BACTERIORHODOPSIN IN MODEL MEMBRANES

A NEW COMPONENT OF THE DISPLACEMENT PHOTOCURRENT IN THE MICROSECOND TIME SCALE

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ABSTRACT A quasi-short-circuit (tunable voltage clamp) measurement method with microsecond time resolution was applied to a bacteriorhodopsin model membrane formed by a novel interfacial technique. A new component (B1) of the displacement photocurrent was recorded: it has no detectable latency at an instrumental time constant of 1.5 μs, and persists at 5°C. In addition, a slower component (B2) of opposite polarity inhibited by low temperature (5°C) and low pH (pH = 3.0) was recorded. The technique is very sensitive for the study of fast capacitative photoresponses in model membranes, and allows the detection of charge displacements in bacteriorhodopsin associated with distinct stages of the photochemical transformation.

INTRODUCTION

Bacteriorhodopsin is a retinal-containing chromoprotein that resembles the visual pigment rhodopsin in chemical composition and photochemistry, and is the only protein component in the purple membrane of *Halobacterium halobium* (for a recent review, see Henderson, 1977). Stoeckenius and co-workers (Oesterhelt and Stoeckenius, 1973; Racker and Stoeckenius, 1974; Lozier, Bogomolni, and Stoeckenius, 1975) have shown that bacteriorhodopsin is a light-driven proton pump: it moves protons from the intracellular space to the external medium. The resulting proton gradient is then utilized for ATP synthesis. Thus, bacteriorhodopsin functions as a photon energy converter similar to a photosynthetic membrane.

Upon illumination, bacteriorhodopsin as well as rhodopsin proceed through a series of conformational transitions characterized by flash spectroscopy and identified by their wavelength of maximum absorbance (Lozier et al., 1975; cf. Kropf, 1972; Yoshizawa, 1972). The formation and decay of the rhodopsin photochemical intermediates have been associated with the generation of the early receptor potential (ERP) in retinal photoreceptor cells (Brown and Murakami, 1964); this has been characterized as a displacement (capacitative) signal with no detectable latency (cf. Cone and Pak, 1971). Recently, Trissl et al. (1977) succeeded in recording directly such ERP signals in a model membrane in which an asymmetrically oriented rhodopsin-lipid monolayer was deposited on a thin Teflon septum sepa-

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rating two aqueous compartments: flashes evoked fast photoelectric signals that originated from charge displacements of oriented rhodopsin upon bleaching. A fast capacitative photosignal was also observed in a similar model membrane containing asymmetrically oriented bacteriorhodopsin (Trissl and Montal, 1977). Hwang et al. (1978) confirmed these results using multilayers of dry purple membranes and lipids sandwiched between two metal electrodes; in addition, they recorded charge displacements associated with each of the photointermediates. Such a capacitative photosignal in bacteriorhodopsin membranes was predicted by Hong (1977) from analysis of data initially reported by Drachev et al. (1974), and recently refined by Drachev et al. (1978) and Herrmann and Rayfield (1978).

The displacement photosignals recorded from both the rhodopsin (Trissl et al., 1977) and the bacteriorhodopsin (Trissl and Montal, 1977) model membranes display a distinct latency of 250–500 μs after illumination. This latency can be attributed to the relatively long duration of the light pulse (1 ms) and the limited sensitivity of the open-circuit method (i.e., infinite access impedance) hitherto used. In the course of studying fast photoelectric effects in metalloporphyrin-containing bilayer lipid membranes, Hong and Mauzerall (1974, 1976) developed a quasi-short-circuit (tunable voltage clamp) method of measurement and introduced the concept of chemical capacitance (related to light-induced transient charge separation and subsequent recombination) to interpret the data so measured. In addition to a microsecond time resolution, this method provides relevant kinetic information that is usually distorted or even masked in conventional open-circuit measurements.

Here, we combined two approaches to examine the fast photoelectric responses of bacteriorhodopsin in a membrane: the monolayer technique to incorporate bacteriorhodopsin in a model membrane, and the tunable voltage clamp method of measurement. We report the observation of a new component of the displacement photocurrent with rise time limited only by the instrumental time constant of 1.5 μs.

MATERIALS AND METHODS

Purple membrane fragments of *H. halobium* were kindly supplied by Dr. N. Nelson (Department of Biology, Technion, Haifa, Israel). The model membranes were formed according to Trissl and Montal (1977) (Fig. 1 A): surface-active material from the aqueous salt suspension of purple membrane fragments was obtained by agitation in a Vortex mixer (Scientific Industries, Inc., Bohemia, N.Y.) until foam formed. About 100 μl of the foam was transferred to one interface of a two-compartment Teflon cell containing a NaCl subphase (2 or 4 M). Thereafter, it was overlaid with about 50 μl of hexane. The surface film in the water-hexane interface was apposed to a vertically mounted Teflon septum (6 μm thick) by slowly raising the water level; this septum has an area of 0.16 cm^2. The chamber on the other side of the partition contains only electrolyte solution. Tunable voltage clamp measurements were made through a pair of carefully shielded Ag/AgCl electrodes connected to a negative feedback amplifier with adjustable gains, instrumental time constants, and access impedances. The circuitry and the principle of its operation are described elsewhere (Hong and Mauzerall, 1976). The output of this amplifier was then fed into a transient recorder (Biomation model 8100, Biomation Corp., Cupertino, Calif.). The analog output of the latter was then recorded on Polaroid films via a cathode ray tube display (Polaroid Corp., Cambridge, Mass.). The light source was a pulsed dye laser (Phase-R model DL-1200V; pulse duration 300 ns) with rhodamine 6G or coumarin dyes (output 590 and 499 nm, respectively; dye solutions purchased from Phase-R Corp., New Durham, N.H.). The light intensity was about 10 mJ/cm^2, delivered without focusing to the entire membrane. The synchronization of the laser discharge and the start of the recorder sweep was controlled by a digital delay generator (Berkeley Nucleonics Corp., Berkeley, Calif.,

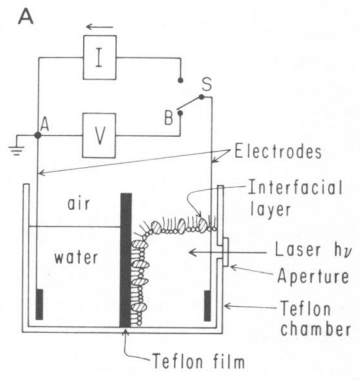

FIGURE 1A

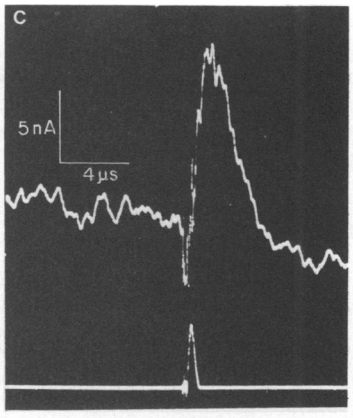

FIGURE 1C

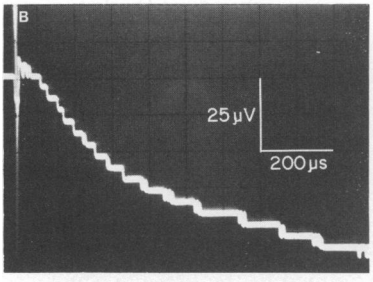

FIGURE 1B

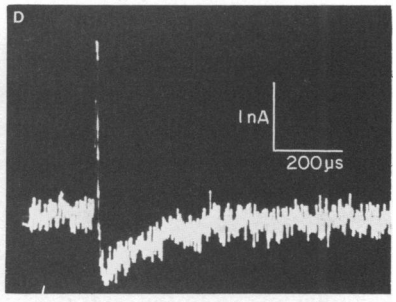

FIGURE 1D

FIGURE 1 Schematic representation of the experimental system and typical photoelectric responses from a bacteriorhodopsin model membrane. Fig. 1 A illustrates the membrane-forming chamber, with two aqueous compartments separated by a thin (6 μm) Teflon film (not drawn to scale). The interfacial layer is shown as a defined lipid-protein monolayer only for illustrative reasons and not to convey information on the molecular array of the layer, which is certainly more complex. The sign conventions of measurements are indicated: the arrow indicates the direction of positive current, and the voltage is measured with electrode A as reference. S is a switch for selecting a quasi-short-circuit (I) or an open-circuit (V) measurement. Fig. 1 B is an open-circuit photovoltage record. The steps are due to digitization of the signal. Fig. 1 C and 1 D were taken from the same membrane measured at an effective access impedance of 1 kΩ and instrumental time constants of 1.5 μs and 15 μs, respectively. The initial negative deflections on the photovoltage record (Fig. 1 B) and on the photocurrent record in Fig. 1 C are artefacts due to the laser discharge. The photocurrent has a positive phase followed by a more prolonged negative phase. The negative phase eventually relaxed to the original dark base line. The light source was a dye laser pulse (499 nm in Fig. 1 B, and 590 nm in Fig. 1 C and 1 D). The laser pulse is shown in the lower trace of Fig. 1 C. The two electrolyte subphases contained 4 M NaCl. Temperature: 20°C. pH: 7.0.

model 7010). The laser pulse was measured with a silicon photodiode detector (EG&G model 560B, EG&G Inc., Electro-Optics Div., Salem, Mass.). Fluctuations in the laser pulse amplitude were less than 10%, and so was the variation of the photosignal amplitudes. Since the observed changes under the various conditions to be described below far exceed the fluctuations in laser power, no attempt was made to normalize the amplitude of individual photosignals with respect to the actual light intensity. Measurements of access impedance and membrane discharging time constants (product of the equivalent access resistance and the membrane capacitance, or product

of the membrane resistance and the membrane capacitance, whichever was smaller) were made by applying a square-wave pulse from a pulse generator (Wavetek, San Diego, Calif., model 801) according to described procedures (Hong, 1976). The open-circuit photovoltage was measured as described by Trissl and Montal (1977), except that a coumarin dye laser pulse at 499 nm was used instead of a millisecond flash. The sign conventions of the current and voltage measurements are indicated in Fig. 1 A.

RESULTS

An open-circuit photoresponse to a dye laser pulse is shown in Fig. 1 B. The time-course reported by Trissl and Montal (1977) is reproduced. In addition, a new peak of opposite polarity is barely discernible in the record; it has a latency of less than 10 μs and is about 30 times smaller in amplitude than the previously reported signal.

Typical photocurrents of a bacteriorhodopsin model membrane responding to a 300–ns laser pulse are shown in Fig. 1 C and 1 D. The photocurrent, as measured at an access impedance of 1 kΩ, exhibits two transient peaks of opposite polarities (Figs. 1 C and 1 D). The initial positive phase has a polarity that is positive on the side where the bacteriorhodopsin lipid layer was deposited. The photocurrent reaches a positive peak in 1.5 μs after the light pulse is delivered, and decays in 2 μs toward a negative peak at t = 21 μs. The negative phase relaxes with an exponential time constant of about 160 μs toward the original dark base line (Fig. 1 D). The polarity is unrelated to the direction of the incident light beam. The two photovoltage signals (Fig. 1 B) display the same polarities as the two photocurrent peaks (Fig. 1 C and 1 D).

Control experiments in membranes without bacteriorhodopsin indicated that the electrical artefact (due mainly to the laser discharge) is confined to the first microsecond after the rise of the light pulse. Long-term reproducibility of the photosignal amplitude is remarkable: the photosignal amplitude shows no tendency to decline over a 5-h period in which hundreds of light pulses had been shone on a membrane. In the present series of measurements, the light intensity is in the range of linear light response for both the positive and the negative peaks. Under a given condition, the time-course of the photosignals does not show noticeable fluctuations from membrane to membrane. The two observed relaxation time constants are, however, strongly dependent on the access impedance of the measurement. As the access impedance is progressively increased from 1 and 20 kΩ, the relaxation time constants are prolonged and the amplitudes are reduced, in agreement with similar observations of magnesium porphyrin lipid bilayers (Hong and Mauzerall, 1976).

The effect of temperature and pH on the two photocurrent peaks was studied. As temperature is lowered from 25 to 5°C, the negative peak decreases in amplitude while the positive peak increases (Figs. 2 A and 2 B). This temperature effect is reversible, as shown in a series of experiments using the same membrane. The effect of low pH (pH = 3.0) is similar to low temperature (5°C), i.e., low pH enhances the positive peak but suppresses the negative peak. The combined effects of pH and temperature are shown in Fig. 2 with data taken from the same membrane to illustrate the reversibility of pH effect. The data are presented in the chronological order in which they were taken over a period of 3 h. As the temperature is lowered from 25 to 5°C at pH 5.8, the photosignal changes as described above (Figs. 2 A and B). Lowering the pH from 5.8 to 3.0 at 5°C has a negligible effect on the photosignal (Fig. 2 C). Raising the temperature back to 25°C while maintaining the pH at 3.0 does not

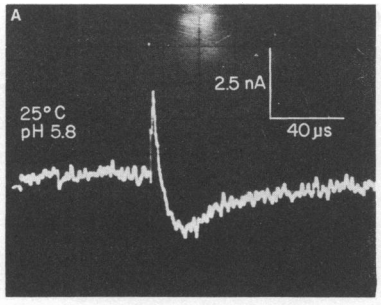

FIGURE 2A

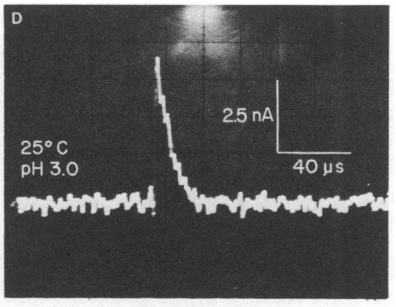

FIGURE 2D

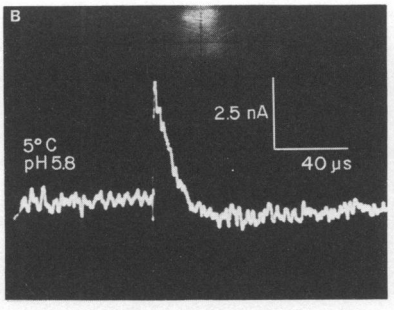

FIGURE 2B

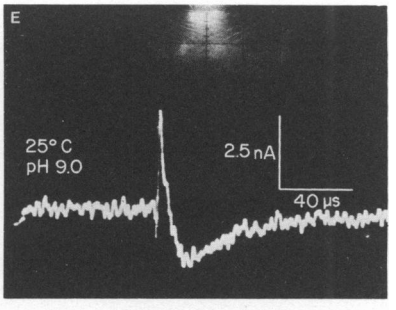

FIGURE 2E

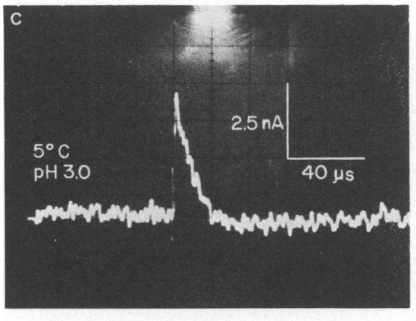

FIGURE 2C

FIGURE 2 Temperature and pH effects on the photocurrent from a bacteriorhodopsin model membrane. All the records were taken from the same membrane in the sequence of presentation. The effective access impedance was 1 kΩ. The instrumental time constant was 15 μs. The light source was a dye laser pulse at 590 nm. The two electrolyte subphases contained 2 M NaCl.

restore the negative peak (Fig. 2 D). However, when the pH is increased to pass neutrality and up to pH 9.0, the negative peak recovers and the positive peak diminishes (Fig. 2 E).

DISCUSSION AND CONCLUSION

The most salient feature of the bacteriorhodopsin photoelectric signal reported here is the apparent lack of a latency: the rise time of the photocurrent is limited by the instrumental

time constant of 1.5 μs. The photocurrents recorded at room temperature and neutral pH display a positive and a negative phase. The relaxation time-course of the photocurrent can be fit with two exponential time constants. The temperature effect suggests two components of displacement photocurrent; the fast positive component is temperature-insensitive and the slow negative component is inhibited by low temperature (5°C). These observations raise the following question: are these two time constants the relaxation time constants of two distinct molecular processes?

A previous study of model pigmented membranes indicated that a fast capacitative photocurrent arising from a single process relaxes almost always in two exponentials of opposite polarities (Hong and Mauzerall, 1974, 1976). The two apparent time constants are not themselves molecular constants, but result from the interaction between the time constants of the intrinsic molecular relaxation and of the passive membrane discharge through a dependence on the access impedance of the measurement. This has been verified in the bacteriorhodopsin system, since the two apparent relaxation time constants increase as the access impedance is increased. At the extreme case of open-circuit conditions (i.e., infinite access impedance), the slower of the two apparent time constants becomes coincident with the membrane-discharging time time constant (Hong and Mauzerall, 1976).

Here we conclude that there are actually two distinct molecular processes involved in the generation of the photoelectric signal. This conclusion is supported by the photovoltage signals presented in Fig. 1 B and based on the following arguments and results. The nature of the model membrane determines that only capacitative photocurrents be observed, since the Teflon septum has a high DC resistance ($>10^{15}\Omega$) and couples the aqueous compartments only capacitatively. Therefore, every relaxation process gives rise to a displacement photocurrent that is biphasic and that has a zero time-integral (Hong, 1976). This latter condition is not fulfilled in the data shown in Figs. 1 C and 1 D, as the area bounded by the negative phase and the base line exceeds the area bounded by the positive phase and the base line. This leads to the conclusion that we are indeed observing a photosignal due to two or more molecular processes. Likewise, to fulfill the zero time-integral condition, we postulate a slow (second) positive phase that follows the negative phase and is not resolved in the present measurement. Thus, there are two components, each relaxing with two apparent time constants and each biphasic in wave form. The fast positive component (B1) has a polarity opposite to that reported by Trissl and Montal (1977), and is thus a new component; the slow negative component (B2) can be readily identified with that previously observed (Trissl and Montal, 1977). A schematic decomposition of the photocurrent response is illustrated in Fig. 3.

This interpretation is consistent with the temperature dependence of the photosignals. The records in Fig. 2 show that the time-integral of the photocurrent at 5°C (pH 5.8) is closer to zero than at 25°C (pH 5.8). This is expected if low temperature preferentially suppressed the B2 component but left the B1 component essentially intact. The apparent amplitude increase of the positive peak can be accounted for by the overlapping of the B1 component decay and the B2 component rise, as illustrated in Fig. 3.

Fig. 2 demonstrates that low pH (pH = 3.0) reduces the B2 component but does not significantly affect the B1 component and that the pH effect is reversible. This finding is consistent with the observations of Trissl and Montal (1977) on the reduction of the amplitude

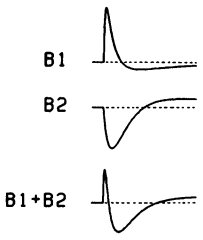

FIGURE 3 Schematic decomposition of a typical photocurrent response of a bacteriorhodopsin model membrane at room temperature and neutral pH. The dotted lines are the base lines. The observed photocurrent is the sum of two components, B1 and B2. The illustration is exaggerated for the sake of clarity. Notice that a second positive phase may or may not be present in the sum, depending on the relationship of the time-courses of B1 and B2 components. Please see text for further explanation.

of the negative phase and the appearance of a very fast positive transient. Presumably, the B1 component masked by the larger B2 component at neutral pH emerges when the B2 component is suppressed by low pH.

The two components of the displacement photocurrent, B1 and B2, are the electrical correlates of the molecular charge displacements associated with the photochemical transformation. A precise assignment of the relaxation kinetics of the photointermediates associated with the B1 and the B2 components is not feasible with the present data, because of insufficient signal-to-noise ratio. Nevertheless, considering in addition the effects of temperature and pH, the B1 component may be tentatively correlated with the formation and decay of the K_{590} intermediate, while the B2 component may be generated by the formation and decay of M_{412} or a still later photochemical intermediate.

The arrangement of the Teflon layer in this model system has the virtue of excluding the more prominent DC photocurrent that would be present otherwise. However, its effect on the kinetics of the system cannot be ascertained until comparable measurements are made with a genuine bilayer, e.g., one formed by apposing two monolayers (Montal and Mueller, 1972).

It is generally believed that fast photoelectric signals, such as the ERP, are generated by intramolecular charge displacement, while the oriented membrane-bound pigment undergoes conformational changes (oriented dipole model) (Cone 1967; Hagins and Rüppel, 1971). However, it was demonstrated in a model system that an alternative mechanism based on reversible surface charge transfer can also generate capacitative photocurrents (interfacial charge transfer model) (Hong, 1976, 1978). The approach reported here with the accompanying kinetic analysis offers a possible way to evaluate the contribution of each mechanism to the generation of the observed photoresponses.

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