Transduction heats in retinal rods: Tests of the role of cGMP by pyroelectric calorimetry

(phototransduction)

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ABSTRACT The sensory dark current of vertebrate retinal rods is believed to be controlled by light activation of a chain of coupled biochemical cycles that finally regulate the cationic conductance of the plasma membrane by hydrolytically reducing the level of cGMP in rod outer segment cytoplasm. The scheme has been tested by measuring heat production by live frog retinas when stimulated with sequences of light flashes of progressively increasing energy. Using pyroelectric poly(vinylidene 1,1-difluoride) detectors that simultaneously measure transretinal voltage and retinal temperature change, four heat effects assignable to known biochemical cycles in rods have been found. As the dark current shuts down after a flash causing 180–1800 rhodopsin photoisomerizations per rod, a heat burst, q_1 , raises the retinal temperature 1–2 μ K. q_1 is closely regulated in size and slightly precedes dark current shutdown. Isobutylmethylxanthine slows and enlarges q_1 , delaying the dark-current response. Increasing cytoplasmic Ca^{2+} stops the dark current without affecting q_1 . Although rod heat production is consistent with splitting of 1–3 μ M of free cytoplasmic cGMP during transduction, the kinetics of the two processes do not match the predictions of current cGMP control models.

A vertebrate retinal rod responds to photons absorbed in its outer segment (OS) disk membranes by briefly reducing the dark current flowing through the overlying plasma membrane, hyperpolarizing the cell (1, 2). Since OSs contain a light-activated cGMP phosphodiesterase and the cationic conductance of OS membranes increases rapidly and reversibly on exposure to concentrations of cGMP $>5 \mu\text{M}$ (3–5), transduction based on control of the dark-current conductance by free cytoplasmic cGMP has been proposed (6, 7). In this work we test this idea by comparing the heat produced by frog retinas during transduction with that generated when cGMP is split by phosphodiesterase. Although cGMP yields 11 kcal/mol of heat on hydrolysis to 5'-GMP (8), the tiny amounts believed split during light responses would yield heats measurable only by radically improved calorimetry. Our method, based on heat detection by pyroelectric poly(vinylidene 1,1-difluoride) (PVDF) films as pioneered by Tasaki and Nakaye (9), extends the range of chemical calorimetry to submicrodegree levels and millisecond times.

EXPERIMENTAL

Dark-adapted bullfrog (*Rana catesbiana*) retinas, removed under infrared illumination, were stored for 10 min to 1 hr in an oxygenated bath containing Ringer's A solution [(in mM) Na^+ , 110; K^+ , 2.7; Ca^{2+} , 1.4; Mg^{2+} , 0.5; phosphate, 0.5; Hepes, 10; glucose, 11; EDTA, 0.01; at pH 7.1]. Cl^- was the main anion. Disks 6.3 mm across were cut from a retina and

attached by their vitreal surfaces to nylon mesh 25- μm -thick (Small Parts, Miami) disks of the same diameter.

Before use, each disk was soaked for 5 min in Ringer's G solution, Ringer's A solution with 10 mM glutamate and 5 mM cysteine added to suppress electrical responses from all retinal cells except the rods, cones, and glial Müller cells (10). Using an infrared viewer, the disks were placed on the detector with receptor layer down (Fig. 1) and excess fluid was blotted away.

Detector. Fig. 1 shows retina and detector. A polyester film 6.8 μm thick (Chemplex Industries, Eastchester, NY), a disk of aluminum foil 6.3 mm in diameter, and a poled PVDF film (Kynar; Pennwalt, King of Prussia, PA) 9 μm thick were bonded together with cyanoacrylate adhesive (3M, type CA-40). The assembly was mounted in a cavity in an Al bar. The Al disk and the Ni-coated PVDF film were connected to two preamplifier inputs with graphite yarn and carbon paint. A polystyrene foam pad was pressed against the lower surface of the detector to reduce sound pickup. An Al cap containing a lens and a fiber optic light pipe closed the chamber top. Flowing moist oxygen replaced the atmosphere every 2 min.

Physical Parameters. Flashes (6 μs) with a spectral width 70 nm centered at 500, 550, or 640 nm from a xenon flash tube were attenuated by a circular graded wedge and fed by a light pipe whose end was projected to a 6-mm circle at the retina. A computerized stepping motor turned the wedge so that flashes of various intensities could be quickly delivered. The light input at the retina was measured with a silicon photovoltaic diode (International Light, Newburyport, MA). Exposures of the OS were calculated for a rod density in the retinal mosaic of $1.76 \times 10^6 \text{ cm}^{-2}$ (unpublished measurements on live frog retinas). Cone and green rod pigments were neglected, and rod porphyropsin (11) was lumped with rhodopsin. Taking the quantum efficiency of rhodopsin photoisomerization (PI) to be 0.6 (12) and its molar absorbance at 550 nm to be $2.0 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$ (13), its isomerization cross-section Z_R was $4.6 \times 10^{-17} \text{ cm}^2$.

The weight fraction of rod OS cytoplasm in retinas was estimated to be 7% from (i) the measured OS lengths ($50 \pm 5 \mu\text{m}$ in four retinas), (ii) the fraction of the retinal mosaic area composed of OS cross-sections (50% in two measurements), and (iii) the water fraction in the OS mass (0.7 ± 0.05 , unpublished electron microprobe measurements of M. C. Foster and W.A.H.).

The enthalpy of hydrolysis of cGMP to 5'-GMP by rod phosphodiesterase was measured by mixing two parts of a suspension of totally bleached frog rod OS membranes harvested from a continuous metrizamide density gradient ($d = 1.01$ – 1.12) with one part of 300 μM cGMP in a solution containing (in mM) K^+ , 80; Na^+ , 40; Cl^- , 80; HOEtSO_3^- , 40; Mops, 10 (pH 7.15); MgSO_4 , 5; CaCl_2 , 0.1; EDTA, 0.02. The

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Abbreviations: OS, outer segment; PVDF, poly(vinylidene 1,1-difluoride); PI, photoisomerization.

FIG. 1. Diagram of apparatus with thicknesses of layers of retina and detector to scale. Operational amplifiers (type OPA 111BM; Burr-Brown, Tucson, AZ) clamped the lower PVDF electrode and the Al foil and upper PVDF electrode at ground potential by current through 220-pF polystyrene capacitors (C_A and C_B). The ≈ 0.5 -pA input currents of the amplifiers caused their outputs to drift steadily. Comparators reset MOSFET switches across C_A and C_B when either output reached ± 800 mV. The capacitance between Al foil and retina was 75 ± 5 pF, while that of the PVDF film was 380 ± 10 pF in series with 400Ω . The thermal sensitivity, measured with a known temperature step generated in a Cu slug touching the detector, was 4 ± 0.3 V/K at point A. Pyroelectric current from the PVDF flowed through C_A and C_B in opposite directions. A weighted sum of voltages V_A and V_B , V_{AB} was adjusted to be independent of temperature but responsive to transretinal potentials through the polyester film capacitance and the grounded wick on the retina. A voltage ramp in series with the wick calibrated V_{AB} . V_A gave the pyroelectric current integrated in C_A . V_A and V_{AB} were differentiated and recorded digitally as θ and V_r . After subtracting preamplifier input currents, θ and V_r were integrated numerically to yield changes in temperature, θ , and in trans-retinal potential, V_r . Noise in θ was $\approx 1 \mu\text{K/s}$ in 0.1–10 Hz, about half due to sound pickup.

rhodopsin concentration in suspension before bleaching was $12 \mu\text{M}$. Heat evolution was measured in an LKB batch calorimeter at 25°C . ΔH observed was 11.2 ± 0.7 kcal/mol of cGMP hydrolyzed ($n = 2$), in agreement with ref. 8.

RESULTS

Detector Response to Internal and External Heat Sources.

When a light flash falls on a detector bearing a retinal disk whose rods have been light-adapted by bleaching 1% of their rhodopsin, the detector's thermal response shows two components, q_d and q_p , with different time courses. For flashes of $<10^8$ incident photons per rod, both are proportional to flash energy. q_d is due to light reaching the Al layer, where $18 \pm 2\%$ is absorbed,[†] converted to heat, and spread through the full thickness of the PVDF film within $100 \mu\text{s}$. The slower q_p is due to photons absorbed in the OS. Since the polyester film has a heat conductivity 1000 times less than Al (14), the first $800 \mu\text{s}$ of the detector response is entirely from q_d and

[†]This fraction was measured by comparing the detector responses to monochromatic flashes when loaded with matched disks punched from gelatin filters that totally transmit (Wratten 2A) or totally absorb incident light (Wratten 96, Density 2).

can be used to remove its contribution (Fig. 2 C and D) to the composite responses by curve stripping. q_p then gives the detector response to a sudden heat burst released in the OS. q_d and q_p show large initial overshoots because the sources are close to the PVDF film. As the heat then spreads up through the retina in a time τ_S of 20–30 ms, the PVDF temperature drops to a plateau, then declines to the ambient with a Newton's law (15) time constant τ_N of about 2 min. τ_N is much shorter for the bare detector (Fig. 2 A and B), whose heat capacity was equivalent to 1.8 mg of water.

For times $\ll \tau_N$ and $\gg \tau_S$, the specimen is almost thermally uniform and adiabatic, so using 650-nm flashes of known energy that heated only the Al layer, the specific heat, C_p , of the sample was estimated from the temperature rise at 2.5 s (Fig. 2 A and B). C_p for the 13.6-mg specimen was 0.86 ± 0.04 cal/(g·K) ($n = 4$). The relative sizes of q_d at 640, 500, and 550 nm produced by equal energy incident flashes express the retina's light transmission. Assuming no OS absorption at 640 nm, the rods absorbed 59% and 50% of incident light at 500 and 550 nm, respectively, yielding $77 \pm 4\%$ and $80 \pm 3\%$ ($n = 2$) of the absorbed light energy as heat (Fig. 2D). These values are near those found by calorimetry on bovine rods (16).

Retinal Heat Sources During Phototransduction. Flashes absorbed in the OS of dark-adapted retinas elicit other heats besides q_d and q_p . By using a rapid series of flashes of increasing energy these can be resolved into components of two types: heat bursts, q , that raise the sample temperature by microdegrees with apparently first-order kinetics and time constants of 100–300 ms, and continuous heat fluxes, r , that cause steady increases (or decreases) in sample temperature for many seconds. Each component saturates in size at an exposure that bleaches far less than 1% of the rhodopsin.

Fig. 3 shows these components in retinas exposed to eight 550-nm flashes at intervals of 5 and then 1.2 s along with the transretinal (PIII) waveform, V_r , recorded together. Curve 1 shows the change in transretinal voltage, curve 2 displays the raw θ transients uncorrected for q_p or q_d , and curve 3 shows the temperature changes, obtained by integrating curve 2

FIG. 2. Thermal responses of detector with 13.5-mg light-adapted retina plus nylon mesh to $6\text{-}\mu\text{s}$ flashes at the wavelengths shown. Incident flash energies in units of $\log_{10}[\text{photons per cm}^2]$. Rates of temperature change θ recorded with a 2-kHz bandwidth (A and C). Temperature changes θ recorded with 10-Hz bandwidth (B and D). Each curve is the mean of 64 trials. (A and B) Solid curves, retina mounted, energy 13.36 units; dashed curves, bare detector, energy 13.45 units. (C and D) Solid curves, retina mounted, energy 13.67 units at 550 nm; dashes, q_d component calculated from curves in A and B; alternating dashes, heat q_p from light absorbed in OS. No heat from the rods is seen at 640 nm.

FIG. 3. Transretinal electrical response (V_r) and thermal responses (θ and $\dot{\theta}$) to a sequence of eight flashes producing indicated numbers of photoisomerizations per outer segment (PI per OS). V_r with rod component V_d and polarization V_p shown roughly by dashes (curve 1). Rate $\dot{\theta}$ of temperature change (curve 2). Time integral of curve 2 (curve 3). Curve 3 corrected for components ($q_d + q_p$) from light absorbed in the photopigments and Al layer of detector as described in text (curve 4). At time Z (curve 4) the gain was reduced and the integrator reset to eliminate the cooling process r_1 . Recording bandwidth, 10 Hz.

numerically. At time Z, the integration restarted with the dc offset (r_1 on curve 2) set to zero, to eliminate the downtrend in θ after flash F₁. Flash F₈ produced $q_p + q_d$ alone. When the F₈ transient, scaled down to the incident flash energy of F₃₋₇ and shifted suitably in time, was subtracted from each transient in curve 2 before integration, curve 4 showing the extra heat components in addition to $q_p + q_d$ was obtained. The heats after F₃₋₇ recovered in 20–40 s, but the small component seen at F₁ and the voltage waveform V_r only recovered after dim flashes like F₁ and F₂.

Fig. 3 shows at least four new light-stimulated heat components: (i) a fast heat burst, q_1 , during the quickly rising phase of V_r , warming the sample by 1–2 μK and completely evoked by F₁; (ii) a steady cooling, r_1 , of about $-5 \mu K/s$ persisting as long as the dark current is suppressed; (iii) a slower heat burst, q_3 , 20–50 μK in size evolving about 300 ms after F₃₋₇; and (iv) a steady warming, r_3 , that increases after F₆ and F₇.

The heats produced by F₃₋₇ are replotted in Fig. 4A after

correcting for r_1 and $q_p + q_d$, to show the incremental heat produced by each flash. Curve F₃ is fitted to the function

$$H(t) = A(1 - e^{-Bt}) + Ct, \quad [1]$$

in which A , B , and C have been adjusted. A is the q_3 amplitude, B is its rate constant, and C measures r_3 . The summed amplitudes G of the q_3 values are plotted against total exposure in Fig. 4B. The points from two samples are fitted to the equation

$$G(E) = K(1 - e^{-E/E_0}), \quad [2]$$

where K is the total q_3 size and E_0 is the flash exposure yielding 0.632 K . E_0 was 5.0 and 6.6×10^{12} photons incident per cm^2 for the two retinas. Eq. 2 portrays activation of a set of targets of cross-section $1/E_0$, each releasing a fixed amount of heat when one or more PIs occur within it. In general, q_3 resembles the “receptor heat” described by Tasaki and Nakaye (9).

Heat Transients During Dark-Current Shutdown. In retinas incubated in Ringer’s G solution with Ca^{2+} reduced to 100 μM , flashes bright enough to suppress the dark current (100–1000 PI per OS) produced a V_r of 30–100 μV and a q_1 of 1–2 μK followed by steady cooling lasting for >20 s or until the dark current resumed (Fig. 5, curves 1). A second flash while the dark current was suppressed (as judged by the absence of change in V_r) gave no q_1 and no change in r_1 (Fig. 3, curves 2 and 3). In 30 experiments, V_r responses, whose amplitudes corresponded to dark currents of 20–50 pA per rod, were accompanied by q_1 bursts of 0.8–2 μK [1.09 ± 0.05 , $n = 24$ (mean $\pm$ SEM)] while r_1 varied from -2 to $-5 \mu K/s$.

q_1 was unchanged by several agents that suppressed the dark current and changed r_1 . Fig. 5 shows the effects of flashes causing 180 PI per rod on retinal temperature and transretinal voltage. Each curve is the average of two to four trials with 60-s intervals between flashes, during which q_1 recovered. The 30 mM Ca^{2+} added to Ringer’s G solution reduced V_r by a factor of 6 and changed the sign of r_1 while q_1 remained $2.0 \pm 0.4 \mu K$ ($n = 4$) (Fig. 5, curves 5). Retinas incubated in 50 μM ouabain for 10 min showed no V_r (curves 2) even though the cells retained 30 mM of cytoplasmic K^+ and produced light-sensitive dark current if the external $[\text{Ca}^{2+}]$ was suddenly lowered to 1 μM (M. C. Foster, S.Y., and W.A.H., unpublished x-ray microanalyses). Nevertheless, a flash causing 180 PI per OS produced a q_1 of $1.6 \pm 0.1 \mu K$ ($n = 3$) and r_1 of 4 $\mu K/s$ (curves 2). q_1 increased to $2.4 \pm 0.4 \mu K$ ($n = 3$) when 20 mM imidazole, a permeant nontoxic pH buffer, was added to the incubation medium and became the main cytoplasmic buffer for protons produced during cGMP hydrolysis (17). Its ΔH of neutralization (7 kcal/mol) adds 3.5 kcal/mol to the total heat from cGMP hydrolysis if OS cytoplasm is mainly buffered by phosphates. Isobutylmethylxanthine (500 μM) suppressed both V_r and q_1 at 180 PI per OS (curves 3). Flashes 10-fold brighter (curves 4) yielded slow V_r waves and delayed q_1 heats of $2.1 \pm 0.1 \mu K$ ($n = 6$).

FIG. 5. Heats (A) and transretinal voltages V_r (B) from retinas incubated in various solutions. Curves: 1, Ringer's G; 2, 50 μ M ouabain in Ringer's G; 3 and 4, 0.5 mM isobutylmethylxanthine in Ringer's G; 5, Ringer's G with 30 mM of CaCl_2 replacing an osmotically equivalent amount of NaCl. All flashes caused 180 PI per OS except those for curve 4, which were 10-fold brighter. Each curve was the mean of two to four trials.

The kinetics of normal $\dot{q}_1$ and $\dot{V}_r$ are compared in Fig. 6 Upper. If $\dot{q}_1$ is produced in the outer segment layer with the same spatial distribution as q_p , the $\dot{\theta}$ curves can be corrected for the overshoot in the detector's q_p response (Fig. 2C, alternating dashes) by deconvolution. This yields thermal traces that would have been observed if the $\dot{q}_1$ heat had been uniformly released throughout the full thickness of the sample. At low exposures (Fig. 6, curves c and d) $\dot{q}_1$ preceded $\dot{V}_r$. At higher levels (curves a and b) $\dot{q}_1$ was unchanged but $\dot{V}_r$ became faster, so that the two processes became almost simultaneous. $\dot{q}_1$ was never slower than $\dot{V}_r$ and always accompanied it.

DISCUSSION

The excitatory cascade (Fig. 7) suggested for phototransduction in rods (6, 7) consists of five coupled cycles: the rhodopsin cycle R, a guanine nucleotide-binding regulatory protein (G protein) cycle G, a phosphodiesterase cycle P, the

cGMP cycle C, and the dark-current cation cycle D. In darkness, the R, G, and P cycles are each poised with their levels of intermediates determined by pileup behind a single strongly inhibited bottleneck reaction. The C cycle is possibly not completely inhibited in the dark (18). A flash that activates a cycle will cause (i) a transient "activation heat" q as the levels of intermediates change from the dark values to those obtaining when the bottleneck reaction is turned on, and (ii) a sustained "cycling heat" r as the intermediates pass through the cycle. r will die away as the bottleneck reaction slows, but in an efficient transduction mechanism the two heats should have widely different time courses. Both q and r can be composites of endo- and exothermic components. Partial activation of a cycle should produce smaller, slower values of q and r .

The activation/flash energy relation for each cycle depends on its coupling gain, M (molecules activated during transduction per active molecule in the preceding cycle), and on the numbers N of each molecular type per OS. For the G and P cycles, in which one Meta II molecule, R^* , can activate M_G G proteins, each then activating one P molecule and splitting a GTP, the activation cross-section Z_P for a P cycle can be predicted by a simple argument. If M_G/N_G is constant as the G proteins are activated by several flashes, Z_P will be $Z_R \times M_R \times M_G \times N_R/N_P$. And the fraction $F(E)$ of P molecules activated by a flash of energy E will be of the form of Eq. 2 in which Z_P will be $1/E_0$. Estimates based on uncited sources (6, 19) predict a Z_P of as much as 80,000 times Z_R for the P cycle in dark-adapted amphibian OS. Fig. 4B shows cross-sections for q_3 of 5600 and 7500 times Z_R . If the summed values of q_3 , 58 and 87 μ K, arose from GTP hydrolysis in the G cycle yielding 4.8 kcal/mol (20), 220 and 330 μ M GTP would be consumed in the OS of the two

FIG. 6. (Upper) Rates of change of transretinal voltage $\dot{V}_r$ (solid lines) and retinal temperature $\dot{\theta}$ (dashes). Mean transients from eight flashes causing 1800 PI per OS (curves a and b) or 180 PI per OS (curves c and d). Curve e is the recorder impulse response. (Lower) Predicted rates of change $\dot{g}_m$ in dark-current conductance accompanying hydrolysis of cytoplasmic cGMP at rate $\dot{c}_g$ (short dashes). Conductance changes were computed from relation 3 with Hill coefficients 2 or 3 assuming that free cGMP concentration approaches zero during a light response at the rate shown (Upper).

FIG. 7. Simplified scheme for control of the dark current by light-induced hydrolysis of free cytoplasmic cGMP (6, 7). N_X , number of molecules of species X per OS; M_X , number of molecules X altered or controlled per active molecule in the preceding cycle. The table gives rough estimates for the values of the M s and N s of frog OS. Bold arrows indicate known exothermic reactions.

samples. The coupling gains M_G are then 290 and 560 GTPs split per PI. Total GTP split by isolated frog OS 20 s after bleaching 1% of their rhodopsin is larger (21), possibly because much is used in reactions producing r_3 .

The cGMP control model and the responses of rod membrane patches to applied cGMP shape expected heat events during dark-current shutdown. Membrane patch conductance g_m depends on the concentration c of free cGMP according to the relation

$$g_m = g_{\max} c^n / (c^n + K_c^n), \quad [3]$$

where $g_{\max}$ is the maximum cGMP-induced cationic conductance, n ranges from 2 to 3, and the dissociation constant K_c is 10–42 μM in various studies (3–5, 22). The cGMP control hypothesis then predicts.

(i) A full size q_1 should be produced by <1 PI per 10 OS disks.

(ii) If the dark current is suppressed by light, more light should yield little more q_1 heat.

(iii) The retinal heating due to cGMP hydrolysis during transduction will be >3.5 μK . This figure follows from Eq. 3 if (a) the usual dark-current conductance is 1/15 of $g_{\max}$ (23), (b) K_c is 10 μM , (c) $n = 3$ (4, 5), (d) the outer segment cytoplasm is 7% of the sample mass, (e) cGMP diffuses freely in OS water during the transduction time¹¹, and (f) ΔH for cGMP hydrolysis is 11.2 kcal/mol.

(iv) Dark-current shutdown during a light response should be faster than q_1 heat evolution, as predicted by Eq. 3 and Fig. 6.

(v) Inhibitors of the cGMP phosphodiesterase should increase the dark level of cGMP, slow its hydrolysis in light, and raise the total q_1 heat.

The properties of q_1 agree with predictions *i*, *ii*, and *v* but not *iii* and *iv*. The slowing effect of isobutylmethylxanthine suggests that phosphodiesterase activity underlies q_1 . If q_1 is not due to cGMP splitting, one must explain the lack of heat expected from prediction *ii*. Neither the present heat measurements nor the patch clamp results conform to the larger light-induced changes in acid-extractable cGMP seen in rods (refs. 24 and 25, but see ref. 26). The discrepancy could be explained if the free energy of hydrolysis of cGMP were conserved in some coupled reaction (27), or cGMP might be bound and released unchanged (28).

How does Ca^{2+} control the dark current? Increasing intracellular Ca^{2+} concentration has long been known to suppress the dark-current conductance quickly, reversibly, and selectively (24, 29, 30). But patch clamp studies (3, 31) find that even millimolar levels of divalent cations have only weak and non-selective inhibitory effects on the conductance of isolated rod membrane patches. So it has been suggested that Ca^{2+} blocks the formation of cGMP (32, 33). The persistence of a normal q_1 when the dark current is inhibited by high external Ca^{2+} or by ouabain argues that calcium controls the dark current in some other way in dark-adapted rods.

The cooling trend, r_1 , which is reversed by high Ca^{2+} and other treatments that suppress the dark current, does not seem to be directly connected with transduction but rather with suspension during light responses of exothermic metabolic processes that sustain the dark current. The size of r_1 suggests that the retina is about 1 mK warmer than the ambient in darkness. The dark current itself has little heating effect since its flow only mixes two dilute neutral ionic

solutions (34). r_1 is clearly a composite and must be analyzed further in future work.

Note Added in Proof. A thermal response similar to q_1 was reported by Tasaki *et al.* (35).

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¹¹6-Carboxyfluorescein, whose molecular weight is close to that of cGMP, diffuses freely in OS with a radial diffusion coefficient >10⁻⁶ cm²/s (S.Y. and K. R. Spring, unpublished results).