HEAT PRODUCED BY THE DARK-ADAPTED BULLFROG RETINA IN RESPONSE TO LIGHT PULSES

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ABSTRACT By using a pyroelectric detector constructed with a polyvinylidene fluoride film, a rapid rise in the temperature of the dark-adapted bullfrog retina induced by light was demonstrated. In the bullfrog retina, as in the squid retina examined previously, the heat generated in response to a brief light pulse was found to be far greater than the amount produced by conversion of the entire radiant energy of the stimulus into heat. The thermal responses consist of the heat generated by the photoreceptor and the postsynaptic elements in the retina, preceded by a small signal reflecting conversion of a portion of the radiant energy of the stimulus into heat. The dependence of the thermal responses on the light intensity, on the wavelength and on a variety of physical and chemical agents was examined. The exothermic process underlying the production of heat by the photoreceptor was found to precede the electrophysiological response of the retina.

INTRODUCTION

In recent years, high time-resolution thermal detectors using pyroelectric material for sensing heat became available (see e.g. Roundy, 1975). Polyvinylidene fluoride (PVDF) is a synthetic polymer which, after poling, has a large persistent polarization (see Murayama, 1975). By using a thermal detector constructed with a thin PVDF film, the production of heat associated with excitation of a crab nerve has been detected with a time-resolution of ~1 ms (Tasaki and Iwasa, 1981), and the existence of a rapid thermal response in the dark-adapted retina of the squid has been demonstrated (Tasaki and Nakaye, 1985). The availability of thermal detectors of this type has opened up the possibility of investigating the sequence of physiological events in the vertebrate retina by taking the evolution of heat as an index.

The structure of the vertebrate retina is more complex than that of the squid retina. In the vertebrate photoreceptor, the visual pigments are bound to the disk membrane known to be separated from the plasma membrane (Cohen, 1970), while in the squid eye the photopigments are located in a specialized portion of the plasma membrane known as microvilli (Zonana, 1961; Cohen, 1972). Furthermore, the vertebrate retina contains, in addition to photoreceptor cells, a variety of neuronal and glial cells. In contrast, the squid retina consists almost exclusively of the photoreceptor cells. Because of the structural complexity of the vertebrate retina, detection and analysis of thermal responses in the bullfrog retina would be expected to be difficult.

The present study shows that it was possible to overcome technical difficulties encountered in recording thermal responses from the excised bullfrog retina. By analyzing a large number of records of thermal signals obtained under a variety of conditions, it was demonstrated that the signals consist of the following components: (*a*) the heat derived from direct conversion of a small portion of the radiant energy; (*b*) the heat reflecting the physiological activity of the photoreceptor cells; and (*c*) the heat generated by the postsynaptic elements. Evidence is presented that the exothermic process in the photoreceptor precedes the production of the electrical response by the receptor. The significance of this and other findings is discussed.

METHODS

Isolated retinae of the bullfrog, *Rana catesbeiana*, were used. After dark-adaptation of the animals, the eyes were removed under illumination with dim red light. Following resection of the cornea and the lens, the retina was carefully detached from the sclera and the pigment epithelial layer in oxygenated Ringer's solution. The composition of Ringer's solution most frequently used contained 111 mM NaCl, 1.5 mM KCl, 1.2 mM $CaCl_2$, 5 mM glucose, 10 mM Hepes, pH 7.4. A rectangular piece of the retina, ~7 × 7 mm^2 in area and weighing 17–20 mg, was used for detection of heat generation initiated by stimulation with a brief light pulse.

The heat evolved by the retina was measured with a detector constructed by using 9-μm-thick polyvinylidene fluoride (PVDF) film which was a gift of Kureha Chemical Co., Horidome-cho, Tokyo. The design of the detector was essentially the same as that employed previously in this laboratory (Tasaki and Nakaye, 1985). A retangular piece of PVDF film, ~7 × 7 mm^2 in area, was glued first to a 25 μm thick platinum plate of the same size by using a very thin (~1 μm thick) layer of Epoxy resin. The platinum plate was glued further to the aluminum-deposited surface of a large Mylar sheet of 5 μm in thickness: the aluminum layer on the Mylar sheet was ~10 nm in thickness. (The purpose of inserting a metal plate between the Mylar and PVDF films was to ensure a uniform rise in the temperature of the PVDF film, as well as to reduce mechanical disturbances arriving at the film.) In the

detector, the Mylar sheet carrying the PVDF film formed a partition that separated the upper (sample) compartment of the detector from its lower compartment containing an operational amplifier. The top of the detector was covered with a plastic plate provided with a glass window. The two aluminum layers on the PVDF film in the lower compartment were connected to the operational amplifier (AD 515) which had a high (10^{10} Ω) feedback resistance and a small capacitor ($\sim 10^{-12}$ farad). The output of the amplifier was led to a signal averager (model 1070; Nicolet Instrument Co.) through a capacity-coupled amplifier with a gain of 100 and a time-constant of 2 s.

The isolated retina was introduced into the upper compartment of the detector with its receptor side making contact with the heat-sensitive portion of the Mylar sheet. Oxygen, humidified by passing through an isotonic salt solution, was circulated above the retina. The stimuli employed were in most cases pulses of quasi-monochromatic light from a 100 W quartz-iodine lamp used in conjunction with an electromagnetic shutter, a heat filter, an interference filter, and neutral-density filters. The light was led to the glass window of the detector with a bundle of optical fibers. The intensity of the light used was calibrated with a photometer (model 550; E G & G Electro-optics, Salem, MA). The light stimuli were repeated usually at intervals of 10–14 s, and records of thermal responses were taken after signal averaging over four to 32 trials.

The amplitude of the signal recorded with a pyroelectric detector is proportional to the rate of change of the temperature (Roundy, 1975; Tasaki and Nakaye, 1985). The rate of temperature change could be converted into the temperature by using the integrating device incorporated in the signal averager employed. The detector was calibrated by using a light pulse of a known intensity absorbed by a dye solution of 400 μm in thickness covering the heat-sensitive area of the detector. The concentration of the dye (a mixture of chlorophenol red and phenol red) was such that ~10% of the light energy was transmitted through the solution (Tasaki and Nakaye, 1985). A pulse of light of a constant intensity generated a signal with a rectangular time-course that was rounded off both at the beginning and at the end of the pulse. The distortion of the recorded signal is associated primarily with the thermal and electrical time-constants of the detector. The electromagnetic shutter also introduced a slight distortion accompanied by a delay of 3 ms. (To improve the signal-to-noise ratio in recordings, a relatively long time-constant, ~10 ms, was adopted in the electrical feed-back system in the present study.) The thermal time-constant was determined primarily by the distance from the source of heat in the retina to the detector surface. For the photoreceptor cells, of which the distal ends are in contact with the surface, the delay caused by conduction of heat is shorter than 10 ms (see Discussion). (Note that the thermal diffusivity of water is 1.44×10^{-3} cm^2/s [Carslaw and Jaegar, 1959, p. 497].)

In studying properties of thermal responses of the bullfrog retina to light stimuli, it was found necessary to elucidate the origin of the signals arising from conversion of the stimulating light directly into heat. To achieve this aim, the spectrum of the light transmitted through the isolated retina was determined by the "opal-glass transmission method" (see Shibata, 1959; see also Liebman, 1962). A dark-adapted retina prepared in normal Ringer's solution was mounted on a sheet of parafilm with its photoreceptor side facing the film, and a continuous flow of humidified oxygen was maintained above the retina. The tip of the light guide was placed ~20 mm above the retina. The light transmitted through the preparation was detected by using another light guide that was placed under the parafilm layer and was led to a photodiode. (An example of the results obtained is presented later; see Fig. 2.)

These measurements have indicated that the light of 650–700 nm in wavelength is attenuated predominantly by light scattering. Around 500 nm, the effect of strong (~75%) absorption by the photopigments is superimposed upon the attenuation by scattering. From these findings it is expected that relatively intense radiation reaches the aluminum layer of the detector when pulses of light with long wavelengths were employed for stimulation. The radiant energy absorbed by the metal layer is converted immediately into heat. It was found that the absorption coefficient of radiant energy by the aluminum layer of the detector was ~20% at 650 nm and ~30% at 500 nm. It will be shown later that, with very brief pulses of 500 nm light, the light absorbed by the photopigments of the retina makes a very little contribution to the short-latency signal designated as "direct heat."

In a series of unsuccessful experiments, attempts were made to detect the heat produced by rod outer segments freshly detached from dark-adapted retinae by the procedure described by Bownds and Brodie (1975). The salt solution in which the outer segments were detached was (*a*) solution B of Bownds and Brodie, (*b*) a 1:1 mixture of normal Ringer's solution and isotonic $MgCl_2$ solution, or (*c*) K^+-rich solution prepared by mixing isotonic K-phosphate solution with Ringer's solution at various ratios. The outer segments detached from two dark-adapted retinae were precipitated on the surface of a 7×7 mm^2 Kimwipe tissue (~1.5 mg) by centrifugation at 2,000 rpm (3–5 min). The precipitate (~15 mg) containing a large number ($\sim 10^6$) of outer segments was brought in contact with the surface of the detector. Only signals representing the heat produced by direct conversion of the radiant energy (i.e., direct heat) was observed under these conditions. The possible significance of this finding is discussed later in the Discussion.

RESULTS

Components of Thermal Responses

Using the pyroelectric detector described under Methods, it was found possible to record signals representing the heat evolved by the dark-adapted retina in response to light stimuli. The light stimuli employed in most of the present study were 500 nm in wavelength and 6 ms in duration. The light intensity was kept usually at a level between 1 and 6 $\mu W/cm^2$. The top trace in Fig. 1 *A* represents the rate of rise in the temperature of the retina evoked by a light pulse. The middle trace, representing the time course of the temperature rise, was derived from the top trace by integration. These records indicate that the heat signal taken from the retina consists of several components.

From the outset, it appeared probable that the radiant energy of the stimulus gives rise to a small artefact. Note that ~7.5% of the radiant energy of 500 nm light is absorbed by the aluminum layer of the detector and that this energy raises the temperature of the PVDF film before the heat is dissipated by conduction into the retina (see Methods). The major portion of the stimulating light is absorbed by the visual pigment, rhodopsin. However, in the absence of exothermic physiological processes within the retina, the temperature rise caused by the radiant energy absorbed by rhodopsin is not expected to exceed the level observed with an inert dye solution introduced into the detector (see Fig. 4 in Cooper and Converse, 1976). The temperature rise observed in the presence of a dark-adapted retina in the detector was found to surpass the level ($\sim 5 \times 10^{-7}$ deg) expected from the total conversion of the radiant energy (6×10^{-6} W/cm^2 6 ms) into heat. Evidently, the slow, upward deflection in record A represents the heat associated with physiological responses of the photoreceptor and of the postsynaptic elements in the retina.

It is known that addition of Mg-salt to the surrounding medium tends to suppress the electrical responses of the

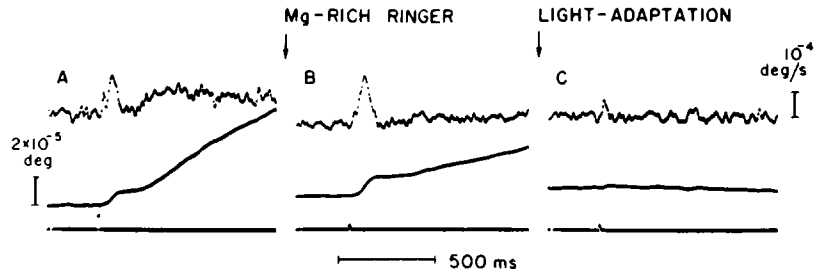

FIGURE 1 Records of the temperature change (expressed in degrees centrigrade per second) and of the temperature (expressed in degrees centrigrade) of a dark-adapted bullfrog retina observed following the delivery of a light pulse (indicated by the bottom trace) of 500 nm in wavelength, 6 ms in duration and ~6 $\mu W/cm^2$ in intensity. The *middle* trace representing the temperature was derived from the *top* trace by time integration. (*A*) Record taken from a retina shortly after isolation in normal Ringer's solution. (*B*) Record taken from the same retina after immersion in Mg-rich Ringer's solution. (*C*) Record taken after 2 min exposure of the retina to room light. Temperature is 20°C.

neurons in the retina by blocking the synaptic transmission between the photoreceptors and the bipolar cells (Winkler, 1972; Pinto and Pak, 1974). Hence, a 1:1 mixture of an isotonic (84 mM) $MgCl_2$ solution and normal Ringer's solution was applied to the preparation for the purpose of distinguishing the heat production by the postsynaptic elements from that generated by the photoreceptor cells. Fig. 1 *B* shows the record obtained after immersion of the retina in the Mg-rich medium for a period of ~10 min. It is seen that the late (sustained) heat production was strongly suppressed by the Mg treatment. Consequently, the late component of the observed signal is interpreted as representing the heat generated by the postsynaptic elements in the retina. The heat evolved after a very short latency is explained, accordingly, as consisting of the direct heat of the stimulus and the heat generated by the photoreceptor cells.

From the previous study of the squid retina (Tasaki and Nakaye, 1985), the heat generated by the photoreceptor cells in response to light is expected to be strongly suppressed by light-adaptation. Fig. 1 *C* shows the records taken after ~2 min exposure of the retina under study to the room light (~50 $\mu W/cm^2$). It is seen that heat production was completely suppressed except during the short period immediately after the delivery of the stimulus. Hence, the earliest heat signal is regarded as being generated by direct conversion of the radiant energy into heat. The dominant signal that was suppressed by light adaptation may now be designated as the "receptor heat." Immersion of the retina in modified Ringer's solution containing 20–40 mM $MgCl_2$ was found to enhance the receptor heat by a factor of 1.7 to 2.

The rate of rise of the recorded signal representing the "direct heat" is determined by the electrical time-constant of the detector. Upon termination of the brief light pulse, the heat generated by absorption of the radiant energy by the aluminum layer of the detector is rapidly dissipated by conduction into the retina; the falling phase of the direct heat signal is determined by this process of heat conduction (see later).

Under the present experimental conditions, the rate of temperature rise associated with the receptor response varied between 7×10^{-5} deg/s and 18×10^{-5} deg/s at the peak and the temperature rise between 3×10^{-6} deg and 9×10^{-6} deg at the end. The receptor response appeared to terminate abruptly. (It was not clear, however, whether the slow temperature rise observed in Fig. 1 *B* is due to the residual heat arising from the receptors or not.) The duration of the response, i.e., the time from the onset to the point of an abrupt change in the slope at the end, was in the range between 95 and 120 ms. The half-maximum duration of the thermal response of the receptor generated by a brief pulse was usually 40–50 ms (at 20°C).

By using inverted retinae so that the vitreal side was in contact with the heat-sensitive surface of the detector, the temperature of the other side was found to gradually rise and approach the temperature of the receptor side 500–600 ms after the delivery of the brief light pulse. This finding indicates that the source of heat in the Mg-treated retina is localized in the layer of the photoreceptor cells.

Effects of Varying the Stimulus Intensity

The dependence of the magnitude of the heat signal on the light intensity was examined in the range between 0.05 and 30 $\mu W/cm^2$ by using light pulses of 500 nm in wavelength and 6 ms in duration. The peak amplitude of the receptor heat varied with the light intensity employed. In the range of intensities 2 $\mu W/cm^2$ at the position of the retina, the response amplitude was found to vary by a factor of 1.3 ±0.1 when the intensity was changed by a factor of 2. The intensity-dependence was found to decrease at high light intensities. However, no clear saturation of the receptor heat was observed even when the intensity of the 6 ms stimulus approached 30 $\mu W/cm^2$. The time intervening between the onset of the stimulus and the peak of the receptor heat was ~63 ms when the intensity was ~6 $\mu W/cm^2$; it decreased to ~42 ms when the intensity was raised to 25 $\mu W/cm^2$. The latency, i.e., the time from the stimulus to the onset of the receptor heat, was too short to be determined with the present experimental setup accurately; it was close to 10 ms or less. As expected, the

magnitude of the direct heat was found to be proportional to the stimulus intensity.

It is known that, in vertebrate retinae immersed in normal Ringer's solution, strong light pulses evoke markedly prolonged electric responses of the photoreceptors (see, e.g., Penn and Hagins, 1972; Gold and Korenbrot, 1980). Under the present experimental conditions, no marked change in the duration of the thermal response of the photoreceptors was observed in the range of intensity employed. No difficulty was encountered in recording thermal response repeated at 10–14 s intervals in the present study. At extremely low light intensities, the response duration was increased.

Dependence of Heat Signal Amplitude on Wavelength

The dependence of the magnitude of the thermal responses upon the wavelength of the light was investigated by using retinae isolated in a 1:1 mixture of an isotonic $MgCl_2$ solution and normal Ringer's solution. The intensity of light pulses at 6 ms in duration was adjusted to a level of ~9 $\mu W/cm^2$ at every wavelength. Five retinae were used for this purpose.

Fig. 2 *A* was constructed from the data thus obtained. Here, the averaged values of the amplitude were plotted against the wavelength of the stimulating light. It is seen in the figure that the action spectrum has a peak at ~500 nm. This spectrum is now compared with the transmission spectrum (curve *B* in the figure) determined by the opal-glass transmission method (see Methods). This spectrum indicated the existence of a distinct peak of absorption at ~500 nm, which is very close to the absorption maximum of rhodopsin in the frog red rods (Harosi, 1975). It is thus clear that the thermal responses of the photoreceptor cells are initiated by absorption of light by the rhodopsin molecules in the red rods.

Effect of Sodium-ion Deprivation

It is well known that the process of receptor potential generation is completely suppressed by elimination of sodium ions in the medium (Furukawa and Hanawa, 1956; Sillman et al., 1969; Winkler, 1972; Hagins and Yoshikami, 1975; Yau et al., 1981; and others). In the present study, the effect of eliminating sodium ions from the medium on the thermal response of the photoreceptor cells was examined by using retinae isolated in a 1:1 mixture of an isotonic $MgCl_2$ solution and normal Ringer's solution.

After recording a thermal response from such a preparation (see Fig. 3), a sodium-free (magnesium-rich) salt solution, prepared by replacing NaCl in the medium completely with $MgSO_4$, was introduced into the upper compartment of the detector. During the following 10–15 min period, the Na-free salt solution (~1.5 ml), in which the retina under study was suspended, was gently stirred by means of 0_2 bubbles. At the end of this period, the retina was placed on the heat-sensitive area again, the major portion of the solution in the detector was removed and a thermal response was recorded. It was found by this procedure that the ability of the receptors to develop thermal responses was not eliminated by Na-ion deprivation.

The effect of Na-ion deprivation on the thermal response was compared with that on the electrical response of the receptors. To record electrical responses, the retina was placed between two compartments of a plastic chamber. The potential difference between the two pools of the Na-containing (Mg-rich) solutions separated by the retina was recorded with a pair of silver electrodes connected to a differential amplifier. The receptor potentials recorded under these conditions were relatively small because of the presence of a high Mg-ion concentration in the medium. After recording a receptor potential, the solution in which the retina was immersed was replaced with the Na-free solution. As can be seen in the lower row in Fig. 3, the

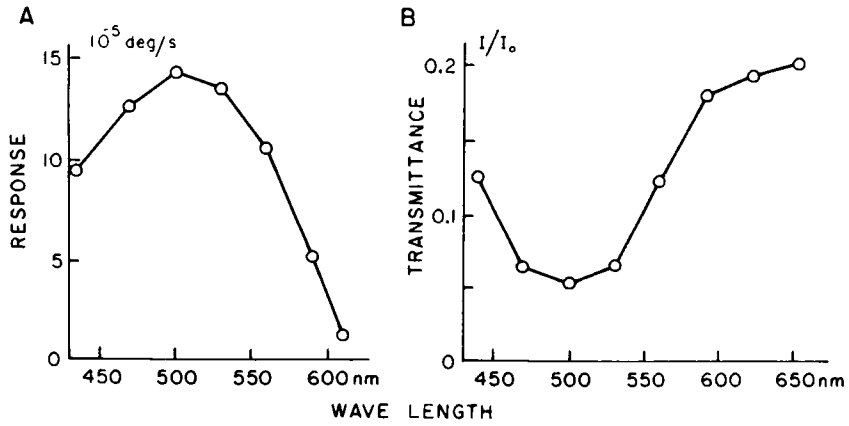

FIGURE 2 (*A*) Dependence of the amplitude of thermal responses of the photoreceptor cells on the wavelength of light. Brief (6 ms in duration) light pulses of 9 $\mu W/cm^2$ in intensity were used for stimulation. (*B*) transmission spectrum of an isolated retina preparation determined by the method described under Method. Temperature is 20°C.

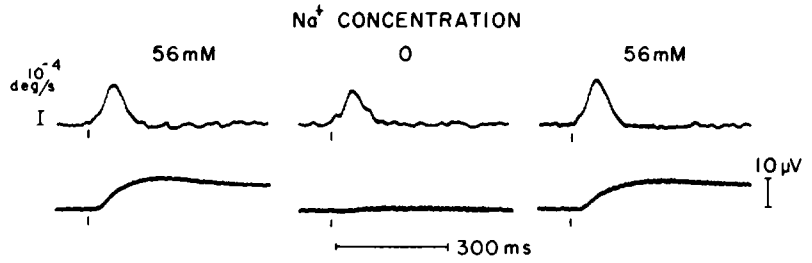

FIGURE 3 Records showing the dependence of thermal responses of the photoreceptors (*top*) and of the receptor potential (*bottom*) on the extracellular sodium-ion concentration. The light stimuli employed were 500 nm in wavelength, 6 ms in duration, and ~12 $\mu W/cm^2$ in intensity. The left records were taken from retinae treated with a 1:1 mixture of a 84 mM magnesium chloride solution and normal Ringer's solution. The middle records were obtained after 10 min immersion of the retinae in a Na-free medium prepared by replacing NaCl in the mixture with $MgSO_4$. The records on the right were taken after re-immersion of the retinae in the original Na-containing medium. Two different retinae were used.

electrical response of the receptor was completely eliminated by this procedure. The effect was reversible.

The sodium salt in the medium could be replaced with choline chloride or sucrose without suppressing the receptor heat. In the range down to ~20 mM, a reduction in the external Na-ion concentration affected the amplitude of the receptor heat not more than 30%. When the Na-ions in the medium were completely eliminated, a reduction in the amplitude of 40–50% was usually observed. (The electrical response of the receptor is known to fall drastically when the Na-ion concentration in the medium is reduced to ~40 mM [see Yau et al., 1981].) The amplitude of the receptor heat was not significantly affected by raising the KCl concentration in the medium to ~50 mM. It was not significantly affected by raising the external Ca-ion concentration to ~20 mM or by lowering to ~1 μM (by using 10 mM nitrilotriacetic acid buffer). The receptor heat was not diminished by addition of cocaine (3 mM) to the medium.

The effect of Na-ion deprivation described above was not wholly unexpected. According to Yoshikami, George, and Hagins (1980) and Gold and Korenbrot (1980), the photoreceptor cells immersed in a low Na^+ medium are capable of releasing Ca-ions in response to light stimulus in the absence of electrical responses of the cells. These authors have shown that the light absorbed by the rhodopsin molecules induces the release of Ca-ions from the disk membranes of the rod outer segments into the medium. It is reasonable, therefore, to assume that the receptor heat derives from an exothermic intermediate process taking place in the disk membranes. The finding that the production of the electrical response of the photoreceptors lags behind that of the thermal response is consistent with this assumption.

Physical and Chemical Agents that Affect Thermal Responses

Effects of several physical and chemical agents on the thermal response of the bullfrog photoreceptor were examined in the hope that such studies might lead to a better understanding of the intermediate process taking place in the rod outer segment. The agents known to prolong the duration of the electrical response of the nerve — cooling and heavy water — were examined in the first series of experiments (Fig. 4). Again, retinae isolated in a Mg-rich medium were used in conjunction with brief pulses of 500-nm light.

To achieve rapid cooling of the isolated retina, the thermal detector (see Fig. 1 in Tasaki and Nakaye, 1985) was modified. The lower compartment of the detector was divided into two parts in such a manner that both the upper

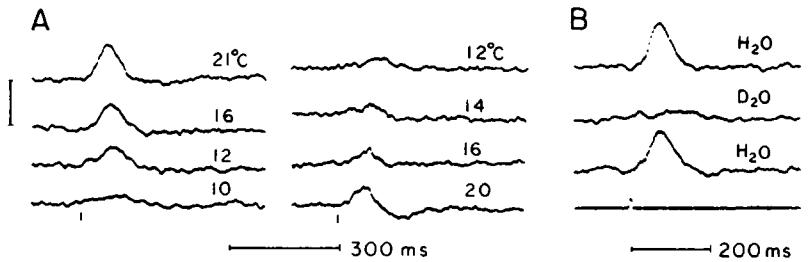

FIGURE 4 (*A*) The effect of changing the temperature of the isolated retina upon the receptor heat. Brief (6 ms) 500 nm light pulse of 15 $\mu W/cm^2$ were employed for stimulation. Retinae taken from bullfrogs acclimated to the laboratory atmosphere (20°C) were used. The time required to lower the temperature of the retina to 10° was 10–15 min. (*B*) The effect of replacing H_2O in normal Ringer's solution with D_2O upon the receptor heat. The stimuli employed were the same as in the temperature experiment. 21°C. The vertical bar on the left, representing 2×10^{-4} deg/s, applies to all the records.

and lower surfaces of the heat sensor could be exposed to the circulating cold oxygen. Using a Teflon cover, the operational amplifier in the lower compartment was kept thermally insulated from the cold atmosphere. The temperature of the retina was estimated by using a thermister placed under the PVDF film.

Fig. 4 *A* shows an example of the records obtained. It is seen that cooling brought about a decrease in the magnitude of the receptor heat without prolonging its duration. The time to peak of the thermal response was slightly longer at low temperatures. Between 8 and 10°C, the magnitude of the receptor heat became unmeasureably small. The effect of cooling was reversible. The finding that the duration of the receptor heat remained unaffected by cooling was quite unexpected. Nonetheless, the present finding appears to be consistent with the result obtained by Winkler (1972) who found that cooling suppresses the electrical response of the rat retina without appreciably prolonging the duration of the response (see also Penn and Hagins, 1972). The strong suppressive effect of cooling seen in the figure is reminiscent of the temperature dependence of the protoplasmic transport. In bullfrog nerves, the velocity of the fast transport of protein molecules is known to fall abruptly toward zero at temperatures below 10°C (Brimijoin et al., 1979).

The record in Fig. 4 *B* shows the effect of replacing H_2O in normal Ringer's solution with 99.8% D_2O. Immersion of the bullfrog retina in D_2O-Ringer's solution for ~5 min was found to suppress the ability of the retina to develop receptor heat. Suppression of the thermal responses by the D_2O-Ringer's solution was prompt and complete; recovery upon immersion of the retina in normal Ringer's solution was equally complete. In Ringer's solution prepared by using 50% heavy water, suppression of the receptor heat was incomplete. This finding does not contradict the result obtained previously by Chirieri et al. (1977); they employed 1-min long pulses of white light to examine the effect of D_2O on the electrical responses of the frog retina (see below). It is known that immersion of the frog nerve fiber in D_2O-Ringer's solution prolongs the action potential of the fiber without reducing its amplitude (Spyropoulos and Ezzy, 1959) and suppresses fast protoplasmic transport (Anderson et al., 1972). In the frog muscle, D_2O-Ringer's solution is known to suppress the contractile responses by inhibiting calcium release (Kamir and Kimura, 1972). It seems possible that, in the frog retina, D_2O-Ringer's solution suppresses calcium release by the disk membrane and/or fast protoplasmic transport of energy-rich materials.

It is to be noted that, in the experiments described above, the effects of D_2O-application on the photoreceptors were examined by using brief light pulses for stimulation. The responses evoked by long light pulses were found to be more resistant to various agents than those triggered by brief light pulses and develop "regeneratively" after the end of the stimulus. When light pulses with a long duration were employed for stimulation, the thermal responses of the photoreceptors were superimposed on the heat generated directly by the stimulus. Hence, analyses of heat signals evoked by long light pulses are somewhat complicated (see below).

Next, anoxia and inhibitors of oxygen utilization, which are known to suppress the production of thermal responses of the squid retina (Tasaki and Nakaye, 1985), were studied. The effect of anoxia on the bullfrog retina was examined by circulating humidified nitrogen above the retina. Under these conditions, the production of thermal responses was completely suppressed within 8–12 min. When oxygen was reintroduced into the detector without appreciable delay, there was a complete recovery. The effect of anoxia could be imitated by cyanide (or azide) dissolved in oxygenated Ringer's solution at a level of ~1 mM. These findings suggest that the process of heat production in the bullfrog photoreceptor is not fundamentally different from that in the squid photoreceptor. In the bullfrog retina, as in the squid retina, the ability to develop thermal responses could not be maintained without D-glucose in the medium. When D-glucose in the Ringer's solution was completely replaced with 2-deoxyglucose, which is known to bind to the same sites in the membrane as D-glucose (Sokoloff et al., 1977), the magnitude of the thermal response was found to fall at an accelerated rate; addition of D-glucose to the medium rapidly restored the ability to generate the receptor heat. There is little doubt, therefore, the fuel required for the maintenance of the ability of the photoreceptor cells to respond to light is D-glucose (see, e.g., Ames and Gurian, 1963).

In the recent literature on the vertebrate retina, the effects of inhibitors of phosphodiesterase on the electrical response of the retina are described. According to Lipton et al. (1977) and Waloga (1983), 3-isobutyl-l-methylxanthine, a potent inhibitor of phosphodiesterase, enhances the receptor potential at a low concentration and causes a loss of electrical activities of rods at a high concentration. In an effort to find whether or not this drug effects the receptor heat, the drug was dissolved in normal Ringer's solution at a concentration of 1–4 mM and was applied to the retina (Fig. 5 *A*). It was found that the drug suppresses the ability of the retina to develop receptor heat in response to brief light pulses. The effect was partially reversible. This finding, by itself, does not prove that guanosine 3′-5′-monophosphoric acid (cyclic GMP), the substrate for phosphodiesterase, is directly involved in the process of generating thermal responses.

Finally, the effects of two pharmacological agents, picrotoxin and d-tubocurarine, that were found to enhance the amplitude of the thermal responses, are described. In studying the effects of these drugs, retinae isolated in normal Ringer's solution were used. The duration of the stimulating light pulses (500 nm in wavelength and ~3 $\mu W/cm^2$ in intensity) was varied between 6 ms and ~1 s; the records obtained by using light pulses of relatively long

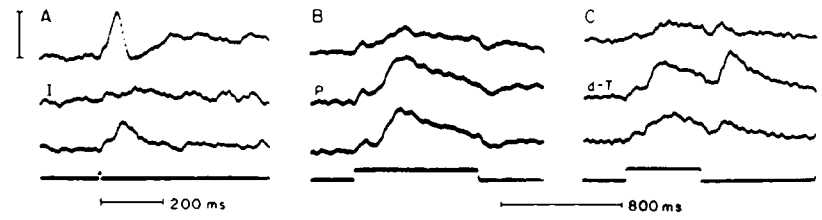

FIGURE 5 (*A*) Records showing reversible suppression of thermal responses of the retina by 4 mM isobutylmethylxanthine-Ringer's solution. The *middle* trace (marked I) was taken after immersion of the retina in the drug solution for ~10 min. The *top* and *bottom* records were taken from the same retina in normal Ringer's solution before and after drug-treatment respectively. Stimuli were 500 nm, 6 ms, and 15 $\mu W/cm^2$. 21°C. (*B*) Records showing the effect of 1 mM picrotoxin (*middle* trace). (*C*) The effect of 1 mM d-tubocurarine (*middle* trace). The stimuli used in taking records in *B* and *C* were 500 nm and 3 $\mu W/cm^2$. Note that the duration of the stimuli for records *B* and *C* was long. 22°C. The bar on the right indicates 2×10^{-4} for *A* and 4×10^{-4} deg/s for *B* and *C*.

duration are reproduced in Fig. 5. The top traces in records *B* and *C* represent the rate of temperature rise evoked by long light pulses. Both the receptor heat and the heat generated by the postsynaptic elements, including the off-responses, are recognizable. The middle trace in Fig. 5 *B* was obtained after treating the retina with 1 mM picrotoxin added to the medium. It is seen that there was a large increase in the amplitude of the thermal response of the postsynaptic elements. Since picrotoxin is known to block the negative feedback, probably at the bipolar-amacrine junction, it seems probable that the observed enhancement of the response amplitude is caused by a weakening of the negative feedback (see Burkhardt, 1972). Fig. 5 *C* furnishes an example of the results obtained with ~1 mM d-tubocurarine. A large enhancement of the response, particularly of the off-response, brought about by this drug is evident in the figure. The effect of d-tubocurarine on neurotransmission within the vertebrate retina has been studied by Ames and Pollen (1969); the activity of off-cells has been shown to increase under the action of the drug.

DISCUSSION

The rod outer segments of the bullfrog retina are known to be 6–8 μm in diameter and 50–80 μm in length (Bownds and Brodie, 1975). Assuming that the absorbance for 500 nm light wave transmitted along the axis of the outer segment is 0.013/μm (see Liebman, 1962), it is found that the radiant energy absorbed by the upper (proximal) half of the outer segments is roughly twice as great as that absorbed by the lower (distal) half. Since, however, the thermal diffusivity of the aqueous phase in the retina is expected to be 1.44×10^{-3} cm^2/s (see Carslaw and Jaegar, 1959), the spatial nonuniformity of the temperature within the outer segments is reduced to an insignificant level within 10–20 ms after the delivery of the brief light pulse. (Note that the measure of the time required for heat conduction across a layer of thickness x may be taken as $x^2/(2D)$, where D is the thermal diffusivity; for $x = 65$ μm, the time required is found to be 14.7 ms.)

The latency of the thermal response of the photoreceptors was found to be ~10 ms or less and the time to peak is 40–70 ms at moderate and high light intensities. The potential difference across the plasma membrane changes more gradually than the temperature. It is known that the light-induced hyperpolarization of the amphibian rod evoked by a brief light pulse has an absolute latency between 30 and 50 ms and the time to peak at moderate and high light intensities is ~100 and 200 ms (see, e.g., Fain, 1976; Woodruff and Bownds, 1979). The light-induced hyperpolarization of the rod is suppressed by elimination of the Na-ions in the medium or by raising the K-ion concentration (see Winkler, 1972; Hagins and Yoshikami, 1975; Yau et al., 1981); the thermal responses of the photoreceptors are not suppressed by these changes in the ionic composition in the medium. Since the loss of electrical excitability of the plasma membrane does not suppress the receptor heat, it is postulated that the stack of disk membranes is responsible for the production of the receptor heat.

When a thermal response is generated by a brief light stimulus, there is a great amplification of the energy involved. As in the squid retina (Tasaki and Nakaye, 1985), the ratio of the heat generated by the photoreceptor to the radiant energy employed for stimulation increases as the stimulus intensity is reduced. The weakest stimuli employed in the present study were 6-ms pulses of 500-nm light of ~0.06 $\mu W/cm^2$. The energy delivered to the retina by a single light pulse was 8.6×10^{-11} cal/cm^2. The thermal energy released was estimated to be between 6 and 10 times 10^{-8} cal/cm^2 (measurements on four retinal preparations). The ratio of the thermal energy generated by the photoreceptor to the energy delivered for stimulation is then between 700 and 1,200. This estimate of the ratio increases further when the scattering of light by the nerve fibers and cells above the photoreceptor layer (see Fig. 4) is taken into consideration. This great amplification is considered to take place in the disk membranes in the outer segments.

Physiochemical and biochemical properties of the rod outer segments have been examined extensively by a number of recent investigators using suspension of outer segments detached from the inner segments. It has been shown that enzymatic reactions involving cyclic nucleotide

phosphodiesterase and guanosine triphosphatase persist during a limited period of time after isolation of the outer segments (see e.g., Woodruff and Bownds, 1979; Robinson and Hagins, 1979; Liebman and Pugh, 1979). Now, the question arises: Do outer segments detached from the mitochondria-rich portion of the receptor cell respond to light stimulus with production of receptor heat?

By the experimental procedure described under Methods, attempts were made to detect heat production that follows "direct heat." So far, no distinct sign of production of receptor heat was observed in response to brief light pulses.

The absence of heat production by detached outer segments may simply indicate the inadequacy of the experimental procedure employed. Alternatively, it may be interpreted as indicating that the ability of the outer segments to generate receptor heat is suppressed after separation of the inner segments much sooner than the enzymatic activity of the constituents of the disk membrane. Note that the light-induced splitting of intrinsic c-GMP (guanosine 3 ,5 -monophosphate) takes place only in freshly isolated outer segments (Woodruff and Bownds, 1979). Liebman (1978) reported that he could not demonstrate significant light-induced Ca^{2+}-release from detached outer segments. It was shown under Results that production of receptor heat (which takes place after the end of the light stimulus) is readily suppressed by various physical and pharmacological agents (see Fig. 4). Therefore, the second, alternative interpretation cannot be ignored at present.

In the rod outer segments, there are several processes that can contribute to the production of receptor heat. Light-induced Ca^{2+}-release (Yoshikami, George, and Hagins, 1980; Gold and Korenbrot, 1980) is one of the possible sources of heat production. (Note that, in a variety of cation-exchangers, replacement of Ca-ions with univalent cations is known to involve heat generation of 1.5–3 kcal/equivant [see p. 278 in Tasaki, 1982].) Hydrolysis of guanosine 3 ,5 -monophosphate (c-GMP) by the light-activated phosphodiesterase (see Woodruff and Bownds, 1979; Greengard et al., 1969) is another possible source. Since the generation of receptor heat involves a large amplification of the energy involved, enthalpy changes associated with various steps in bleaching of rhodopsin (see Cooper and Converse, 1976) are not expected to make any positive contribution to the receptor heat. The limited time-resolution of the methods of detecting Ca^{2+}-release and of c-GMP splitting appears to constitute a major obstacle in the way of identifying the source of heat. It seems difficult to explain the time-course of the receptor heat in terms of phosphodiesterase activity.

Finally, the heat produced by the postsynaptic elements in the retina, which was treated only superficially in the present paper, is considered. Since the peripheral nerve fibers are known to evolve heat when excited, there seems little doubt that the nerve fibers and cell bodies in the retina evolve heat during excitation. There is a possibility that excitation processes at synapses also generate heat, since Ca-ions may be released by the receptor proteins (see Chang and Neumann, 1976). An invention of a new method for identifying the heat produced by various cells among the postsynaptic elements is required to carry our further studies.

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