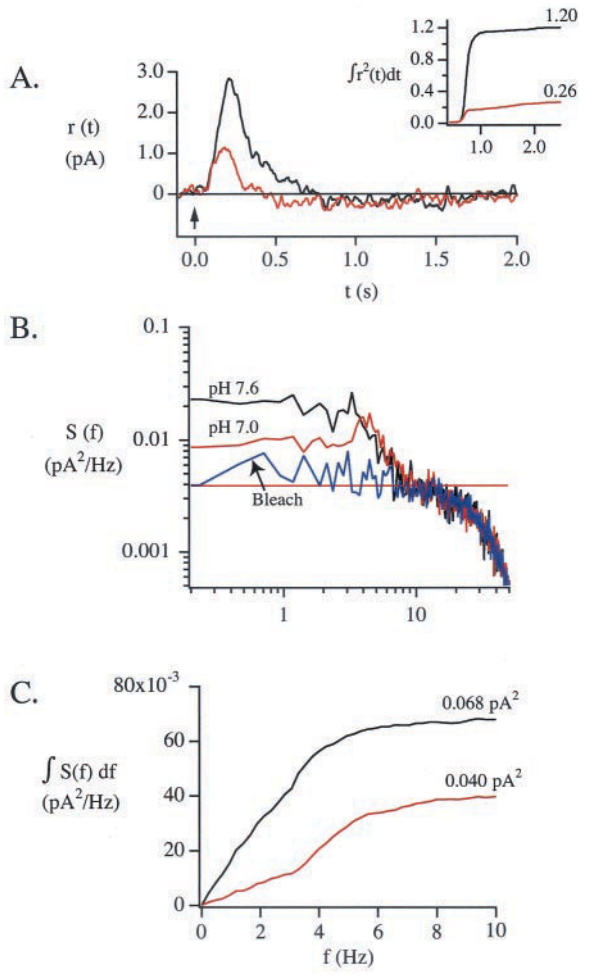

FIGURE 5 Pigment noise at pH 7.6 and 7.0. (*A*) Responses at pH 7.6 (*black*) and pH 7.0 (*red*) to a dim flash delivering 380 photons μm^{-2}. This cell was preloaded with BAPTA-AM (see Materials and Methods). The response shortened at pH 7.0. The dark current at pH 7.6 was 24.4 pA, and at pH 7.0 it was 22.0 pA. (*Inset*) Integrals of the squared dim flash responses. (*B*) Power spectra of the dark noise measured at pH 7.6 (*black*), 7.0 (*red*), and after >99% pigment bleach (*blue*). (*C*) Integrals of the power spectra at each pH with the spectrum after bleaching subtracted.

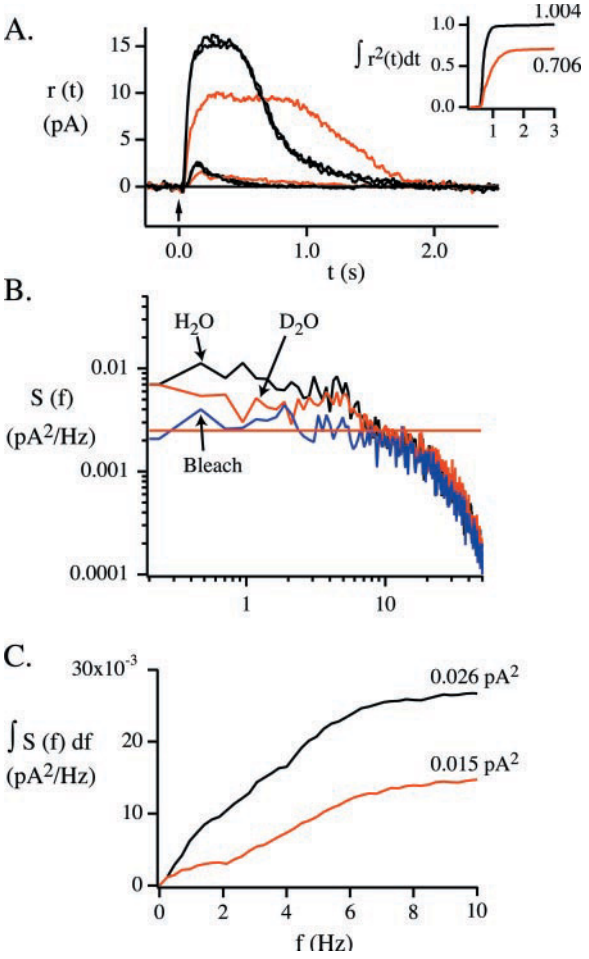

FIGURE 6 Pigment noise in Ringer and heavy water Ringer. (*A*) Flash responses in normal Ringer (*black*) recorded before and after the response in heavy water Ringer (*red*). The flashes delivered 770 photons μm^{-2}. (Inset) Integrals of the squared dim flash responses, indicating the factor by which the noise variance should change as a result of alterations in the dim flash response. (*B*) Power spectra of dark noise in normal Ringer (*black*), in heavy water Ringer (*red*), and after a >99% bleach (*blue*). (*C*) Integrals of the power spectra in *B* with the postbleach noise subtracted.

The relative reduction in the amplitude of the dim flash response at pH 7.0 was greater than the relative reduction in the dark current. Furthermore, acidifying the extracellular solution accelerated the recovery phase of the response, shortening the integration time to ~50% of its value in standard Ringer.

Solvent isotope effect in heavy water ringer

As a further test of the Barlow et al. (1993) model we attempted to alter the number of molecules with unprotonated Schiff base linkages by changing the pKa of the Schiff base with heavy water. This method does not depend upon the assumption that the accessibility of the pocket to the intracellular medium is negligible, since the intracellular water will be replaced rapidly by heavy water (Korenbrot and Cone, 1972). When the aqueous solution (H_2O) is replaced by heavy water (D_2O), the solvent isotope effect will make the mean dwell time of deuterons (D) on the Schiff base nitrogen (N) longer than that of protons (H). This will occur because the N-D stretching vibration is less energetic than the N-H vibration, making dissociation slower. Approximately a sevenfold increase in the mean occupancy time on the Schiff base is predicted (Lowry and Richardson, 1981), corresponding to an increase in the pKa of the Schiff base by 0.8 units. This change should decrease the thermal activation rate by sevenfold on the Barlow et al. (1993) model.

Fig. 6 shows results from an experiment to measure the dark noise in D_2O Ringer. Happily, the photoreceptor continued to transduce in D_2O. With the outer segment bathed in D_2O, flash responses were smaller and slower, and the dark current was

reduced (Fig. 6 *A*). The mechanisms producing these changes are unknown; they may involve the collective effects of increased occupancy of many proton-binding sites within the outer segment and/or the increased viscosity of the D_2O solution. Whatever the mechanism, the change in the kinetics of the dim flash response provides evidence that the intracellular H_2O was indeed replaced with D_2O. Power spectra of the dark noise of a cone in normal Ringer and D_2O Ringer are shown in Fig. 6 *B*. D_2O Ringer reduced the dark noise at low frequencies. The integrals of the spectra reveal that the noise variance in the bandwidth 0 to 10 Hz was 1.8-fold lower than that in standard Ringer (Fig. 6 *C*). However, this reduction in dark noise was consistent with the 1.4-fold reduction expected from the change in the elementary response (Fig. 6 *A*, inset), indicating no significant change in the rate of occurrence of elementary noise events. Similar results were obtained from five cells. On average there was a 1.4 ± 0.5-fold reduction in dark noise variance in D_2O. A 1.4 ± 0.5-fold reduction in dark noise variance was predicted from the change in the elementary response.

Fig. 7 presents a compilation of all the results from the solution change experiments. The three columns show: 1) the measured change in noise variance in each solution, 2) the change in noise variance expected solely on the basis of the change in the elementary response, and 3) the prediction of the Barlow et al.(1993) model. None of these solutions significantly altered the thermal event rate, suggesting that the rate of the thermal activation process did not vary with the fraction of molecules having unprotonated Schiff base linkages.

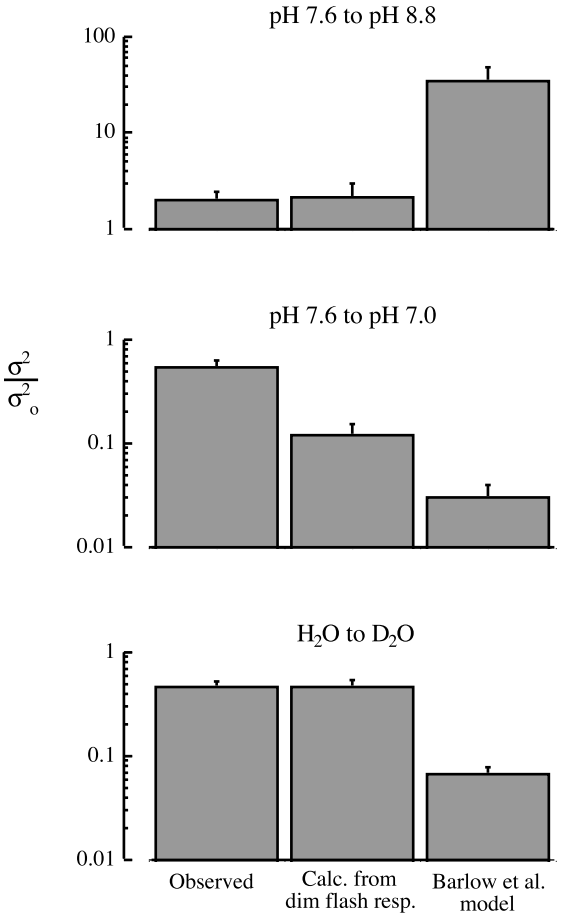

FIGURE 7 Summary of pigment noise parameters during solution changes. The dark noise variance in each solution relative to that at pH 7.6 is plotted for changes to pH 8.8, pH 7.0, and to heavy water Ringer. The leftmost column is the observed change in dark noise computed in the bandwidth 0 to 10 Hz. The middle column is the predicted change in dark noise based solely on alterations of the dim flash response. The rightmost column is the prediction of the Barlow et al. (1993) model, determined by dividing the middle column by the relative change in proton concentration, or the expected change in Schiff base occupancy for deuterons.

DISCUSSION

Mechanism of production of L-cone noise

From the temperature dependence of the rate of occurrence of dark noise events we obtained an Arrhenius activation energy E_a of 25 kcal/mol, a value similar to that for thermal activation of rhodopsin in red rods (Baylor et al., 1980) and green rods (Matthews, 1984) of the toad retina. In contrast, activation of visual pigments by light has a much higher E_a (Koskelainen et al., 2000). The difference in the values of E_a for activation by light and heat may be explained in the following way. Light absorption raises an electron to a higher energy level, and this allows *cis-trans* isomerization of the chromophore to take place. Thermal activation presumably does not involve electronic excitation but instead results from nuclear vibrations that produce *cis-trans* isomerization. The derived values of E_a for thermal activation in rods and L cones are comparable to the enthalpy difference between the ground state and the catalytically active intermediate Meta II (~27 kcal/mol) determined by Cooper (1981). In thermal activation the pigment molecule apparently reaches the Meta II state without passing through the high energy intermediates (e.g., Bathorhodopsin and Lumirhodopsin) of light-driven activation.

Our derived value for the preexponential factor for the L-cone pigment was 4.5×10^{13} s^{-1}, compared with a value for rhodopsin of 6×10^{6} s^{-1}. The different preexponential factors are formally explained by the different entropy changes the pigments undergo during thermal activation. The entropy change for rhodopsin is −0.035 kcal/mol K (Baylor et al., 1980), whereas that for the L-cone pigment was close to zero. A general interpretation is that thermal activation proceeds from a particular configuration state that is highly improbable in rhodopsin but probable in the L-cone pigment. Indeed the Arrhenius preexponential factor A for thermal activation of the L-cone pigment is comparable to the theoretical maximum of kT/h (or ~6.2×10^{12} s^{-1} at 25°C). Our value of A was derived from the rate of occurrence of thermal activation events, ν, the Arrhenius activation energy E_a, and the total number of pigment molecules N in the outer segment. If only a subpopulation

N/n of the pigment molecules (e.g., those with an unprotonated Schiff base nitrogen) were capable of thermal activation, the preexponential factor for these "special molecules" would have to be nA to be consistent with the derived values of ν and E_a. Exceeding kT/h becomes increasingly unphysical as n grows. For example, suppose that $n = 100$, so that only 1% of pigment molecules could undergo thermal activation. The preexponential factor A would then exceed kT/h by 100-fold. These considerations on their own argue against the idea that only unprotonated molecules, or indeed any special subpopulation of pigment molecules, give rise to thermal activation events.

Although our experiments do not specify the structural basis of thermal activation, the general statements above are consistent with available structural information about rhodopsin and the L-cone pigment. Electron density maps of the chromophore-binding pocket in rhodopsin indicate a sharply defined pocket that tightly encases the 11-*cis* retinal chromophore and constrains its movement (Palczewski et al., 2000). This constraint may then give a low probability for the chromophore to assume conformations that permit thermal activation. The tight wrapping of the chromophore may also explain why a small molecule like hydroxylamine is unable to penetrate the chromophore-binding pocket and degrade rhodopsin in darkness but is able to when the pigment molecule has become activated (Wald and Brown, 1950). While structural information about cone pigment molecules is not available, there is convincing evidence that the chromophore-binding pocket is open for the diffusion of ions and small molecules (see Materials and Methods); the open pocket may constrain the chromophore less, allowing thermal isomerization to occur more easily.

Experiments with chromophore analogues further support the idea that the chromophore in rhodopsin is more tightly constrained than that in the L-cone pigment. Thus, small changes in the 11-*cis* retinal chromophore, such as the removal of methyl groups in 9-desmethylretinal and 13-desmethylretinal, can have large affects on the catalytic activity and active lifetime of rhodopsin, but have little or no effect on the catalytic activity of the L-cone pigment. For example, incorporation of 9-desmethylretinal into rhodopsin produces a substantial slowing of the shutoff of the pigment after light activation (Corson et al., 1994), and 13-desmethylretinal activates the transduction mechanism when it enters the chromophore-binding pocket (Corson et al., 2000). Conversely, incorporation of 9-desmethylretinal into the L-cone pigment has no effect (Corson and Crouch, 2001), and 13-desmethylretinal shuts off the transduction mechanism (Corson et al., 2000). Similarly, β-ionone increases the catalytic activity of the bleached pigment in rods (Kefalov et al., 1999), but quiets transduction in L cones (Jin et al., 1993). Thus, the cone pigment can assume the dark configuration even when the chromophore structure is altered, suggesting that the pocket is looser.

Barlow (1957) suggested that the thermal stability of visual pigment molecules might vary inversely with their wavelength of peak sensitivity (λ_{max}). He proposed that as λ_{max} becomes longer the energy barrier for thermal activation becomes lower, so that visual receptor cells with pigments sensitive at longer wavelengths would be intrinsically noisier. Whereas the rate of thermal activation of the L-cone pigment is indeed higher than that of S-cone pigment (Rieke and Baylor, 2000) and rhodopsin (Baylor et al., 1980), the energy barrier we have derived for thermal activation of L-cone pigment is comparable with that for thermal activation of rhodopsin (Baylor et al., 1980); the large difference in thermal activation rates of L-cone pigment and rhodopsin instead reflects a much larger preexponential factor for the L-cone pigment.

Protonation of the Schiff base and dark noise

Barlow et al. (1993) proposed a possible explanation for the low value of the preexponential factor in the rate constant for thermal activation of rhodopsin. The hypothesis was that only the minute fraction of molecules with unprotonated Schiff base linkages are allowed to attempt thermal activation. An extension of their idea is that cone pigments with a lower Schiff base pKa (Liang et al., 1994) would have a larger rate constant for thermal activation. Direct tests of this hypothesis in L cones, however, failed to support it. When we changed the external proton concentration by ~100-fold, the observed change in the dark noise variance was consistent with small alterations in the elementary noise event but little change in the rate of occurrence of events. In addition, when we superfused the cone outer segment with D_2O Ringer, a manipulation expected to increase the pKa of the Schiff base by 0.8 units, we observed no change in the thermal event rate. This negative result cannot be explained by failure to deuterate the Schiff base nitrogen, because spectroscopic experiments indicate that the Schiff base hydrogen exchanges for deuterium within 7 ms (Deng et al., 1994). We therefore suggest that the fraction of molecules with the unprotonated Schiff base linkage has no bearing on the overall rate of thermal activation and that thermal activation probably proceeds from the protonated state.

The strong pH dependence of thermal activation in Limulus photoreceptors reported by Barlow et al. (1993) suggests that different mechanisms control spontaneous activation of the pigment in these cells. This may not be entirely unexpected given the bistable nature of the Limulus pigment (Hubbard and St. George, 1958; Lisman and Sheline, 1976). Nevertheless, our results in salamander L cones argue against the notion that protonation of the Schiff base nitrogen is a general mechanism that prevents thermal activation of the chromophore in visual pigments.

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NOTE ADDED IN PROOF

Firsov et al. (*J. Physiol.* 539:837–846, 2002) recently reported a lack of pH dependence for thermal noise events in toad rods.

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