Supplemental Information: Materials and Methods

Norianne T. Ingram, Gordon L. Fain, and Alapakkam P. Sampath

RECORDING

Methods for recording from mouse cones have been previously described (1, 2). In brief, mouse retinas (C57BL6/J) were sliced in cold HEPES-buffered Ames' medium bubbled with 100% O_2, which contained 2.38 g HEPES per liter and was balanced with 0.875 g NaCl per liter to give an osmolarity of 284 ± 1 mOsm (pH 7.35 ± 0.5). During physiological recordings, retinal slices were superfused at 2 ml/minute with Ames' medium in the recording chamber while maintained at 35 ± 1.0 ºC. This solution was continuously bubbled with 95% O_2/5% CO_2 and buffered with 1.9 g per liter sodium bicarbonate to maintain pH between 7.3 and 7.4. The mM concentrations of the principal ions in Ames' medium are as follows (3): Na^+, 143, K^+, 3.6; Ca^{2+}, 2.3; Mg^{2+}, 2.4; Cl^-, 125.4; HCO_3^-, 22.6; and SO_4^{2-}, 2.4. This medium also contains 6 mM glucose. In some experiments, channel-blocking compounds were added to the external medium (4). These compounds and their applied concentrations were as follows: isradipine (10 μM; ISR; Sigma) to block L-type voltage-gated Ca^{2+} channels at the photoreceptor synaptic terminal; niflumic acid (200 μM, NFA, Sigma), to block Ca^{2+}-activated Cl^- channels; and tetraethylammonium chloride (25 mM, TEA, Sigma), to block sustained voltage-dependent K^+ channels.

The standard internal solution for recording pipettes was a potassium aspartate (K-Asp) solution consisting of (in mM): 125 K-Asp, 10 KCl, 10 HEPES, 5 *N*-methyl-D-glucamine (NMG)-HEDTA, 0.5 $CaCl_2$, 0.5 $MgCl_2$, 0.1 ATP-Mg, 0.5 GTP-TRIS, 2.5 NADPH (pH 7.3 ± 0.02 with NMG-OH; 280 ± 1 mOsm). In some measurements, the internal solution was a cesium methane-sulfonate solution consisting of (in mM): 110 $CsCH_3O_3S$, 12 TEA-Cl, 10 HEPES, 10 EGTA, 2

www.pnas.org/cgi/doi/10.1073/pnas.2001776117

QX-314-Br, 11 ATP-Mg, 0.5 GTP-Tris, 0.5 $MgCl_2$, 1 NAD^+ (pH 7.3 with CsOH; 280 ± 1 mOsm). This solution was used to block potassium conductances including i_h (5, 6). Measured values of cone membrane potential were corrected by subtracting the liquid junction potential (7), which was measured to be approximately 10 mV for these internal solutions. Light stimuli were provided by ultra-bright LEDs driven with a linear feedback driver (Opto-LED; Cairn Research) and were monochromatic at 405 nm, a value near the isosbestic point of the S-cone and M-cone pigments (8). As a result, the number of cone pigment molecules activated by light was nearly independent of the ratio of the two pigments in any particular cone from which we recorded (9). Light intensities were calibrated with a photodiode (Graseby Optronics 268R) and are given as effective photons at the λ_{max} of the photopigment. This meant that, for cones stimulated with 405-nm light, the number of photons in the stimulating light was multiplied by 0.2, to account for the decrease in pigment absorption for either the S or M pigments at their λ_{max}'s. This adjustment was unnecessary for rod responses, which were obtained with light at 500 nm near the peak spectral sensitivity of the rod pigment. Intensities can be converted into nominal values of rhodopsin molecules bleached (Rh*) or cone pigment molecules bleached (P*) by using a collecting area of 0.2 μm^2 for rods and 0.07 μm^2 for cones (1). Data were filtered at 500 Hz (8-pole Bessel, Frequency Devices 900), sampled at 10 kHz, and recorded in an open-source, MATLAB-based program called Symphony Data Acquisition System (http://www.open-ephys.org/symphony/). Data traces were analyzed with custom scripts written in Matlab.

CALCULATIONS

Na^+ Influx Through cGMP-gated Channels of Outer Segment. The total flux as current into the rod outer segment is the sum of the current passing through the cyclic-nucleotide gated (CNG) channels and the current carried by the Na^+/Ca^{2+}-K^+ exchanger,

$$i_T = i_{Ch} + i_{Ex} \tag{1}$$

For rods, the current through the channels was taken directly from suction-electrode recordings. For cones, the current through the channels was calculated from the change in conductance measured from voltage-clamp recordings at the resting potential, together with the driving force estimated from the voltage at each of the light intensities, together with published measurements of the current-voltage curve of the conductance and the reversal potential of the light response (2). The part of this current carried by Na^+ is the sum of the current carried by Na^+ though the channels plus the current carried by Na^+ through the exchanger,

$$i_{Na(T)} = i_{Na(Ch)} + i_{Na(Ex)} \tag{2}$$

We calculate the Na^+ current carried through the channels first. It is approximately equal to the total current minus the exchange current minus the fraction of the inward current through the channels carried by Ca^{2+},

$$i_{Na(Ch)} = i_T - i_{Ex} - i_{Ca(Ch)} \tag{3}$$

If we ignore any Ca^{2+} diffusing from the outer segment into the inner segment, the value of the Ca^{2+} current entering the rod at steady state will be equal to twice the exchange current, and we have

$$i_{Na(Ch)} = i_T - 3i_{Ex} \tag{4}$$

And because 4 sodium ions are transported inward by the exchange transporter for each net charge transported inward (10), the value of the Na^+ flux as a current transported inward is four times the value of the exchange current,

$$i_{Na(Ex)} = 4i_{Ex} \tag{5}$$

The total current carried by Na^+ is therefore given by

$$i_{Na(T)} = i_T - 3i_{Ex} + 4i_{Ex} = i_T + i_{Ex} \tag{6}$$

It is not possible with present methods to measure the exchange current of a mammalian cone, because the light response rises so rapidly that the component of current produced by the exchanger cannot be separated from that produced by the closing of the CNG channels. We used instead the value of 10% (i.e. $i_{Ex} = 0.1 i_T$) from the measurements of Perry and McNaughton for salamander cones (11), where the kinetics of the light response are much slower than in mammals. This value would change in background light as a result of changes in Na^+ and Ca^{2+} driving forces and the voltage dependence of Na^+/Ca^{2+}-K^+ transport (10), but these effects are likely to be small. Even doubling the fraction of the exchange current to 20% would only increase the Na^+ influx by 1.2/1.1 or about 10%, which would not substantially alter the curves in Fig. 2 and would, if anything, only increase the difference between rods and cones.

Our calculation of the current carried by Na^+ ignores the small fraction of the current through the CNG channels carried outward by K^+. We estimated this fraction from the GHK current equations (12),

$$I_{Na} = P_{Na} V_m \frac{F^2}{RT} \frac{[Na^+]_o - [Na^+]_i e^{FVm/RT}}{1 - e^{FVm/RT}}, \quad I_K = P_K V_m \frac{F^2}{RT} \frac{[K^+]_o - [K^+]_i e^{FVm/RT}}{1 - e^{FVm/RT}} \tag{7}$$

The ratio I_K/I_{Na} is then given by

$$I_K/I_{Na} = \frac{P_K}{P_{Na}}\left\{\frac{[K^+]_o - [K^+]_i e^{FV_m/RT}}{[Na^+]_o - [Na^+]_i e^{FV_m/RT}}\right\} \quad (8)$$

If we assume that P_K/P_{Na} is the same for mouse cone CNG channels as for channels in other cells and is about one (see 13), Eq. (8) gives a value of -0.14 at the membrane potential and ionic conditions of our recordings. And since the current carried by Na^+ and K^+ is given by

$$I_{Na} + I_K = I_{Na} + I_{Na}\frac{I_K}{I_{Na}} = I_{Na}(1 + \alpha) \quad (9)$$

with α = -0.14, the value of the sodium current would be underestimated by about 16% at the resting membrane potential of a cone in darkness. This error will decrease as the membrane potential hyperpolarizes and the driving force for K^+ decreases in the light.

We have also ignored the fraction of current carried by Mg^{2+}, which is unknown for a mouse cone but has been estimated as about one-third the value of the Ca^{2+} current in toad rods (14). It may therefore represent about 3% of the total current and would lead us to overestimate the part of the current carried by Na^+ by approximately this percentage.

Na^+ Entering Inner Segment Through HCN channels (i_h). The net current through the HCN channels at any voltage is given by

$$I_t = I_{Na} + I_K = I_K\frac{I_{Na}}{I_K} + I_K = I_K(\alpha + 1) \quad (10)$$

where α is now the ratio of the sodium current to the potassium current, calculated as a function of voltage from Eqs. (7) and (8) and from the previously determined value of P_{Na}/P_K of 0.35 (2). The value of I_K was then obtained by dividing the total current by (α + 1), and I_K was subtracted from I_t to give the value of the current carried by Na^+.

ATP Consumption by Guanylyl Cyclase. The rate of the guanylyl cyclase is regulated by the guanylyl-cyclase activating proteins (GCAPs) and is maximal when the Ca^{2+} concentration is minimal, and minimal when the Ca^{2+} concentration is maximal. The rate of the cyclase cannot be measured in mouse cones because there are too few photoreceptors to make biochemical measurements. The cyclases and GCAPs are however similar in the two kinds of photoreceptors (15), so we have assumed that cyclase in cones behaves much like cyclase in rods, for which biochemical measurements are available (16, 17). For rods, the dark rate of the cyclase when outer segment Ca^{2+} is minimal is about 3 x 10^6 cGMP synthesized per rod per second (for details of this calculation, see SI of 18). The rate of synthesis in the light is 0.1 of this value (16), or 3 x 10^5 cGMP s^{-1}. Since 2 ATPs are required to regenerate the GTP from which cGMP is synthesized by guanylyl cyclase, the expenditure of ATP for cGMP synthesis would range from about 6 x 10^5 ATP s^{-1} in darkness to 6 x 10^6 ATP s^{-1} in bright light.

We have estimated the dependence of the cyclase on light intensity in the following way. We assumed that the free-Ca^{2+} concentration in the cone outer segment is proportional to the outer segment current, as seems to be true from experiments on salamander cones (19) and rods of both salamander (20) and mouse (17). The free-Ca^{2+} concentration was then estimated from the equation

$$[Ca]_{free} = \left([Ca]_{dark} - [Ca]_{light}\right)\left(1 - i_{step}\right) + [Ca]_{light} \qquad (11)$$

where $[Ca]_{free}$ is the free-Ca^{2+} concentration in the outer segment, $[Ca]_{dark}$ and $[Ca]_{light}$ are the values of the free-Ca^{2+} concentration in darkness and in a light bright enough to close all of the channels, and i_{step} is the mean normalized photocurrent response of a mouse cone to steps of light from Fig. 1B. The values of $[Ca]_{dark}$ and $[Ca]_{light}$ were assumed to be the same as for mouse rods

(17), namely approximately 240 nM in darkness and 50 nM when all the channels are closed by saturating light.

The cyclase activity could then be calculated at each light intensity from the free-Ca^{2+} concentration and the equation

$$A = \frac{(A_{\max} - A_{\min})}{1 + ([Ca]_{free} / K_m)^n} + A_{\min} \qquad (12)$$

as given in Olshevskaya et al. (16), where A is the activity of the enzyme, A_{max} and A_{min} are the maximum and minimum activities (3 x 10^6 cGMP s^{-1} and 3 x 10^5 cGMP s^{-1}), $[Ca]_{free}$ is the free-Ca^{2+} concentration taken from Eqn. (11), and K_m and n are constants equal to 70 nM and 2.2 (16). The enzyme activities were then multiplied by 2 in order to calculate the ATP expenditure required to synthesize cGMP, and then multiplied by 10 because cyclase is more highly expressed in cones (see 21). Because cyclase contributes only a small amount to the overall ATP consumption of either a rod or a cone (Figs. 3A and 3B), our estimates may be in error by a factor of 2 – 3 without significant effect on our conclusions.

Visual Pigment Regeneration. The number of pigment molecules bleached in bright, constant illumination is relatively small. The fraction bleached at steady state F can be shown to be given by $(IP\tau)/(1+ IP\tau)$, and the rate of bleaching by (1-F)IP, where I is the intensity of light, P the photosensitivity, and τ the time constant of regeneration. The maximum rate at steady state for large I is therefore given by the rate of bleaching, which can be no greater than the rate of regeneration or $1/\tau$. For rods, τ is several thousand seconds in mouse (22) and 400 s in man (23), giving a maximum Rh* s^{-1} at steady state even in the brightest light of about 10^4 Rh* s^{-1} in mouse and perhaps 10-fold greater in man. Even at 10 ATPs per rhodopsin, the energy required for rod pigment regeneration is unlikely to exceed 10^6 ATPs per second. For cones, the rate of

regeneration in the intact eye is unknown for mouse but unlikely to exceed the value of 120 seconds measured for human (24), giving between 10^5 – 10^6 pigment molecules regenerated per second. Some fraction of chromophore re-isomerization is done by light itself and would require minimal ATP turnover (25). Even ignoring this fraction, the ATP required for regeneration would be relatively small by comparison to ion transport, which in bright light is much larger for cones than for rods (Fig. 2C of article).

REFERENCES

1. Ingram NT, Sampath AP, & Fain GL (2019) Voltage-clamp recordings of light responses from wild-type and mutant mouse cone photoreceptors. *Journal of General Physiology* 151:1287-1299.
2. Ingram NT, Sampath AP, & Fain GL (2020) Membrane conductances of mouse cone photoreceptors. *Journal of General Physiology* In press.
3. Ames A, 3rd & Nesbett FB (1981) In vitro retina as an experimental model of the central nervous system. *J Neurochem* 37(4):867-877.
4. Van Hook MJ, Nawy S, & Thoreson WB (2019) Voltage- and calcium-gated ion channels of neurons in the vertebrate retina. *Prog Retin Eye Res*.
5. Fain GL, Quandt FN, Bastian BL, & Gerschenfeld HM (1978) Contribution of a caesium-sensitive conductance increase to the rod photoresponse. *Nature* 272(5652):466-469.
6. Yagi T & MacLeish PR (1994) Ionic conductances of monkey solitary cone inner segments. *Journal of Neurophysiology* 71(656-665).
7. Neher E (1992) Correction for liquid junction potentials in patch clamp experiments. *Methods Enzymol* 207:123-131.
8. Nikonov SS, Kholodenko R, Lem J, & Pugh EN, Jr. (2006) Physiological features of the S- and M-cone photoreceptors of wild-type mice from single-cell recordings. *J Gen Physiol* 127(4):359-374.

9. Applebury ML*, et al.* (2000) The murine cone photoreceptor: a single cone type expresses both S and M opsins with retinal spatial patterning. *Neuron* 27(3):513-523.
10. Cervetto L, Lagnado L, Perry RJ, Robinson DW, & McNaughton PA (1989) Extrusion of calcium from rod outer segments is driven by both sodium and potassium gradients. *Nature* 337(6209):740-743.
11. Perry RJ & McNaughton PA (1991) Response properties of cones from the retina of the tiger salamander. *J Physiol (Lond)* 433:561-587.
12. Hodgkin AL & Katz B (1949) The effect of sodium ions on the electrical activity of giant axon of the squid. *J Physiol* 108(1):37-77.
13. Kaupp UB & Seifert R (2002) Cyclic nucleotide-gated ion channels. *Physiol Rev* 82(3):769-824.
14. Nakatani K & Yau KW (1988) Calcium and magnesium fluxes across the plasma membrane of the toad rod outer segment. *J Physiol (Lond)* 395:695-729.
15. Ingram NT, Sampath AP, & Fain GL (2016) Why are rods more sensitive than cones? *Journal of Physiology* 594:5415-5426.
16. Olshevskaya EV*, et al.* (2004) The Y99C mutation in guanylyl cyclase-activating protein 1 increases intracellular Ca2+ and causes photoreceptor degeneration in transgenic mice. *J Neurosci* 24(27):6078-6085.
17. Woodruff ML*, et al.* (2007) Constitutive excitation by Gly90Asp rhodopsin rescues rods from degeneration caused by elevated production of cGMP in the dark. *J Neurosci* 27(33):8805-8815.
18. Okawa H, Sampath AP, Laughlin SB, & Fain GL (2008) ATP consumption by mammalian rod photoreceptors in darkness and in light. *Curr Biol* 18(24):1917-1921.
19. Sampath AP, Matthews HR, Cornwall MC, Bandarchi J, & Fain GL (1999) Light-dependent changes in outer segment free Ca^{2+} concentration in salamander cone photoreceptors. *Journal of General Physiology* 113:267-277.
20. Matthews HR & Fain GL (2003) The effect of light on outer segment calcium in salamander rods. *Journal of Physiology* 552:763-776.
21. Takemoto N, Tachibanaki S, & Kawamura S (2009) High cGMP synthetic activity in carp cones. *Proc Natl Acad Sci U S A* 106(28):11788-11793.

22. Lamb TD & Pugh EN, Jr. (2004) Dark adaptation and the retinoid cycle of vision. *Prog Retin Eye Res* 23(3):307-380.
23. Alpern M (1971) Rhodopsin kinetics in the human eye. *J Physiol* 217(2):447-471.
24. Alpern M, Maaseidvaag F, & Ohba N (1971) The kinetics of cone visual pigments in man. *Vision Research* 11:539-549.
25. Morshedian A*, et al.* (2019) Light-Driven Regeneration of Cone Visual Pigments through a Mechanism Involving RGR Opsin in Muller Glial Cells. *Neuron* 102(6):1172-1183 e1175.