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Thermal activation of the visual transduction mechanism in retinal rods

INVERTEBRATE photoreceptors show electrical changes which apparently result from isomerisation of single rhodopsin molecules by light or thermal energy[1–3]. Observation of corresponding phenomena in vertebrates has been prevented by intercellular electrical coupling, which averages membrane potential over many photoreceptors[4–6]. Recently, however, recordings of membrane current from individual rod outer segments have revealed responses to single photons[7,8]. Here we report that similar electrical events occasionally occur in darkness, perhaps because of thermal isomerisation of rhodopsin.

Membrane current was recorded from single rod outer segments in toad (*Bufo marinus*) retina using a method described previously[9]. Pieces of thoroughly dark-adapted retina were placed in a chamber containing oxygenated Ringer and viewed in a compound inverted microscope. Retinal isolation and subsequent procedures were carried out under infrared illumination with an IR/visible image converter. Under visual control the outer segment of a rod in one of the pieces of retina was drawn by gentle suction into the tip of a glass micropipette. A low-noise amplifier connected to the inside of the pipette gave an output voltage proportional to membrane current. This current signal, the output of a light stimulus monitor, and synchronising pulses were led to an FM tape recorder for later analysis in a PDP 11/34 computer. For 'dark' recordings the preparation was enclosed in a black box which attenuated the very dim light (low scotopic level for humans) in the experimental room by a factor of 10^6. Heated or cooled water could be circulated through a jacket surrounding the central Ringer pool in the experimental chamber; temperatures were measured with a calibrated thermistor about 0.5 mm from the tip of the recording electrode.

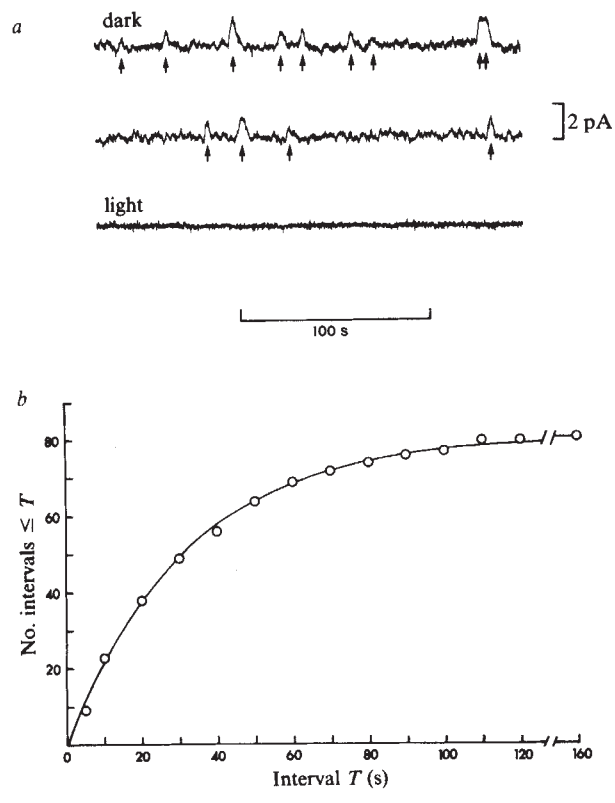

Fig. 1 *a*, Sample records of rod outer segment current in darkness (two upper traces, sequential records) and bright light (bottom trace). Arrows below traces indicate times at which current exceeded a criterion level 0.5 pA above the baseline; these times are taken as occurrences of discrete events, discussed in the text. Using this method a total of 82 events were counted over a period of 2,631 s. In light the photocurrent reached a saturating amplitude of 19 pA and no events were evident in the observation period of 1,106 s. Records were low-pass filtered at 5 Hz with a 6-pole filter. Temperature 22 °C. *b*, Cumulative distribution of intervals between successive events in the same cell shown in *a*. Circles are experimental points; curve drawn according to equation (1) in text with $\tau = 32$ s, $N = 81$.

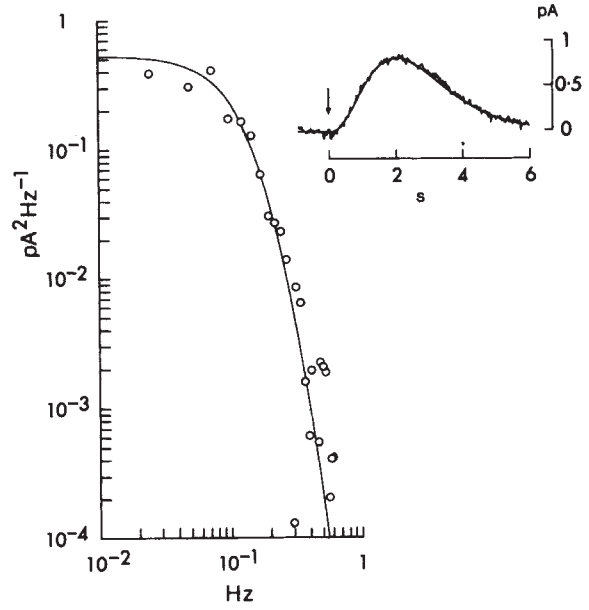

Fig. 2 Comparison of power spectral densities of dark events and single photon response in a rod. ○, Difference power spectrum of dark events obtained as spectrum of all dark sweeps minus spectrum of dark sweeps not containing events. Experimental spectra based on 43 sweeps, each 40.96 s long, digitised at 20-ms intervals after low-pass filtering at 15 Hz. Continuous spectrum calculated from the Fourier transform of equation (2) in the text, with $\alpha = 1.33\ s^{-1}$. Vertical scaling of theoretical spectrum, chosen to give best fit to points, corresponds to a mean rate of $0.022\ s^{-1}$ assuming dark events had the same amplitude (1 pA) as this cell's single photon response. Event frequency determined by counting was $0.023\ s^{-1}$. Temperature 21.6 °C. Inset, jagged curve is the average of 80 responses of the same cell to dim flashes delivering an average of 0.8 effectively absorbed photons per flash. Flash timing shown by arrow. Smooth curve is equation (2), with $\alpha = 1.33\ s^{-1}$.

Figure 1*a* shows records of outer segment current in darkness (upper traces) and bright light (bottom trace). The noise in light was of instrumental origin, and the dominant component had a magnitude predicted by the measured value of the leakage resistance between electrode wall and outer segment membrane. The excess fluctuation in the dark records consisted of two components: (1) occasional discrete events (indicated by arrows below dark traces) of about 1 pA amplitude, and (2) a continuous small-amplitude fluctuation, not discussed further here. The discrete events resembled the average response to a single photon and in this cell were estimated to occur at a mean rate of $0.031\ s^{-1}$. In the experimental conditions, however, the expected rate of photoisomerisations from stray light was at least two orders of magnitude below the observed event rate. This suggests that the events were not triggered by photons

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but reflected spontaneous activation of the transduction mechanism.

If random independent excitations occurred in the rhodopsin molecules or intracellular disks of the outer segment the intervals between events should be exponentially distributed. Figure 1*b* compares an experimental interval histogram with this prediction. The continuous curve was drawn according to

$$n = N(1 - \exp(-T/\tau)) \quad (1)$$

where n is the number of intervals less than or equal to T, τ is the average interval, and N is the total number of intervals observed. The satisfactory agreement between experimental results and equation (1) supports the interpretation that the occurrence of events is a Poisson process describable by the single parameter, τ, the average interval between events. Similar agreement between experimental results and theoretical curves was observed in seven other experiments, with a range of values for τ of 76–17 s at temperatures of 15–26 °C.

The amplitude of the dark events was similar to the amplitude of the same cell's single photon response. To compare the kinetics of dark events and single photon responses we examined their power spectra; previous work[8] has shown that the current noise evoked by dim steady light has the spectrum of the average single photon response. The spectrum of the dark events was obtained as the spectrum of all dark sweeps minus the spectrum of dark sweeps not containing events. The average single photon response in each experiment was fitted by the relation[9] (Fig. 2 inset)

$$r(t) = k(\alpha t)^3 e^{-\alpha t} \quad (2)$$

where r is the photocurrent as a function of time t after photon absorption, k is a scaling constant and α is a rate constant characteristic of the cell. This equation would apply if photoisomerisations cause electrical action by a process involving a series of four first-order delays[10,11]. The points in Fig. 2 are the spectrum of the dark events, and the curve is the calculated spectrum of the same cell's single photon response. Similarly close agreement between observed and calculated spectra was seen in experiments on eight other cells. The ability of the equation for the flash response to predict the spectrum of the dark events suggests that they are triggered by fluctuations at the input of the cascade. A simple explanation would be that the dark events result from a thermal configuration change in rhodopsin itself.

Figure 3 shows the temperature dependence of the rate of occurrence of dark events. The slope of the Arrhenius plot gives an activation energy E_a of 20.9 kcal mol^{-1} for the thermal excitation process. In similar experiments on five cells the value of E_a was 23.2 ± 3.2 kcal mol^{-1} (mean ± s.d.). The mean frequency of events in these experiments was 0.021 s^{-1} at 20 °C.

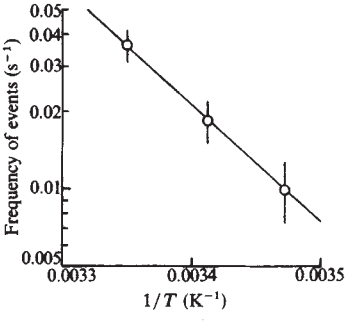

Fig. 3 Arrhenius plot of frequency of occurrence of dark events in a rod (log scale) against reciprocal absolute temperature. Detection of events in the continuous background noise was facilitated by digitally processing the current records with a 'matched filter' (ref. 15). In this procedure equation (2) was fitted to the rod's single photon response and then cross-correlated with the dark recordings. Vertical bars are based on the square root of the number of events counted. Slope of linear regression line corresponds to activation energy E_a of 20.9 kcal mol^{-1}.

Assuming that the rod outer segment contains 2×10^9 rhodopsin molecules and that thermal fluctuations in rhodopsin cause the dark events, the estimated rate constant for thermal excitation of rhodopsin is 10^{-11} s^{-1}. This corresponds to a molecular half life of the order of 1,000 yr. Additional parameters for the thermal excitation of rhodopsin at 20 °C were calculated as: Gibbs free energy of activation $\Delta G\ddagger = 31.9 \pm 0.13$ kcal mol^{-1}, entropy of activation $\Delta S\ddagger = -31.7 \pm 11.2$ e.u.

Ashmore and Falk[12], by analysis of membrane noise in neurones postsynaptic to dogfish rods, estimated values of rate constant and $\Delta G\ddagger$ similar to our own but found E_a to be 36 kcal mol^{-1} and $\Delta S\ddagger$ to be +13 e.u. The differences may reflect different experimental methods. Chemical studies show that thermal isomerisation of 11-*cis* retinal in aqueous digitonin solution[13] is associated with a small negative activation entropy of −12.5 e.u. and E_a of 24.5 kcal mol^{-1}, whereas for thermal denaturation of cattle rhodopsin in rod fragments Hubbard[14] found $\Delta S\ddagger = +214$ e.u. and $E_a = 100$ kcal mol^{-1}. Furthermore, extrapolation to 20 °C gives a rate constant for denaturation in rod fragments three orders of magnitude below the excitation rate constant derived here. Isomerisation is thus the more likely trigger of dark events. In a variety of solvents, however, derived rate constants for thermal isomerisation of 11-*cis* retinal alone are two to three orders of magnitude larger than the spontaneous excitation rate constant. This implies that linkage with opsin stabilises the 11-*cis* configuration of the chromophore.

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Photoinduced electron transport across phospholipid wall of liposome using methylene blue

PHOTOSENSITISED electron transport across membranes is a useful model reaction for photosynthesis. We have previously reported[1] the photoredox reaction in/on liposomes. Other similar studies have used chlorophyll as a sensitiser[2,3], but this compound is easily broken and difficult to purify. The surfactant analogues of metal complexes (particularly tris-(2,2′-bipyridine) Ru^{2+})[4–6] are also widely used, but with such photosensitisers which possess long alkyl chains, electron transport does not occur in the absence of electron carriers such as quinones or carotenes. We report here the occurrence of photosensitised electron transport across the single phospholipid wall of the

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