Fast Electrical Potentials Arising from Activation of Metarhodopsin in the Fly

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ABSTRACT The cellular origin and properties of fast electrical potentials arising from activation of Calliphora photopigment were investigated. It was found by intracellular recordings that only the corneal-negative M1 phase of fly M potential arises in the photoreceptors' membrane. This M1 phase has all the accepted characteristics of an early receptor potential (ERP). It has no detectable latency, it survives fixation with glutaraldehyde, it is linear with light intensity below pigment saturation, and it is linear with the amount of metarhodopsin activated by light. The Calliphora ERP was found, however, to be exceptional because activation of rhodopsin, which causes the formation of metarhodopsin in 125 μs (25°C), was not manifested in the ERP. Also, the extracellularly recorded ERP was not proportional to the rate of photopigment conversion. The corneal-positive M2 phase of the M potential was found to arise from second-order lamina neurons (L neurons). Intracellular recordings from these cells showed a fast hyperpolarizing potential, which preceded the normal hyperpolarizing transient of these cells. This fast potential appeared only when metarhodopsin was activated by a strong flash. The data indicate that the intracellularly recorded positive ERP, which arises from activation of metarhodopsin, elicits a hyperpolarizing fast potential in the second-order neuron. This potential is most likely the source of the corneal-positive M potential.

INTRODUCTION

Two principal methods have been used to study the kinetics of fast visual pigment changes in situ: photometric, by means of flash photolysis; and electrophysiological, by recording the fast electrical potential, which arises directly from the visual pigment and is called the early receptor potential (ERP) (Brown and Murakami, 1964; Cone and Pak 1971). In the intact retinas of vertebrates a good correlation was found between specific pigment transitions measured photometrically and those measured by an extracellularly recorded ERP (Cone, 1969; Ostroy, 1977). In the Limulus ventral eye, on the other hand, the ERP only partially correlated with fast photometric measurements of the visual pigment, because photometrically observed pigment transition from a thermolabile intermediate could not be detected with the ERP (Fein and Cone, 1973).
Recently, Kirschfeld et al. (1978) measured photometrically the kinetics of pigment transition between rhodopsin and metarhodopsin in dipteran flies at 5°C. They found that the time constant of the formation of metarhodopsin when extrapolated to room temperature was 125 μs. The formation of rhodopsin at that temperature was at least 10-100 times faster. This result was confirmed by measurements at room temperature (Stark et al., 1979).

In this work we have examined whether the fast pigment transitions observed photometrically in the fly by Kirschfeld et al. (1978) are also recordable in an ERP measured under similar conditions.

The visual pigment system in the peripheral (R1-6) photoreceptors of the fly is a bistable one in which the blue rhodopsin ($\lambda_{\text{max}} \approx 480$ nm) and the orange metarhodopsin ($\lambda_{\text{max}} \approx 570$ nm) are photointerconvertible (Stavenga et al., 1973). After blue adaptation, which shifts a maximal fraction of pigment to the metarhodopsin state in R1-6 receptors, an intense orange flash elicits a fast biphasic potential in the electroretinogram (ERG) of the fly. This potential was called the $M$ potential because it apparently arises in some way from activation of metarhodopsin (Pak and Lidington, 1974). The $M$ potential was later separated into a corneal-negative phase ($M_1$ phase) and a corneal-positive phase ($M_2$ phase) (Grabowski and Pak, 1976). The $M_2$ but not the $M_1$ phase could be abolished by killing the animal or by application of CO$_2$ or hypertonic KCl (Grabowski and Pak, 1976; Minke and Kirschfeld, 1979). In addition, in several Drosophila mutants where the lamina layer is defective, the $M_2$ phase was missing (Pak and Lidington, 1974).

The claim that the $M_2$ phase arises from activation of metarhodopsin was based on the similarity of the absorption spectrum of fly R1-6 metarhodopsin to the criterion action spectrum of the $M_2$ phase (Pak and Lidington, 1974; Minke and Kirschfeld, 1979). Pak and Lidington (1974), furthermore, found that activation of rhodopsin gave no fast electrical potentials in Drosophila.

Recently, Stephenson and Pak (1978) suggested that in Drosophila the $M_1$ phase is the ERP, which arises from activation of metarhodopsin, and that this ERP elicits another fast potential in the second-order lamina neurons (L neurons), which are the origin of the $M_2$ phase. We therefore, investigated the cellular origin of the $M_1$ and $M_2$ phases in the ERG by intracellular recordings from photoreceptors and L neurons. We examined whether the $M_1$ phase has the characteristics of an ERP and tried to find out whether the fast pigment transitions found by photometric measurements in the fly also give rise to an ERP.

**METHODS**

**Preparation**

Intracellular and extracellular (ERG) recordings were made in white-eyed in vivo preparations of the flies Calliphora and Chrysomya which were immobilized by means of wax. For the ERG recordings we used two broken micropipettes filled with fly Ringer's solution. One electrode was placed on the thorax and the other on the cornea, both embedded in a drop of conducting paste. For intracellular recordings we used 2 M KCl-filled micropipettes of resistances on the order of 60-100 MΩ. These electrodes were lowered into the retina via a small hole in the cornea which had been
covered by petroleum jelly (Vaseline). The indifferent electrode was a broken pipette filled with fly Ringer's solution and was placed either onto the thorax or into the hole in the cornea.

**Stimulation and Recording**

The stimulating and recording systems have been described elsewhere (Minke and Kirschfeld, 1979). The adapting blue light was delivered by a xenon light source (XBO, 150 W, Osram, München, W. Germany), in conjunction with a monochromatic 457-nm interference filter (Schott-Depal, Mainz, W. Germany) and two KG1 (2 mm) heat filters (Schott). 1 s of 457-nm adapting blue light was sufficient to bring the visual pigment system into photoequilibrium. For the test stimulus we used a photographic flash light (Braun, type F 900, Frankfurt, W. Germany) in conjunction with either a 590 OG cut-on filter or a broad-band BG-12 blue filter as well as KG2 heat and neutral density (NG) filters (Schott). The orange and blue test flashes of maximal intensity were strong enough to bring the pigment system into photoequilibrium. Nevertheless, for orange adaptation we used two flashes of maximal intensity with the 590 OG filter.

The responses were recorded on an oscilloscope (Tektronix, Inc., Beaverton, Oreg.), in the memory of an averaging computer (NIC-527, Nicolet Instrument Corp., Madison, Wis.), and on a pen recorder (Gould, Inc., Cleveland, Ohio). The responses in the memory of the averager were later transferred to an X-Y plotter (Hewlett-Packard, Inc., Frankfurt, W. Germany).

**RESULTS**

**Fast Components in the ERG Observed When Pigment Is Shifted from Metarhodopsin to Rhodopsin**

At the start of the ERG induced by a strong orange test flash after blue adaptation, a biphasic, fast voltage appears; the first, corneal-negative phase is called $M_1$ and the second, corneal-positive phase $M_2$ (Pak and Lidington, 1974). In the experiments illustrated in Fig. 1, we tried to separate the two phases from each other by application of CO$_2$. In this way we expected to get the undistorted rise time and shape of the extracellularly recorded ERP (see below).

Fig. 1 (left column) shows the initial part of the ERG recorded in white-eyed Calliphora, under normal (no additional CO$_2$) conditions. Trace A shows a response to an orange test flash of maximal intensity after a saturating blue (457 nm) adaptation that shifted a maximal fraction of the pigment into the metarhodopsin state. The various components of the response are marked by numbers: (1) stimulus artifact, (2) a negative phase with no detectable latency—the $M_1$ phase, (3) a positive phase—the $M_2$ phase, (4) a positive phase which is the on-transient of the ERG arising from activation of the L neurons (reviewed by Goldsmith and Bernard, 1974), and (5) initial negativity which represents the beginning of the late receptor potential (LRP) recorded extracellularly. Trace B shows that both the $M_1$ and $M_2$ phase can be abolished by saturating orange preadaptation, which shifted almost all the pigment molecules into the rhodopsin state.

Traces E and F (right column) show that it is possible to separate the negative phase ($M_1$) from the positive phase ($M_2$) by application of a low
The CO₂, which was gently blown onto the abdomen of the fly, very quickly (in less than 1 min) abolished the $M_2$ phase and the on-transient of the ERG, but left unmodified the $M_1$ phase and the LRP phase, which both had the same amplitude and shape before, during, and after the application of the CO₂. This fact ensures that the CO₂ level was low enough that the photoreceptors were not affected. A high level of CO₂ can abolish the
LRP of the fly (Wong et al., 1976). Thus trace E shows the shape of the extracellularly recorded $M_1$ phase undistorted by the $M_2$ phase. This phase has a rise time of 0.8 ms. Trace F shows that the $M_1$ phase is abolished by saturating orange adaptation as in trace B. The small positive phase in trace F has been reported before by Minke and Kirschfeld (1979). Its origin is unknown. In this work we recorded this phase, with the same positive polarity, in the intracellular recordings (Fig. 3 C), which indicates that this phase does not arise from the photoreceptor membrane.

Fast Components in the ERG Observed When Pigment Is Mainly Shifted from Rhodopsin to Metarhodopsin

In Fig. 1 we tried also to find fast electrical potentials during a strong flash that shifted pigment mainly from rhodopsin to metarhodopsin.

Trace C in Fig. 1 shows the response to a broad-band blue flash (BG-12) after saturating orange adaptation that shifted almost all the pigment into the rhodopsin state. This blue flash shifted a maximal amount of pigment into the metarhodopsin state, as indicated in trace D, by the full $M_2$ phase, which was induced by a following orange test flash. A surprising result is observed in trace C: the blue flash which shifted pigment mainly in the direction opposite to that caused by the orange flash (Traces A, D) gives a response with a similar (negative-positive) shape to that in trace A, though with smaller amplitude. It is well established that selective activation of metarhodopsin gives a biphasic negative ($M_1$)-positive ($M_2$) signal in the ERG (Pak and Lidington, 1974; Grabowski and Pak, 1976). Because the blue flash converts some pigment from metarhodopsin to rhodopsin simultaneously with a larger pigment conversion in the opposite direction, it seems as if only the fraction of pigment converted from metarhodopsin to rhodopsin gives rise to the fast response, and that the conversion in the opposite direction, that is, from rhodopsin to metarhodopsin, gives no response at all. This impression was confirmed by intracellular recordings (see below).

The $M_2$ Phase Does Not Arise in the Photoreceptors

In order to locate the cellular origin of the $M_1$ and $M_2$ phases in the ERG, we compared the timing of the various phases in the extracellular signals with that of the phases observable in intracellular recordings from the R1-6 photoreceptors.

Fig. 2 shows the intracellularly recorded initial responses to strong flashes of different colors. The various components of the response are indicated by numbers in trace A: (1) stimulus artifact, (2) positive phase with no detectable latency with a timing similar to the $M_1$ phase of the ERG, (3) a second positive phase with timing similar to the $M_2$ phase, and (4) a third positive phase which is the initial LRP of this cell. The first two phases, like the $M_1$ and $M_2$ phases in the ERG, are abolished by saturating orange adaptation (traces B and D). When we compare the intracellularly recorded response to the ERG recordings, it is immediately obvious that the $M_1$ phase and the LRP reverse polarity when they are recorded intracellularly. However, the
Figure 2. Initial responses to various colors of adaptation and stimulation all recorded intracellularly in single R1-6 receptors of white-eyed Calliphora. (A) When the indifferent electrode was placed on the thorax three phases could be detected: (1) stimulus artifact; (2) the $M_1$ phase which reversed polarity relative to the ERG; (3) the $M_2$ phase which has the same polarity as in the ERG recordings; (4) LRP. The response was elicited by an orange flash of maximal intensity after saturating 457-nm blue adaptation. (B) Response to an orange test flash after saturating orange adaptation in the same experimental conditions as in trace A. The $M_1$ and $M_2$ phases are abolished as a result of the orange preadaptation. (C) Response to the same stimulation adaptation paradigm as in trace A, but after the indifferent electrode was placed in the photoreceptor layer (cornea). In this recording the $M_2$ phase vanished, which indicates that the $M_2$ phase does not arise in the photoreceptors. (D) The same conditions as in trace C but after orange adaptation. (E) Response to a broad-band blue flash (BG-12) of maximal intensity after orange adaptation. Although the flash shifts pigment mostly from rhodopsin to metarhodopsin, the polarity of the $M_1$ phase is the same as in trace C where pigment is mostly shifted to the metarhodopsin state. (F) The same as in trace E but after the blue flash adaptation. The square pulses which precede traces C and F are calibration pulses of 5-mV amplitude and 2-ms duration. The same calibration pulse is also presented in Figs. 4, 5, 9, and 10.
$M_2$ phase (Fig. 2, trace A, phase 3) remained positive in the intracellular recordings as in the ERG. This fact suggests that this phase, in contrast to the other two, does not arise across the photoreceptor membrane. In order to confirm this possibility, we succeeded in three cases in changing the location of the indifferent electrode during the intracellular recording from the thorax to just beneath the cornea of the eye. The response in this recording situation to the orange test flash after a saturating blue adaptation is illustrated in trace C. This response obviously no longer shows the $M_2$ phase. The remaining $M_1$ phase could be abolished by orange adaptation (trace D). In trace E we show, in a manner similar to trace C of Fig. 1, that a blue flash after saturating orange adaptation (which shifts the pigment mostly from rhodopsin to metarhodopsin) gives a positive $M_1$ phase similar (though smaller) to that of trace C when pigment is mostly shifted in the opposite direction.

We found a considerable difference in the latency of the LRP under the various experimental conditions (Figs. 2 and 3). However, in the present work we did not investigate these changes.

Fig. 2 shows that the $M_2$ phase cannot be an ERP in that it does not arise in the photoreceptor membrane.

Trace F of Fig. 2 shows the $M_1$ phase in response to a blue flash after blue preadaptation. The similarity of the $M_1$ phase amplitudes in traces E and F (which are recorded in the same cell) indicates that the initial distribution of the pigment between rhodopsin and metarhodopsin does not affect the amplitude of the $M_1$ phase, when the stimulus is the strong blue flash. This fact indicates that one blue flash was strong enough to bring the pigment system into photoequilibrium and that the amplitude of the $M_1$ phase in traces E and F is the amplitude reached in photoequilibrium in response to a blue flash after saturating blue adaptation. The difference in the amplitudes of the $M_1$ phases on traces C and F probably arises from the fact that the color and intensities of the test flashes were different in the two traces.

Fig. 3 shows, in a similar manner to Figs. 1 and 2, that another species of fly, *Chrysomya*, has similar $M_1$ and $M_2$ phases in the ERG (trace E), which are abolished after orange adaptation (trace F). It also shows that the $M_2$ phase can be abolished by CO$_2$ (trace D). Phases $M_1$ and $M_2$ were both observed in an intracellular recording (trace A) when the indifferent electrode was placed on the thorax, but only the $M_1$ phase reversed polarity in the intracellular recordings relative to the ERG. The $M_2$ phase disappeared when the indifferent electrode was placed onto the cornea (trace B). The only minor difference between the two species of flies was that the positive, photostable components (Fig. 1 F) were found only occasionally in *Calliphora* but always in *Chrysomya* (trace C).

*The $M_1$ Phase Is the ERP of the Fly*

The $M_1$ phase in the ERG (Fig. 1) and the initial positive phase in the intracellular recordings (Figs. 2 and 3 B) have several characteristics in common with the known characteristics of the ERP: (a) it has no detectable latency (Fig. 2); (b) it changes polarity from negative to positive in the ERG relative to the intracellular recordings (Figs. 1 and 2); (c) it is resistant to CO$_2$
and to hypertonic KCl (Grabowski and Pak, 1976; Minke and Kirschfeld, 1979).

In order to make sure that the $M_1$ phase is an ERP, we further examined (a) its resistance to extreme changes of the medium, (b) its linearity with light intensity up to pigment saturation, and (c) its linearity with the amount of pigment activated.

![Diagram](image)

**Figure 3.** The components of the initial responses recorded intracellularly in a single cell (left column) and extracellularly from the ERG (right column) in a single white-eyed *Chrysomya*. (A) The initial intracellular response to an orange test flash of maximal intensity after 457-nm equilibrating blue adaptation. The three phases, as already illustrated in *Calliphora* (Fig. 2 A), were observed when the indifferent electrode was placed on the thorax. (B) The same as in trace A but with the indifferent electrode in the photoreceptor layer. The $M_2$ phase disappeared as in *Calliphora*. (C) The same as in trace B but after orange adaptation. The initial positive phase is a photostable component which was found in all experiments with this species. (D) Initial ERG under low level of CO$_2$ which abolished the $M_2$ phase and left the $M_1$ phase undistorted. (E) The same as in trace D but 5 min after CO$_2$ application has been interrupted. The response recovered within 1 min. (F) The same as in trace E but after saturating orange adaptation.

(a) RESISTANCE TO EXTREME MEDIA Trace A of Fig. 4 is an intracellular recording of a response to a strong orange test flash after saturating blue adaptation. In the lower traces of Fig. 4 we see the responses of the same cell after application of 25% glutaraldehyde to the eye. The resting potential is greatly reduced within 1 min, and the LRP disappeared within 3 min. The slow response at the end of traces B and C is the remaining LRP. This phase completely disappeared in traces D and E, which were recorded 2 min later.
However, the $M_1$ phase was resistant to this medium as indicated in traces B and D. It could be abolished reversibly in a normal fashion by shifting the pigment to the rhodopsin state by equilibrating orange light (Fig. 4, traces C, E). Results similar to that of Fig. 4 were obtained in two other flies.

Figure 4. Effects of extreme media on the $M_1$ phase. The figure shows the effects of application of one drop of 25% glutaraldehyde into the hole in the cornea during an intracellular recording in a white-eyed Calliphora. The indifferent electrode was in the photoreceptor layer. (A) Control trace which shows the response to the orange test flash of maximal intensity after an equilibrating 457-nm blue adaptation before the application of the glutaraldehyde. (B) The same as in trace A but 1-min after application of the glutaraldehyde: The resting potential was greatly reduced. The slow positive phase at the end of the trace is the residual LRP. The $M_1$ phase is still clearly present although reduced in amplitude. (C) The same as in trace B but after orange adaptation. (D, E) The same as in traces B and C in the same cell but 2 min later. The $M_1$ phase of the response to the orange flash after blue adaptation still exists, but the LRP has completely disappeared.

(b) LINEARITY WITH LIGHT INTENSITY AND WITH THE AMOUNT OF PIGMENT ACTIVATED In Figs. 5 and 6 experiments are illustrated in which we examined whether the $M_1$ amplitude increases linearly with the light intensity (up to pigment saturation) and with the amount of visual pigment activated. Data shown in both graphs (Figs. 5 and 6 were measured in a single cell which was
Figure 5. The dependence of the normalized amplitude of the $M_1$ phase (intracellular recordings) on the relative intensity of the orange test flash. Each point was obtained after an equilibrating 457-nm blue adaptation. All points of this figure and of Fig. 6 were recorded from one single R1-6 cell in a white-eyed Calliphora. The maximal intensity was four times weaker than in the experiments illustrated in other figures. The amplitudes of the responses were measured at a fixed time as indicated (arrow on inset). The continuous line that fits the points indicates that the $M_1$ phase is linear with light intensity until pigment saturation. This line was calculated from the equation $M_1 / M_{1\text{max}} = 1 - e^{-it/A}$ (see text).

Inset: sample of responses used in the plot.

completely stable for more than 2 h of intracellular recording. The experiment was done in the following way: after saturating 457-nm blue adaptation, two orange flashes separated by a 1-min dark interval, were given. The first flash, with variable intensity gave responses (Fig. 5, inset) whose amplitudes measured at a fixed time after the onset of the stimulus (arrow) are plotted in Fig. 5 as a function of the relative light intensity on a linear scale. The second one (with constant maximal intensity) measured the amount of pigment left in the metarhodopsin state after the fractional shift of pigment by the first one (Fig. 6). The continuous line in Fig. 5 is a plot of the function:

$$M_1 / M_{1\text{max}} = 1 - e^{-it/A},$$

where $M_1$ is the amplitudes of the various $M_1$ phases at various light intensities, $I$, $t$ is the constant duration of the flash, and $M_{1\text{max}}$ is the amplitude of the $M_1$ phase at the maximal stimulus intensity. $A$ is the negative slope of the graph in Fig. 6. When $A$ is measured in absolute scale of cm$^2$/photons, it is equivalent to the sum of the photosensitivities of rhodopsin and metarhodopsin (see Minke and Kirschfeld, 1979). Since the stimulus intensity in Fig. 5 is in fact the adaptive light intensity in Fig. 6, $A$, the slope of the graph in Fig. 6, is the relative orange light intensity needed to shift $1 - 1/e$ of the shiftable pigment.
molecules. From Fig. 6 we could therefore, measure the constant \( A \) which was needed to draw the theoretical curve of Fig. 5.

The fit of the theoretical curve in Fig. 5 to the experimental points shows that the \( M_1 \) phase is linear with light intensity and that it begins to saturate when the test flash causes appreciable pigment shift.

Fig. 6 shows the normalized response amplitude to the constant orange test flash of maximal intensity after various amounts of pigment have been shifted from metarhodopsin into rhodopsin. This normalized amplitude \( M_1(I_t) / M_1(0) \) is plotted as a function of the intensity of the first adapting flash which was given after an equilibrating blue adaptation. \( M_1(I_t) \) is the amplitude of the \( M_1 \) phase to a constant orange test flash after variable intensities \( I_t = \text{constant} \) of orange flashes were given. \( M_1(0) \) is the \( M_1 \) phase amplitude if no adapting orange flash has been given before. The figure shows that the amplitude of the response to the test flash decreases exponentially with the increase in the intensity of the adapting orange flash, which shifted increasing amounts of pigment from the metarhodopsin state into the rhodopsin state. The exponential dependence of Figs. 5 and 6 is consistent with the notion that the \( M_1 \) phase reflects linearly the changes in metarhodopsin concentration (see also below). Fig. 6 also shows that 90% of the shiftable pigment is shifted by the maximal intensity orange flash which has a relative intensity of 1.0 in the graph. For technical reasons (an arrangement that allows insertion of neutral
density filters in the beam path), this intensity is one-fourth of that of the maximal orange flash intensity used in the other experiments.

This result allows comparison of the rise time of the ERP (M1 phase) with the amount of pigment shift during the test flash as follows. The rise time of the extracellularly recorded ERP is 0.8 ms (Fig. 1 E). From the integral over the light monitor (Fig. 1) it can be estimated that after 0.8 ms one-half of the energy of the test flash has been given. Inasmuch as the light intensity in the experiment illustrated in Fig. 1 has been four times more intense, we find the amount of pigment shifted after 0.8 ms by extrapolation of the line in Fig. 6 to twice the maximal relative light intensity. The outcome is that 99% of pigment would be shifted after 0.8 ms. Therefore, the 0.8-ms rise time of the ERP can be well accounted for by the amount of pigment shift during the test flash. However, it is clear from Fig. 1 E that the rise time of the flash stimulus is shorter than 0.8 ms. The fact that the extracellular ERP amplitude continued to increase when the intensity of the flash already decreased indicates that the amplitude of the extracellular ERP is not proportional to the rate of pigment shift from the metarhodopsin state.

(c) THE M1 PHASE ARISES FROM ACTIVATION OF METARHODOPSIN In Fig. 7 we tried to further establish the fact that the M1 phase arises from activation of metarhodopsin and not from activation of rhodopsin. In order to do so we made use of the evidence which had already shown that the M2 phase arises from activation of metarhodopsin. This evidence consists of the following: (1) the criterion action spectrum (CAS) of the M2 phase in Drosophila and Calliphora fits the absorption spectrum of metarhodopsin (Pak and Lidington, 1974; Minke and Kirschfeld, 1979); (2) the dependence of the fractional concentration of metarhodopsin at photoequilibrium on the wavelength of the adapting light fits the dependence of the amplitude of the M2 phase in response to a constant flash on the wavelength of the adapting light (Minke and Kirschfeld, 1979).

The fractional concentration of fly metarhodopsin at photoequilibrium \( f_M(\infty, \lambda) \) as a function of the adapting wavelength \( \lambda \) is given by

\[
f_M(\infty, \lambda) = \frac{K_R(\lambda)}{K_M(\lambda) + K_R(\lambda)},
\]

where \( K_M(\lambda) \) and \( K_R(\lambda) \) are the photosensitivities of metarhodopsin and rhodopsin, respectively (Hamdorf et al., 1973; Stavenga, 1976). The display of \( f_M(\infty, \lambda) \) as a function of wavelength was called the photoequilibrium spectrum.

Fig. 7 shows the photoequilibrium spectrum measured in Calliphora by means of the amplitude of \( M_1 \). The ordinate is the normalized amplitude of \( M_1 \), which was elicited by a constant orange test flash of maximum intensity. The abscissa is the wavelength of the adapting light which has been used to put the pigment system into photoequilibrium. The data were obtained in four different cells from four different flies by means of intracellular recording. The continuous curve is a replot of the photoequilibrium spectrum that was measured by means of the \( M_2 \) phase in Calliphora (Minke and Kirschfeld,
Since the $M_2$ phase arises from activation of fly metarhodopsin, the good fit between the spectra measured by the $M_1$ and $M_2$ phases shows that the $M_1$ phase obviously also arises from activation of fly metarhodopsin.

The Cellular Origin of the $M_2$ Phase

Although it is clear from Figs. 1–3 that the $M_2$ phase is not an ERP, we tried to find out its cellular origin inasmuch as the $M_2$ phase had been used several times to measure changes in metarhodopsin concentration in the fly (Kirschfeld et al., 1977; Stark et al., 1977; Minke and Kirschfeld, 1979).

![Graph](image)

**Figure 7.** The photoequilibrium spectrum measured from the $M_2$ phase. The ordinate is the normalized amplitude of the $M_1$ phase recorded intracellularly at a fixed time from the onset of the orange test flash of maximal intensity. The abscissa is the wavelength of the adapting light that brought the pigment system to photoequilibrium. The continuous line is a replot of the photoequilibrium spectrum measured by the $M_2$ phase in *Calliphora* (Minke and Kirschfeld, 1979). The figure shows that the $M_1$ phase, like the $M_2$ phase, arises from activation of the metarhodopsin state of the visual pigment. The vertical bars are standard errors of the mean which were calculated from four different R1-6 cells of four different white-eyed *Calliphora* flies.

Following the suggestion of Stephenson and Pak (1978), we tried to locate the origin of the $M_2$ phases in the second-order L neurons by intracellular recordings from the lamina of *Calliphora*. We found two types of intracellular responses in the various penetrations within the lamina region. (a) The more frequent response type is illustrated in Fig. 8 (right). This response has a shape similar to the LRP recorded from the photoreceptors. After blue adaptation these cells show (in response to a strong orange test flash), the normal positive $M_1$ phase. We therefore concluded that this response arises from the axons of the R1-6 photoreceptors that synapse in the lamina (Trujillo-Cenoz, 1965;
Boschek, 1971). (b) The second type of response is illustrated in Fig. 8 (left) as a function of variable intensities of blue light after initial saturating blue adaptation. These cells always had a resting potential smaller than that of the receptor axons, even with recordings that have been stable for more than 30 min. The response of these cells to a pulse of light is initially hyperpolarizing and then depolarizing with a noisy steady state, especially at medium light intensity levels. At the cessation of the light they give a depolarizing off-response. The response characteristics of these cells are very similar to those of

![Figure 8](image-url)

**Figure 8.** A comparison of the L neuron response to that of a photoreceptor. Left: intracellular recordings from the same lamina neuron, further responses of which are also illustrated in Fig. 10 D and E. The stimulus (bottom trace) was 457-nm blue light of various intensities (as indicated in a relative scale) after equilibrating blue preadaptation. The response is similar in shape to lamina cell responses recorded by others except that the steady state is mostly depolarizing. This is interpreted as due to lateral inhibition which is exceptionally strong since diffuse light and white-eyed *Calliphora* have been used for the experiments. Right: intracellular recording presumed to be from axons of R1-6 receptors in the same fly of the same adapting stimulation paradigm as the corresponding L neuron response. The shape of the response is typical for a receptor potential.

the L neurons as recorded by Zettler and Järvi-lehto (1972) from *Calliphora* within the “cartridges” of the lamina. At low intensity levels the cells show a small hyperpolarizing steady-state response (−logI = 5). At higher intensity levels the cells recorded in our experimental situation gave—in contrast to the hyperpolarization as usually reported for these neurons—a depolarizing response. This depolarization is obviously due to our stimulation conditions: we stimulated white-eyed flies by illumination of the whole eye, so that all receptors are activated simultaneously. In this case, according to the results of
Zettler and Järvilehto (1972), a strong lateral inhibitory effect is to be expected, which (after a short latency) overcomes the usually recorded hyperpolarization and leads to a depolarization as shown in Fig. 8.

Figs. 9 B and C and 10 show the responses of this type of cells to a strong orange test flash after blue (Figs. 9 C and 10 A-D) and orange (Figs. 9 B and 10 E) adaptations in fast recordings. The figures show that the responses after blue adaptation, in contrast to the photoreceptor response (Fig. 9 A), have an initial latency of ~2 ms, which is followed by an initial hyperpolarizing phase with a timing similar to that of the $M_2$ phase in the ERG and then by an additional hyperpolarization which has a timing similar to that of the on-

transient of the ERG. This second phase is the typical hyperpolarizing response of the L neurons which is initiated by the LRP of the photoreceptors. The first hyperpolarizing phase is abolished by saturating orange adaptation, like the $M_2$ (and the $M_1$) phase (Figs. 9 B and 10 E).

The initial positive phase that appears only after orange adaptation has a similar latency to the LRP in the photoreceptors and therefore may arise from an invasion of an electrical LRP into the L neurons.

We conclude from the intracellular recordings of the lamina neurons that the origin of the $M_2$ phase in the ERG is most likely from the initial hyperpolarizing response recorded from these cells after blue adaptation.
DISCUSSION

The Cellular Origin of the $M_1$ and $M_2$ Phases in the ERG

The comparison of the ERG with the intracellular recordings shows that the cellular origins of the $M_1$ and $M_2$ phases are very different because the $M_1$ phase and the LRP reversed polarities when recorded intracellularly but the $M_2$ phase did not. Moreover, the existence of the $M_2$ phase in the intracellular recordings strongly depends on the position of the indifferent electrode which indicates that the $M_2$ phase arises from a tissue structure outside the photoreceptor membrane. The $M_2$ phase and the on-transient of the ERG both have the same polarity when they are recorded from the surface of the eye, and both reverse polarity at the same depth when the electrode is advanced deep in the laminar region (Stephenson and Pak, 1978). In that it is known that the on-transient of the ERG arises from the response of the L neurons in the

Figure 10. Intracellular recordings from four different lamina neurons to orange stimuli of maximal intensity after blue (457 nm) and orange adaptation in white-eyed Calliphora. Traces A-D are from four different flies. The responses to the orange flashes after blue adaptation show (in a similar manner to Fig. 9, but with a faster sweep speed) the typical initial response of lamina cells that we observed: a short latency of <2 ms and an initial hyperpolarizing phase which most likely is the origin of the $M_2$ phase in the ERG. (E) The same as in trace D recorded in the same cell but after orange adaptation. The initial negative phase now is missing. The vertical bars indicate 5 mV.
lamina (Goldsmith and Bernard, 1974), it is very likely that the $M_2$ phase also arises from these cells (Stephenson and Pak, 1978).

The corneal-positive on-transient of the ERG arises from the initial (intracellularly recorded) hyperpolarizing transient of the L neurons. We therefore expect that the response of the L neurons, which may give rise to the $M_2$ phase, will also be a fast hyperpolarization. We further expect that this response will appear after a short latency due to a synaptic delay between the R1-6 receptor axons and the L neurons, which is 1.0 ms (Järviilehto and Zettler, 1971). This initial hyperpolarization should precede the normal hyperpolarizing transient of these cells which arises from the input of the LRP.

The responses of Figs. 9 and 10 meet all these expectations. After blue adaptation the responses to orange test flashes from four different cells all show a fast hyperpolarizing phase with a timing similar to the appearance of the $M_2$ phase in the ERG. This phase is followed by the normal hyperpolarizing response of these cells which arises from the LRP input. The fact that this initial hyperpolarization can be abolished (like the $M_2$ phase) by equilibrating orange adaptation (Figs. 9 B and 10 E), further supports our suggestion that this phase is manifested as the $M_2$ phase in the ERG. The identity of these cells as L neurons can be concluded from the general characteristics of the responses (Fig. 8) that were recorded from the same cell that gave the responses in Figs. 9 B and C and 10 D and E. Responses similar to those of Fig. 8 were obtained from three other cells whose responses are illustrated in Fig. 10. All these responses have the typical shape of L neurons (Zettler and Järviilehto, 1972), except that the steady-state phase (Fig. 8) is mostly depolarizing, which probably arises from the use of white-eyed Calliphora illuminated with a diffuse light, giving a strong effect of lateral inhibition.

The above data are consistent with the following hypothesis first suggested by Stephenson and Pak (1978). The $M_1$ phase, which is the ERP of the fly and has a positive polarity (intracellularly), releases a transmitter in the normal way and activates the L neurons, which normally give a fast hyperpolarizing response in response to receptor cell depolarization.

The fact that an ERP can elicit a response in the second-order neurons has been reported before from the turtle (Hodgkin and O'Bryan, 1977), where the negative ERP in the turtle's cones elicited a hyperpolarizing response in the horizontal cells.

The L neurons in the fly amplify small signals coming from the photoreceptors (Järviilehto and Zettler, 1971). Thus the ERP is conveniently amplified by the L neurons and can be used to measure changes in metarhodopsin of the fly in vivo in the ERG.

**Correlation between the ERP and Photometric Measurements of the Visual Pigment Activation**

**THE $M_1$ PHASE IS AN ERP RESPONSE** The $M_1$ phase, which is a corneal negative potential in the ERG and reverses polarity when recorded intracellularly, has all the characteristics of an ERP. It has no detectable latency
(Figs. 2 and 3); it survives fixation with glutaraldehyde, which abolishes all the physiological responses (Fig. 4). It is linear with light intensity (Fig. 5) and with the amount of metarhodopsin activated (Figs. 6, 7). Also, the polarity of the M₁ phase (positive) is the same as the polarity of the ERP arising from activation of metarhodopsin in other invertebrates: barnacle (Hillman et al., 1972; Minke et al., 1973, 1974), Limulus ventral eye (Lisman and Sheline, 1976), squid (Hagins and McGaughy, 1967), and scallop (Cornwall and Gorman, 1976).

**THE ERP OF THE FLY CORRELATES ONLY PARTIALLY WITH PHOTOMETRIC MEASUREMENTS OF THE PIGMENT CHANGES** In the outer segment of vertebrate cones and in the microvilli of invertebrate rhabdomeres, the visual pigment is an integral part of the surface membrane (reviewed by Eakin, 1972). Accordingly, the generation of the ERP in these preparations can be explained as follows (Cone, 1969): upon illumination the dipole moment of the photopigment molecules usually changes from one intermediate state to the next. Because the molecules are electrically in parallel with the membrane capacitance, a rapid transition produces a rapid change in the polarization of the membrane that contains the visual pigment. This polarization then discharges passively throughout the membrane capacitance of the rest of the cell until the entire membrane is equally polarized. The dipole current can be recorded extracellularly as the ERP, which has a rise time equal to the time of change in the polarization of the membrane as a result of the changes in the visual pigment. The intracellularly recorded ERP is approximately the external ERP integrated by the time constant of the cell membrane (Murakami and Pak, 1970; Hodgkin and O'Bryan, 1977). The extracellular ERP recorded in the vertebrates is correlated very well with photometric measurements of the visual pigment changes (Cone, 1969; see review by Ostroy, 1977). It had been shown by Cone (1969) that the rise time of the R₁ and R₂ phases in the albino rat ERP have the same time constant and kinetics as the formation of metarhodopsin I and II, respectively, as measured photometrically. Similar results had been obtained by Gedney et al. (1971) in the frog. Fein and Cone (1973) also showed in the Limulus ventral eye that there is a good fit between the recovery of an intracellularly recorded negative phase in the ERP which arises from activation of rhodopsin, and the disappearance of a thermolabile intermediate with peak absorption at 465 nm. The thermolabile intermediate, however, gave no ERP response.

The ERP of the fly, according to our results, seems to be very different from those of other species for the following reasons. (a) The amplitude of the extracellularly recorded ERP is not proportional to the rate of visual pigment conversion, as expected. This is indicated in Figs. 1 E and 3 D, which show that the ERP amplitudes continues to increase when the intensity of the test flash already decreases. (B) It seems that activation of rhodopsin molecules (or the formation of metarhodopsin) gives no ERP in the fly, in contrast to the other species mentioned above, where activation of rhodopsin gives a hyperpolarizing ERP in intracellular recordings. In the fly, orange adaptation brings almost all the pigment molecules to the rhodopsin state. Therefore,
after orange adaptation a blue flash (Figs. 1 C, 2 E) initially activates only rhodopsin molecules which should give a hyperpolarizing intracellular ERP. However, only a depolarizing ERP was observed (Fig. 2 E). The positive ERP response to the blue flash after orange adaptation, which most likely arises from activation of metarhodopsin, should show a latency since initially there is no metarhodopsin to be activated. However, no latency was observed. This can be explained by the rapid transition time constant of 125 μs measured by Kirschfeld et al. (1978) which brings pigment molecules to the metarhodopsin state during the rise time of the blue flash. Figs. 1 C and 2 E thus show that the ERP and the photometric technique both indicate that isomerized rhodopsin molecules in the fly complete the photochemical cycle via the metarhodopsin state during the flash within ~ 130 μs.

There is a very good fit between the photoequilibrium spectrum as measured with the positive ERP and the photoequilibrium spectrum measured by the M2 phase, which has a spectral sensitivity of fly metarhodopsin. Therefore, we concluded that the positive ERP arises only from activation of metarhodopsin without any rhodopsin contribution.

Hagins and McGaughy (1967) showed that the ERP of the squid in photoequilibrium had a biphasic positive-negative ERP, with a zero-integrated area, as one would expect from a closed pigment system. Similar results were obtained by Minke et al. (1974) in the barnacle where a model of a closed pigment system fitted the ERP waveforms. In the fly, on the other hand, this integral is never zero because even in photoequilibrium the ERP elicited by means of a blue flash after blue adaptation (Fig. 2 F) is always monophasic.

We do not know yet why the ERP of the fly is so exceptional. The unusual characteristics of the ERP of the fly might be related to the differences between the pigment system of the fly and the other invertebrate species like the Limulus and the barnacle. In these species the time constants of the transitions between the two stable states rhodopsin and metarhodopsin are at least 10 times slower than in the fly. Moreover, the time constant of the transition between rhodopsin and metarhodopsin is at least 10 times faster than the transition between metarhodopsin and rhodopsin (for review see Ostroy, 1977). In the fly the situation is just the opposite. There are several possible explanations for the unusual ERP of the fly, but we have no evidence for any of them:

(a) The existence of a slow undetectable phase in the ERP of the fly with opposite polarity to the M1 phase could explain our failure to find a zero-integrated area in the ERP. Such a slow phase could arise from a thermolabile intermediate in the transition between rhodopsin and metarhodopsin. The existence of such an intermediate pigment state was in fact deduced from the photometric kinetic study in the intact fly (Kirschfeld et al., 1978). However, the lifetime of this intermediate was found to be too short (125 μs at 25°C) to account for a slow ERP component.

(b) The model of the visual pigment of the fly suggested by Hamdorf and Razmjoo (1977) (see also Hamdorf, 1979), which assumes three different states
of metarhodopsin all with similar absorption spectrum and relatively slow transitions among them, perhaps could account for our ERP data. In such a model one can assume similar dipole moments for rhodopsin and the first metarhodopsin state and different dipole moments for the other two metarhodopsin states. However, as explained above, it is impossible with this model to explain the results of Figs. 1 C and 2 E as interpreted above.

(c) Finally, there may exist a part in the visual pigment molecule which gives an additional ERP response with the same polarity after photoisomerization in either direction. This additional component might cancel the ERP phase expected from the activation of rhodopsin but would add up and enhance the ERP phase arising from activation of metarhodopsin. However, such an accurate cancellation of the hypothetical negative phase is very unlikely.

In any case we have to conclude that the fly ERP is very different from the ERP of other species, because a fast pigment transition from rhodopsin to metarhodopsin with a 90-nm red shift in the absorption spectrum and an isomerization of the chromophores are not manifested at all in the ERP, and because the amplitude of the extracellularly recorded ERP is not proportional to the rate of visual pigment converted by light.

The linearity of the ERP amplitude with light intensity and with the amount of metarhodopsin activated, nevertheless, indicates that this response in the fly does arise from a photopigment process which still has to be clarified.

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