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Rhodopsin in model membranes: Charge displacements in interfacial layers

(visual excitation/early receptor potential/protein conformation/surface potential/photoelectric signals)

H.-W. TRISSL*, A. DARSZON*, AND M. MONTAL*†

Departamento de Bioquímica, Centro de Investigación y de Estudios Avanzados del Instituto Politecnico Nacional, Mexico 14, D.F., Mexico

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ABSTRACT **A model membrane was developed in which interfacial layers of rhodopsin were reoriented onto one side of a thin Teflon film separating two aqueous compartments. Flashes evoked fast photoelectric signals (1 ms) that originated from capacitative charge displacements of oriented rhodopsin upon bleaching. The photoelectric responses of rhodopsin in the model membrane are compared with the early receptor potential of photoreceptor cells; it is concluded that the signals in both systems originate from the same mechanism.**

Vertebrate as well as invertebrate retinas are capable of generating fast photopotentials evoked by a short flash (1, 2). These potentials are called "early receptor potentials," ERPs, and precede the late receptor potential (for review see ref. 3). Usually the ERP is biphasic, consisting of a corneal positive R_1-phase followed by a corneal negative R_2-phase (4, 5). There are also ERP measurements that show a latency instead of the R_1-phase (6, 7). The overall potential transient lasts about 2 ms. In retinas displaying both the R_1-phase and the R_2-phase, either one exhibits action spectra close to the rhodopsin absorption spectrum (8). Furthermore, the response amplitude is proportional to the content of unbleached rhodopsin (5).

The origin of the ERP is commonly attributed to conformational changes within the rhodopsin molecule during its bleaching sequence. These changes are associated to charge displacement in the molecule and, because rhodopsin is in a highly ordered state in photoreceptor cells, the individual effects sum up to a macroscopically measurable photovoltage.

Because of its amphipathic nature, rhodopsin would be expected to form an oriented layer at a polar/apolar interface. Such a layer, having virtually infinite resistance, when arranged between two conducting aqueous phases, can be considered as a capacitor. Since all rhodopsin molecules can be synchronously excited by a short intense flash, it is clear, therefore, that any charge displacement consequent to rhodopsin bleaching would generate a capacitative photocurrent. This current should flow through an external low-resistance measuring circuit, or, if no current flow through the external circuit is allowed, as might be done by introducing a high input impedance electrometer, a photovoltage should be detected.

In this paper we report the formation of such oriented, high-resistance, rhodopsin layers and their light-induced responses. Our model system allows us to define the minimum molecular components responsible for the ERP, with the advantage of controlling parameters that are otherwise inaccessible in retinal photoreceptors.

Abbreviations: ERP, early receptor potential; ERC, early receptor current.

* Present address: Departments of Physics and Biology, University of California, San Diego, La Jolla, Calif. 92093.
† To whom correspondence should be addressed.

METHODS AND MATERIALS

The experimental set-up used to measure the model ERP and the model early receptor current (ERC) is illustrated in Fig. 1. The cell where the layers are prepared consists of two black Teflon halves pressed together with a 12.5 μm thin Teflon film in between; this septum has an area of 0.16 cm^2. Two silver/silver chloride electrodes, carefully light shielded, connect the two half-cells alternatively with an electrometer (Keithley 610) or an ammeter (Keithley 427). The instrument outputs lead to a storage oscilloscope. The photostimulator consists of a commercial electronic flash (Braun Hobby F 18 LS) mounted in a metal box. In order to avoid electronic noise produced by the flash discharge, a fiber optics light guide delivers the light to the windows of the cell. The optical bundle cuts off the UV region of the flash light. The cell is enclosed inside a light-tight aluminum box, placed on a shockproof table. For measuring the action spectrum narrow band interference filters (Balzers B40) are slipped into the light pathway.

Cattle rod outer segments from dark-adapted bovine retinas (Geo. A. Hormel Co.) were isolated by sucrose flotation and purified in a discontinuous sucrose gradient (9). Cetyltrimethylammonium bromide was used to extract rhodopsin (10). Retinal-free opsin was prepared according to Hubbard *et al.* (11).

The rhodopsin proteolipid was prepared as follows (12, ‡): detergent-solubilized rhodopsin (equivalent to 0.75 mg) was incubated with Bio Beads SM-2 (90 mg) for 5–7 min at 24°, and then mixed with a dispersion of partially purified soybean phospholipids in 1.0 ml of 0.1 M KCl, 0.01 M imidazole·HCl buffer, pH 7.0. The mixture was sonicated by immersion of the test tube in a water-bath sonicator for 4 min at 4°. Then, 0.1 ml of 100 mM $CaCl_2$ and 1.0 ml of hexane were added to the suspension in rapid succession. The tube was vigorously mixed for 4 min and the two phases were separated in a clinical centrifuge for 1 min. The hexane phase was removed and 1.0 ml of diethyl ether was added; the suspension was mixed for 3 min and the two phases were separated by centrifugation. The absorption spectra of 0.5 ml aliquots of the first hexane extraction and the second ether extraction were measured. Under these conditions the hexane extract exhibits a mean ΔA (difference between the absorbance in the dark and bleached states) at 500 nm of 0.022 $\pm$ 0.021 (n = 20) with a phospholipid to rhodopsin molar ratio of about 3000, whereas the ether extract presents a mean ΔA of 0.183 $\pm$ 0.043 (n = 20) with a ratio of about 400 phospholipid molecules per rhodopsin. The detergent content of the two extracts, determined with ^{14}C-labeled cetyltrimethylammonium bromide (Amersham), is also different: of the original

‡ M. Montal, A. Darszon, and H.-W. Trissl, unpublished data.

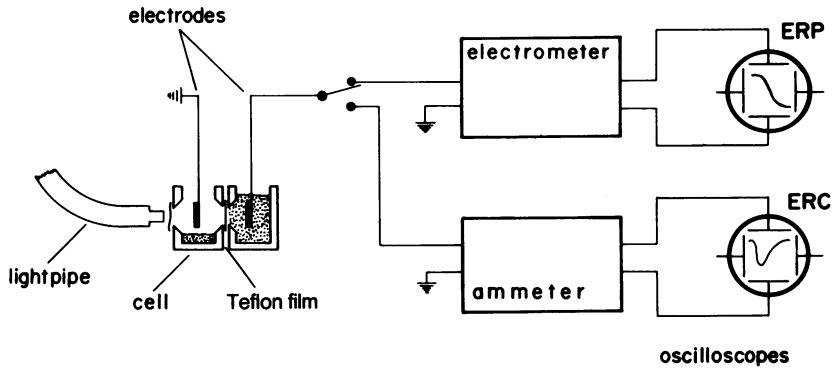


FIG. 1. Experimental set-up for the model early receptor potential (ERP) and early receptor current (ERC) measurements.

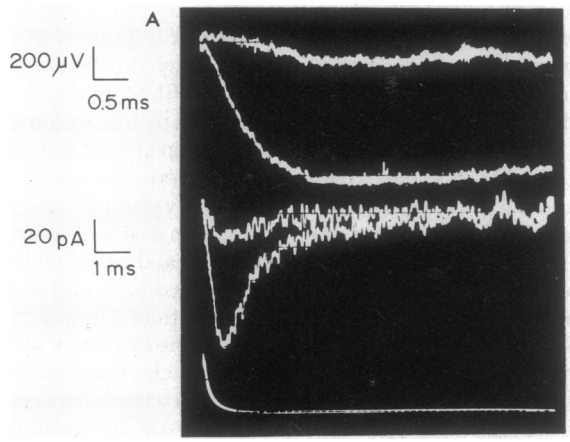
cetyltrimethylammonium bromide concentration in rhodopsin (50 mM), 40% is removed by the beads, 18% appears in the first extraction, and 10–15% appears in the second one. The proteolipid of the second extraction, when it is bleached, can be regenerated in the dark with 9-*cis*-retinal up to 70% of the value prior to bleaching (to be published elsewhere).

The procedure to form the layer is the following: one compartment of the cell is routinely filled up with electrolyte, whereas the water level in the other compartment is low enough so that it does not touch the septum. Two drops, approximately 30 μl, of the rhodopsin-proteolipid in ether are then spread over the lower electrolyte surface. After evaporation of the ether the surface is carefully overlaid with hexane, approximately 60 μl. The water level is then raised to the top of the cell. As the results prove, this procedure guarantees the apposition of the initially horizontal layer to the vertically mounted septum. Alternatively, a suspension of retinal rod disk membranes is spread by the rod method of Trurnit (13). The electrolyte consisted of 1 M NaCl with 5 mM imidazole buffer at pH 7.0 and the temperature was 24° ± 1°, unless otherwise stated.

RESULTS

Because the rhodopsin layers are formed in only one compartment the whole system is strongly asymmetric. The Teflon septum between the two half cells acts as an electrical insulator and couples the compartments only capacitatively. The effect of light flashes on such asymmetric layers is illustrated in Fig. 2A. Two layers of rhodopsin proteolipid are prepared under identical conditions in order to record the photovoltage (top trace) and the photocurrent (middle trace) separately. The lowest trace is the light stimulus. The large-amplitude signals are generated by the first white flash and the small-amplitude signals, by the second flash. The polarity of the signals is negative in the compartment where the rhodopsin layers are formed. Following a flash, a photovoltage develops with a distinct latency period of about 150 μs, achieving a steady-state value within 1.5 ms. In contrast, the photocurrent increases to a maximum within 1 ms and then decays to zero. The signals produced by a second flash follow the same time course but with an amplitude equivalent to 15% of the initial responses. It is noteworthy that the waveform of the signals is not affected or limited by the amplifier time constants. An analysis of the photocurrent time course shows it to be proportional to the first time derivative of the photovoltage.

The photoresponses do not depend on the light direction, since rhodopsin layers formed in the opposite compartment



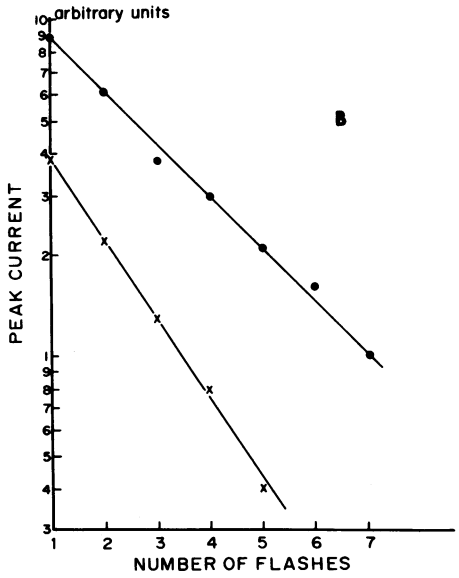


FIG. 2. (A) Photoresponses of rhodopsin-proteolipid layers at pH 7. Top trace: time course of the photovoltage generated by two subsequent white flashes. The amplifier time constant is set to 0.1 Hz–10 kHz. Middle trace: time course of the photocurrent generated by two subsequent white flashes. Control baselines with completely bleached layer or a layer without rhodopsin show a random noise of 15 pA peak-to-peak amplitude. The small waves at the right end of the trace are due to microphonics. The amplifier bandwidth is set on dc to 30 μs. Bottom trace: time course of the light flash. Notice the different time scale for the photovoltage trace. (B) Semilogarithmic plot of the photocurrent peak amplitude as a function of the number of sequential flashes. The lower line displays the result of a rhodopsin-proteolipid layer. The upper line presents the results with a layer from retinal rod disk membranes. The flash intensity was deliberately reduced to bleach gradually within a range that could be measured well. All measuring points result from identical flash intensities.

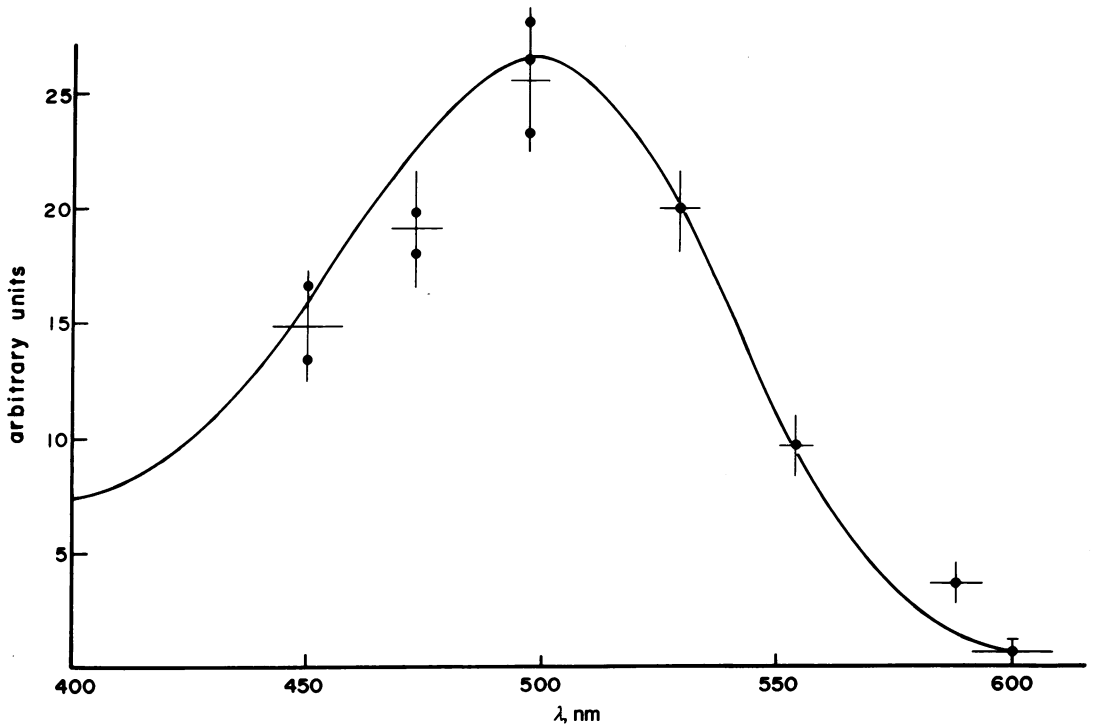


FIG. 3. Action spectrum of layers made from retinal rod disk membranes. The solid line is the absorption spectrum of rhodopsin dissolved in digitonin; its amplitude is adjusted to the data points.Each point derives from the maximal ERP amplitude of a flash at the given wavelength, normalized to the number of quanta within the flash. The figure includes photoresponses from different layers which are normalized to the amplitude of the response to a second full intensity white flash. The highest ERP created by a colored flash came to 10% of the white flash. The horizontal bars indicate the filter bandwidth at 50% transmittance and the vertical bars represent the estimated experimental error.

display signals of inverted polarity.

The dependence of the photocurrent peak amplitude on the number of flashes is illustrated in Fig. 2B, in which disk and proteolipid layers are examined. The straight lines in the semilogarithmic plot indicate that the signal is proportional to the number of unbleached rhodopsin molecules, much in the same way as the ERP signal recorded from albino rat retinas (3, 4).

Fig. 3 shows the action spectrum of the model ERP obtained from layers of retinal rod disk membranes. The photovoltage in arbitrary units is compared with the absorption spectrum of rhodopsin. The good match identifies rhodopsin as the molecular species responsible for the photoresponses.

The ERP and ERC evoked from proteolipid layers differ slightly from those of the rod disk membrane layers. Whereas the proteolipid layer photoresponse shows a latency of $\simeq$150 μs, that of "disk layers" is almost 300 μs. Furthermore, the maximal normalized time derivative of the photovoltage of proteolipid layers is approximately 50% greater than that from disk layers. The amplitudes that can be achieved with both kinds of layers are equivalent.

The dependences of the model ERP on ionic strength, pH, and temperature were investigated with rod disk membrane layers. If the photopotential arises from the exchange of free ions between interface and aqueous phase, a strong dependence of the signal amplitude on the ionic strength would be expected. For this purpose a layer was formed in a medium with high ionic strength (1 M NaCl) and after the water level was raised the electrolyte was exchanged for distilled water. The amplitude and time course of the photovoltage displayed under this condition was not significantly different from that in Fig. 2A.

The signal amplitudes as well as their shapes do not depend on pH between the values 5 and 8. pH values lower than 5 and higher than 8 cause a drop in the response amplitudes and also alter the shape. The model ERP is stable up to 50°. At 0° it is completely abolished.

Control experiments with layers composed of mixtures of 9-*cis*-retinal or all-*trans*-retinal and phospholipids do not display a photoresponse. In addition, a proteolipid prepared from retinal-free opsin is unresponsive.

DISCUSSION

The photoeffects measured in rhodopsin layers establish the presence of excitable and oriented molecules at a polar/apolar interface. It has been proved that the model ERP arises from a capacitative charge displacement, since no ion flow through the layer is possible. Furthermore, it is shown that the photopotential is generated within the rhodopsin molecule itself rather than from ion movement in the aqueous interface. Such movement should depend on ionic strength, which is not the case in these experiments. It can also be concluded that protons are not involved in the generating mechanism, since a change in the H^+ concentration of 3 orders of magnitude does not affect the signals. These results strongly suggest that the model ERP is due to a conformational change connected with a charge redistribution within the rhodopsin molecule.

The ERP from photoreceptor cells normally exhibits a biphasic waveform. However, the rhodopsin layers have not displayed an initial R_1-phase; instead, a distinct latency period is observed. This observation does not imply a discrepancy between the ERP from photoreceptor cells and the model ERP because monophasic photopotentials with a latency have also been recorded from photoreceptor cells (6, 7). For instance, the R_1-phase in the human retina ERP is mainly generated in the cone but not in the rod cells (7).

The question arises as to how the model ERP is related to the

spectroscopically defined intermediates in the bleaching sequence of rhodopsin. A comparison of the time course of the photoreceptor ERP with the model photovoltage suggests that the latter is equivalent to the R_2-phase. The R_2-phase is generally assumed to reflect the metarhodopsin I/metarhodopsin II transition. Because this transition can be frozen at 0°C and the R_2-phase also vanishes at this temperature, just as the model ERP does, it could be that the metarhodopsin I/metarhodopsin II transition has to have taken place in order for the photoresponse to be measured.

In excitable nerve membranes, the opening and closing of the sodium channels is associated with a capacitative current, the gating current (15). The capacitative photocurrent generated in the rhodopsin layers arises from charge displacements in the protein as it goes through the conformational transitions. Because the consequence of the conformation change is the formation of a transmembrane channel (12, ‡), we are tempted to suggest that the photocurrent measured may be to the rhodopsin membranes what the gating current is for the Na^+ channel in nerve membranes.

The approach used in this work can, in principle, be extended to any excitable membrane system that can be oriented as well as synchronously excited. For instance, we have also detected capacitative photoresponses in bacteriorhodopsin layers (unpublished results).

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