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The effects of temperature changes on rod photoresponses were studied by recording the aspartate-isolated mass receptor potential in the dark-adapted retina of the frog *Rana temporaria*. The amplitude of saturating responses, indicating the magnitude of the dark current, increased linearly with temperature in the measured range 6–26 °C, extrapolating to zero dark current at 0 °C. Sensitivity was maximal around 18 °C but the decrease towards lower temperatures was shallow. The results show that rod phototransduction in the frog *Rana temporaria* is adapted to lower temperatures than in the tropical toad *Bufo marinus*. Responses to dim flashes were, approximately up to peak, well fitted by the same 'independent activation' model with four delay stages as have been found to best describe current responses from single toad rods. The kinetics (reciprocal time-to-peak) showed Arrhenius-type temperature-dependence with apparent activation energy 12.4 kcal mol^{-1} and $Q_{10} = 2.1$.

Key words: adaptation, amphibians, photoreception, temperature effects, vision.

Warming accelerates the photoresponses of vertebrate photoreceptors and increases the dark current (Penn & Hagins 1972, Baylor *et al.* 1974). The temperature-dependence of the photocurrent of single rods in the toad *Bufo marinus* has been studied in detail by Baylor *et al.* (1983) and Lamb (1984), who found that the dark current depended approximately linearly on temperature over at least the range 10–30 °C; extrapolating to zero at 4–5 °C. The responses to dim flashes were found to get faster with rising temperature, but to preserve their characteristic waveform. Sensitivity was found to peak around 22 °C.

In the present work we determine the effects

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of temperature on rod photoresponses of the frog *Rana temporaria*, which lives in a considerably cooler environment than *Bufo marinus*. It hibernates in lakes at temperatures which may even be lower than those where the extrapolated dark current of the toad drops to zero. Our main object was to investigate in what respect adaptation to lower temperatures is evident in the photocurrent of *Rana temporaria*.

Since we recorded the aspartate-isolated mass receptor potential across the intact retina, the present work also gives information on the non-receptor contribution to the mass receptor potential at different temperatures. We confirm that in dim flash responses the rising phase and the amplitude are almost purely determined by receptor activity, while in saturating responses a considerable fraction of the amplitude may be due to glial currents.
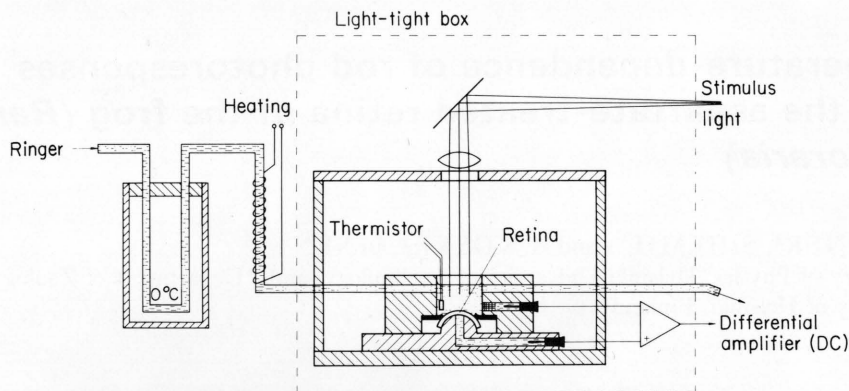
Fig. 1. The specimen chamber and the system for temperature regulation.

MATERIALS AND METHODS

Animals. The experiments were done on isolated, dark-adapted retinas of common frogs (*Rana temporaria*). The frogs had been caught in October in southern Finland and stored in basins at 3–4 °C without feeding (hibernating conditions). The frogs were slowly ($\approx$ 15 h) warmed to about 16 °C before dissection. Both the retinal rods and ganglion cells and the visual behaviour of these frogs display very high dark-adapted sensitivity to light (Hemilä 1977, Aho *et al.* 1987, Donner 1988).

Preparation, perfusion, recording and stimulation. The excised eye was cut open and the cornea, iris and lens were removed under dim red light. The retina was isolated in cooled Ringer and receptors placed upwards in a specimen holder (Fig. 1; cf. Donner & Hemilä 1985). The upper (receptor) side was perfused by frog Ringer solution containing (in mM): NaCl 95; KCl 3; CaCl$_2$ 0.9; MgCl$_2$ 0.5; sodium phosphate buffer 12 (pH 7.5); glucose 10 and sodium aspartate 2. The addition of aspartate serves to isolate the receptor responses by breaking synaptic transmission to second-order neurons (Sillman *et al.* 1969), but it does not, unfortunately, abolish all other light-evoked currents (Hemilä 1983).

The stimulus light passed through a narrow-band 493 nm interference filter. Stimuli were 0.1 s flashes. The intensity was controlled with neutral density filters and wedges; intensities were originally calibrated against a spectrophotometrical measurement of the rate of rhodopsin bleaching.

The mass receptor potential was recorded with two Ag/AgCl electrodes, one connected to the Ringer space beneath the retina and the other in chloride solution connected to the perfusion fluid through a porous plug. The DC-amplified signal was recorded on slow and fast pen recorders.

Signal analysis. The recorded signal is the transretinal voltage drop caused by extracellular radial currents in the retina (see Hagins *et al.* 1970). In order to obtain the temperature-dependence of those *currents* one has to correct the voltages by the conductivity of the extracellular fluid at the appropriate temperature. Since the conductivities of ionic solutions are nearly linearly related to temperature in our range of temperatures, 6–26 °C, the response amplitudes were scaled by the linear conductivity factor $1 + \alpha(\theta - \theta_0)$, where $\theta_0 = 12$ °C is the chosen reference temperature, θ is the response temperature, and α is 0.026 °C^{-1} (see CRC Handbook of Chemistry and Physics).

At each temperature the stimulus–response function was determined by recording responses at three intensity levels: a response to a dim flash, an approximately half-saturating flash and a flash giving about 85 % of the saturating amplitude U_{max}. In a log U *vs.* log I plot (U = response amplitude, I = intensity) the best-fitting stimulus–response curve from a family of z-curves (one type of generalized Michaelis curves, see Bäckström & Hemilä 1979, Hemilä 1987) was fitted to the points. From the apex point of that curve log U_{max} and the logarithm of the relative sensitivity to dim flashes, $S = (U/U_{max})/I$, are obtained. The *absolute* dim-flash sensitivity (i.e. response amplitude/isomerization) is then $S_a = U/I = SU_{max}$, or in logarithmic form

$$\log S_a = \log S + \log U_{max}. \tag{1}$$

When the *temperature-dependences* of U_{max} and S_a are considered, we use normalized values U_{max} (rel) and S_α, scaled to unity at 12 °C:

$$\log S_\alpha = \log \frac{S_a(\theta)}{S_a(12 \,°C)}$$

$$= \log \frac{S(\theta)}{S(12 \,°C)}$$

$$+ \log \frac{U_{max}(\theta)}{U_{max}(12 \,°C)} \tag{2}$$

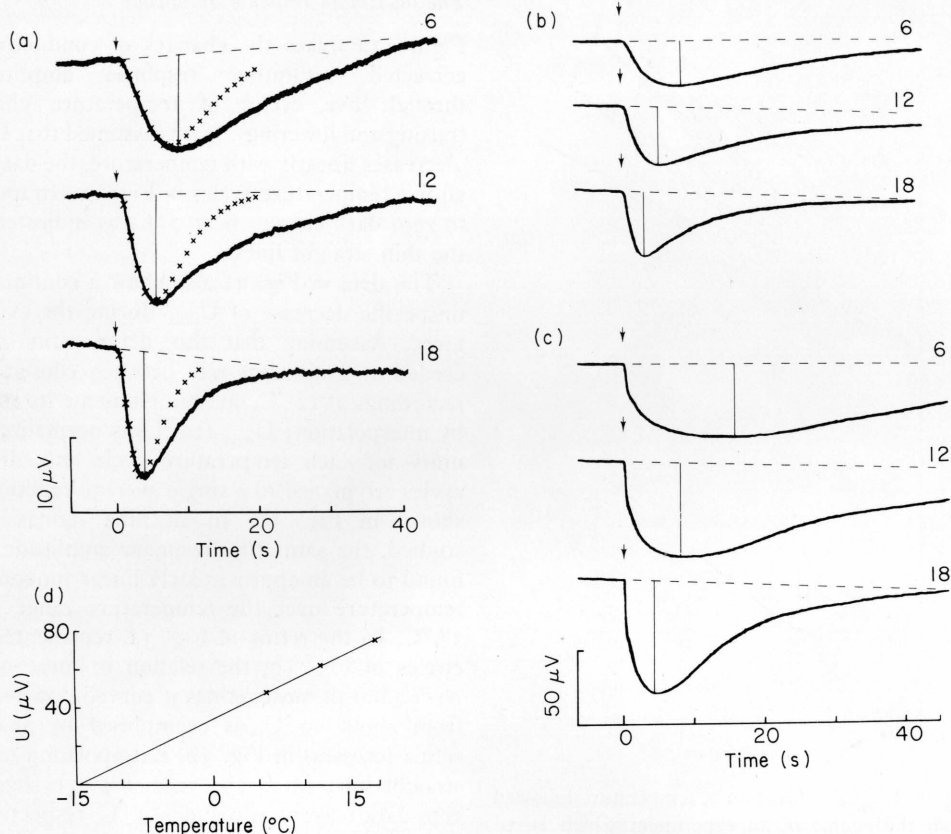*Temperature changes.* The temperature of the Ringer solution perfusing the retina could be varied in

Fig. 2. Photoresponses at three temperatures: 6 °C, 12 °C and 18 °C (a) responses to dim flashes, log I = 0, which corresponds to ca. seven photo-isomerizations per rod; (b) about half-maximal responses, log I = 0.7; (c) nearly saturating responses, log I = 2.0. (d) the amplitudes of the nearly saturating responses as a function of temperature.

Crosses in (a) are theoretical responses calculated according to the independent activation model, using $n = 4$ and choosing the rate constant α and the sensitivity S_a so that the rising phase of the calculated response fits the recorded waveform.

the range 6–30 °C with an estimated accuracy of 0.5 °C. Temperature was monitored with a small calibrated thermistor positioned about 1 mm above the retina. Control measurements with a thin-wired thermocouple showed that even during changes of temperature the spatial temperature variation of the perfusing fluid in the specimen holder was negligible. After a change of the current passed through the heating coil (see Fig. 1) it took 2–4 min before the temperature stabilized; until the recording baseline was reasonably stable, usually for 4–5 min. By repeated recordings of responses to the same intensities we checked that the rods were in a stationary condition. The inter-stimulus interval was 2 min at low temperatures and 1.5 min above 12 °C.

RESULTS

Qualitative effects of temperature on photoresponses

Figure 2 shows original recordings of photoresponses obtained from one dark-adapted retina at three different temperatures (6, 12 and 18 °C). Warming from 6 to 18 °C increased U_{max} 1.5-fold (Fig. 2c). As seen in the inset (d) of Fig. 2, direct linear extrapolation of the near-saturating response amplitude would indicate zero response about −15 °C. However, if response amplitudes are corrected for the temperature-
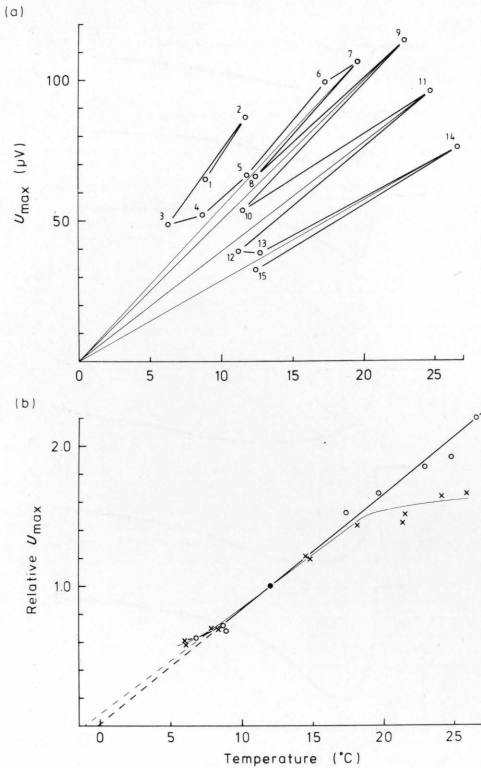
(a)

(b)

The maximum response amplitude

Figure 3a traces the changes of conductivity-corrected saturating response amplitudes through five cycles of temperature change (raising and lowering). If it is assumed that U_{max} decreases linearly with temperature, the data for all five temperature cycles in Fig. 3a extrapolate to zero dark current near o °C, as indicated by the thin straight lines.

The data in Fig. 3a also show a continuous, unspecific decrease of U_{max} during the experiment. Assuming that this deterioration proceeded at a constant rate between consecutive recordings at 12 °C, one can eliminate its effects by interpolation; U_{max} (12 °C) is normalized to unity for each temperature cycle and all the cycles are pooled to a single average function as shown in Fig. 3b. In all nine retinas thus studied, the saturating response amplitude was found to be an approximately linear function of temperature over the temperature range 8 to 18 °C. In the retina of Fig. 3a, represented by circles in Fig. 3b, the relation is linear up to 26 °C, but in most retinas it curved downwards from about 20 °C, as exemplified by another retina (crosses) in Fig. 3b. Extrapolation of the straight lines fitted by eye in Fig. 3b suggests zero dark current at o and −1 °C, respectively. The mean temperature of extrapolated zero dark current was o ± 0.5 °C ($n = 9$). However, at the lowest temperature where responses were actually recorded, near 6 °C, the saturating amplitude observed was invariably larger than expected from the straight-line relationship.

Sensitivity

Unspecific drift of sensitivity during experiments was eliminated in the same way as for U_{max}, by always referring sensitivity to its value at 12 °C, which was scaled to unity. This normalized absolute sensitivity S_α is given by Equation (2).

Figure 4 shows log S_α as a function of temperature for a typical retina. The sensitivity function has a broad maximum, in this case around 17 °C (mean value 18 °C, range 16–22 °C in six retinas). From there, S_α fell off with accelerating steepness towards higher temperatures. Sensitivity also decreased towards lower temperatures, but the function always levelled off about 12 °C, then remained nearly constant down to the lowest temperature measured (6

Fig. 3. (a) U_{max} as a function of temperature followed through the course of an experiment which lasted nearly 3 h. The order in which the photoresponses were recorded is indicated beside the points, which have been connected with bold straight lines. Thin straight lines have been drawn from the origin to each high temperature point. Correction has been made for the changes in ionic conductivity with temperature. (b) U_{max} (rel) (i.e. U_{max} scaled to unity at 12 °C, see Text) as a function of temperature. Circles: the same responses as in Fig. 3a. Crosses: another retina showing deviation from linear relation at temperatures above 18 °C. The temperatures corresponding to extrapolated zero current are o °C (circles) and −1 °C (crosses).

dependence of ionic conductivities (see Methods), a steeper temperature-dependence obtains (cf. Fig. 3).

Figure 2 also shows that increasing temperature from 6 to 18 °C accelerated response kinetics. In responses to dim flashes (Fig. 2a) the time to peak fell from about 9.3 s to 3.7 s and the decay got steeper. Even in the nearly saturating responses (Fig. 2c) both the rise and the decay were steeper at the higher temperatures.
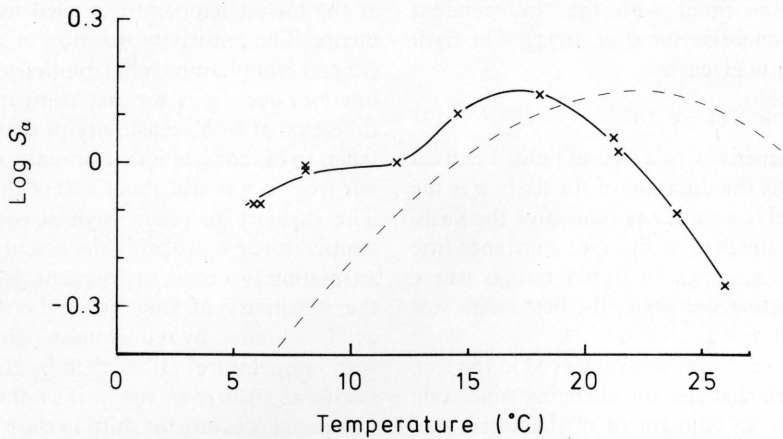
Fig. 4. Crosses and full-drawn curve: the temperature-dependence of sensitivity (i.e. response amplitude/isomerization) in a typical frog retina with maximum sensitivity at 17 °C. Ordinates: log normalized sensitivity, log S_α. Interrupted curve: the temperature-dependence of sensitivity in *Bufo marinus*, taken from Fig. 11 in Lamb (1984). This curve has been positioned vertically to the same peak level as for the full-drawn curve.
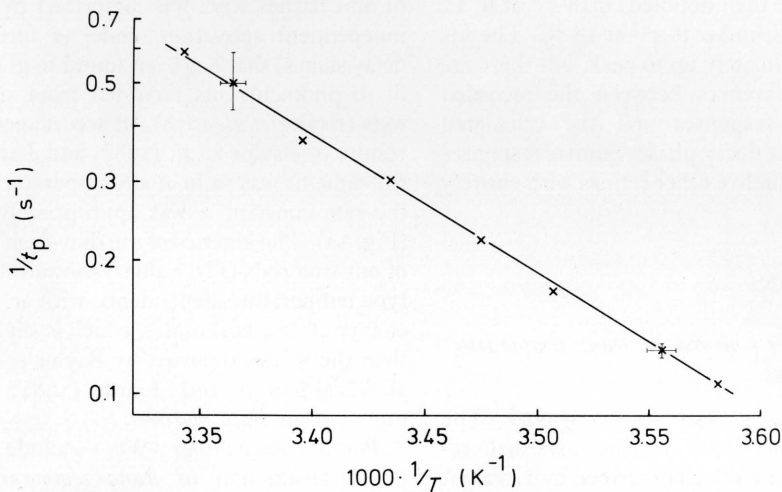

Fig. 5. Log $1/t_p$ as a function of $1/T$ for one retina (Arrhenius plot). The activation energy E_a = 14.2 kcal mol^{-1}.

°C). On average, S_α at 6 °C was still about half of its maximum value.

Kinetics of the photoresponse

The time from the onset of a flash to the peak of the response, t_p, is a useful, directly accessible measure of the speed of the response. Warming makes response faster, implying that t_p decreases.

Figure 5 is an Arrhenius plot of log $1/t_p$ as a function of $1/T$ (T = absolute temperature) from a typical retina. The points fall approximately on a straight line over the whole range from 6 to 26 °C. The slope gives the activation energy, for which we obtained the mean value 12.4 kcal mol^{-1} (range 9.3–14.8 kcal mol^{-1}, six retinas). Mean Q_{10} (determined between 10 and 20 °C) was 2.1.

To investigate possible temperature effects on the waveform of the dim flash response, the

rising part was fitted with the 'independent activation' model (Baylor *et al.* 1974). The flash response R(t) is given by

$$R(t) = S_s I \Delta t\, n e^{-\alpha t} (1 - e^{-\alpha t})^{n-1} \qquad (3)$$

where S_s is sensitivity to a step of light, I is flash intensity, Δt is the duration of the flash, n is the number of delay stages, t is time after the flash, and α is the smallest of the rate constants (the others being $2\alpha, \ldots n\alpha$). In all five retinas where this type of fitting was done, the best result was obtained with $n = 4$.

This basic waveform was preserved in the face of temperature changes, the changing time scale requiring only an adjustment of the single rate parameter α. In Fig. 2a, the dim flash responses recorded at 6, 12 and 18 °C are compared with theoretical responses calculated according to Equation (3). ($n = 4$, parameters S_s and α chosen for optimal fit to the rising phase of the response). In this temperature range the rate constant α more than doubled: $0.18\ s^{-1}$ at 6 °C, $0.26\ s^{-1}$ at 12 °C, and $0.38\ s^{-1}$ at 18 °C. The fits are good approximately up to peak, but there are pronounced differences between the recorded mass receptor responses and the calculated curves during the decay phase. Similar response-fitting was done in five other retinas with entirely similar results.

DISCUSSION

Temperature effects on rods in Rana temporaria and Bufo marinus

The amplitude of saturating responses. The temperature-dependence of the maximal response amplitude was well described by a straight line, typically over the range from 8 to 20 °C. This basically agrees with the results of Baylor *et al.* (1983) and Lamb (1984). However, the temperature-dependence extrapolated to zero dark current at about 0 °C in *Rana temporaria*, which is significantly lower ($P < 0.001$, *t*-test) than the lowest value, 4 °C, reported from *Bufo marinus*. Moreover, below 8 °C frog U_{max} values were always larger than predicted by the straight line.

Sensitivity. The sensitivity function likewise indicates that phototransduction in *Rana temporaria* is adapted to low temperatures. The decrease of log S_α with cooling was shallow and levelled off to approximately constant sensitivity

at the lowest temperatures used in our experiments. The sensitivity function of *Bufo marinus* (Fig. 11 from Lamb (1984), plotted as interrupted line into our Fig. 4 for easy comparison) is very different: at 6 °C, sensitivity in that species had fallen to ca. 20 % of its maximum value, while in our frogs it was still about half of the maximum. The capacity to retain high sensitivity at low temperatures is probably the essential feature in adaptation to a cool environment. It appears that the sensitivity of frog vision between 10 and 20 °C is limited by retinal noise (which increases with temperature) rather than by the size of rod responses (Aho *et al.* 1988). If so, there can be no functional pressure for shifting the rod sensitivity *peak* to low temperatures. Indeed, in our experiments, sensitivity peaked about 18 °C on average, which is considerably higher than the frog's usual body temperature, although still ca. 4 °C lower than in *Bufo marinus* (Lamb 1984).

Kinetics. The rising phases of photoresponses to dim flashes were well described by the same independent activation model (a filter of four delay stages) that has been found to give the best fit to photocurrents recorded from single toad rods (Baylor *et al.* 1979). In accordance with the results of Baylor *et al.* (1983) and Lamb (1984) the same fit was valid at all temperatures, if only the rate constant α was appropriately adjusted (Fig. 2a). The kinetics of the dim-flash responses of our frog rods ($1/t_p$ values) showed Arrhenius-type temperature-dependence with an activation energy of 12.4 kcal mol^{-1}, which is slightly lower than the values reported by Baylor *et al.* (1983; 16.8 kcal mol^{-1}) and Lamb (1984; 13.8 kcal mol^{-1}) from *Bufo marinus*.

Possible mechanisms. We conclude that rod phototransduction of *Rana temporaria* hibernating in Finland is indeed adapted to lower temperatures than that of the tropical toad *Bufo marinus*. At this stage, however, one can merely speculate on the mechanisms. Both the rise and the decay of light responses are determined by interlocked chains of reactions involving diffusion of molecules in the disc and plasma membranes and in the cytoplasm. Thus, changes (and differences) in the viscosity of the membranes may be the most obvious candidate. It is known that acclimation to a cold environment can be associated with increased membrane fluidity (e.g. Cossins 1977) and also that membrane viscosity compared across species is in general inversely related to body temperature

(Cossins & Prosser 1978). The Arrhenius-type behaviour found for response kinetics is consistent with such a mechanism which could affect all the rate constants in the transduction cascade equally. The difference between activation energies in our frogs and *Bufo marinus* goes in the right direction, but appears too small.

However, it is also known that the substrate affinities of enzymes vary with temperature in specific manners, possible effects of which can be neither predicted nor excluded in the present case. It is worth bearing in mind that dark current (reflected in U_{max}) and sensitivity are determined by partly different mechanisms, and do indeed show quite different temperature-dependences.

The glial contribution at different temperatures

The goodness of fit of the independent activation model to the rising phases of our dim flash responses suggests that this model (dotted curves in Fig. 2a) represents the true time course of the receptor part of the mass receptor potential; the difference between the later phases of recorded and calculated responses represents the glial contribution. Above 10 °C there is an appreciable difference only *after* t_p, indicating that both the amplitude and the time-to-peak of small responses are practically free from glial intrusion (c.f. Donner & Hemilä 1985). At equal temperatures the t_p values of *Rana* mass receptor responses and *Bufo* single-rod current responses are similar. From Fig. 5 of the present paper, t_p of dim flash responses is seen to be about 2.0 s at 24 °C, while the typical t_p observed by Lamb (1984) at 24 °C in toad was 1.6 s.

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