KINETICS OF THE LIGHT-DRIVEN PROTON MOVEMENT IN MODEL MEMBRANES CONTAINING BACTERIORHODOPSIN

G. W. RAYFIELD
Physics Department, University of Oregon, Eugene, Oregon 97403

ABSTRACT The short-circuit photoresponse of model membranes containing bacteriorhodopsin to short (35 ms) and long (3.5 s) light pulses is described. It is shown that if the light pulse is short compared with the charging and discharging times of the model membrane, the temporal response of the light-driven proton pump can be measured. Photoactive planar model membranes were formed both from biomolecular lipid membranes and from solid 6-μm thick Teflon septa coated with lipid and bacteriorhodopsin. The kinetic response of the pump is independent of the planar model membrane system in which it is incorporated. Experimental evidence indicates that the shape of the leading and trailing edges of the photoresponse curve for the pump deviates from simple exponential behavior. The short-circuit photoresponse of spinach chloroplast in a planar model membrane was also studied for comparison purposes.

INTRODUCTION

Bacteriorhodopsin, a protein derived from *Halobacterium halobium,* functions as a light-driven proton pump. Extensive reviews (1–3) describe in detail the role of bacteriorhodopsin in photophosphorylation of *H. halobium,* the structure of the protein and the photochemistry associated with it.

Recent studies of the photocurrent response of model membranes incorporating bacteriorhodopsin (BR)[1] have been reported (4–11). A variety of methods have been used to form the photoactive model membrane: purple membrane sheets can be added to the salt solutions that bathe the bimolecular lipid membrane (BLM) (4, 5, 9); vesicles containing bacteriorhodopsin can be added to the bathing solutions (10); bacteriorhodopsin can be "plated" onto a solid thin Teflon septum separating two aqueous compartments (7, 8), or membranes can be formed from air-water-interface films (11). A typical short-circuit photoresponse to a long (3.5 s) light pulse is shown in Fig. 1.

Two qualitative observations of photoactive membranes formed by vesicle fusion have led to a model (10) in which charging and discharging of the membrane takes place: (*a*) when the membrane permeability to protons is increased with a protonophore such as carbonylcyanide *m*-chlorophenylhydrazone (CCCP), there is an increase in the steady-state short-circuit current; (*b*) a large overshoot (displacement current) is observed when the light is turned on. Furthermore, the decay time of this overshoot is sensitive to the membrane's permeability to protons. Fig. 2 *A* is a schematic drawing of this model. This model is somewhat speculative, particularly with regard to the trapped electrolyte region within the dielectric. The movement of charge (either bound or free) within the protein BR requires charge movement in the external circuit to maintain the boundary conditions at the dielectric-electrolyte interfaces. A corresponding displacement current is registered by the ammeter in the external circuit. Under steady illumination of the membrane, a potential builds up in the interior of the fused vesicles. This leads to the development of a leakage current through the dielectric and additional charge movement in the external circuit.

An electrical equivalent circuit for Fig. 2 *A* is shown in Fig. 2 *B*. This equivalent circuit is used to describe the observed photoresponse on a "long" time scale; that is, times comparable to the charging and discharging times of the membrane (see below). The response of the membrane to a long light pulse (Fig. 1) is reproduced by choosing appropriate relative values for R_1, R_2, C_1, and C_2 and by using a nonideal (voltage-dependent) current generator, I_p, that has an instantaneous on-off response (10). R_1, R_2, C_1, and C_2 vary from one membrane to the other and R_1 and R_2 can be altered by adding ionophores to the membrane bathing solutions. The time constant associated with the negative current transient in Fig. 1 is the membrane (RC) time constant and varies from 1 to 3 s (10).[2] The initial

[1] *Abbreviations used in this paper:* BLM, bimolecular lipid membrane; BR, bacteriorhodopsin; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; ODA, octadecylamine; PC, phosphatidylcholine; PS, phosphatidylserine.

[2] This time is consistent with the known electrical properties (i.e., resistance and capacitance) of lipid bilayers (13) and Fig. 2*A* since the RC time constant is area independent.

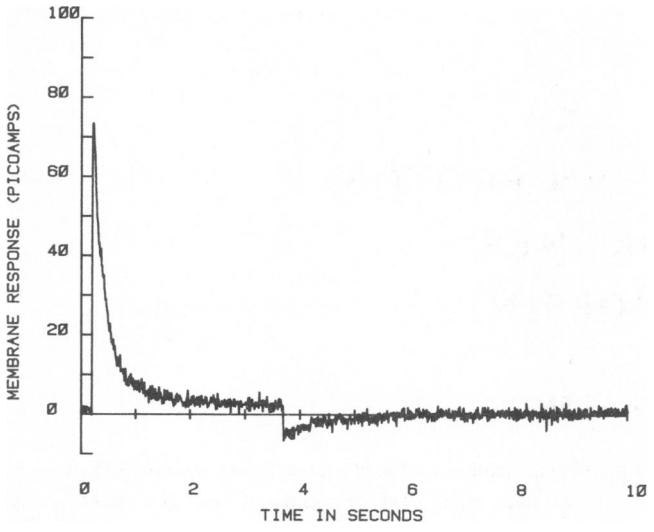

FIGURE 1 The short-circuit photoresponse of a BLM with fused sonicated BR vesicles is shown. The membrane was illuminated with a long light pulse (3.5 s) with a maximum intensity of 18 mW/cm^2. The BLM was formed from PS-azolectin (in the ratio 3:7) and the vesicles contained both lipids in the same ratio. The bathing solution was 100 mM NaCl, 10 mM pipes pH 7 and 10 mM $CaCl_2$.

decay after the peak photocurrent exhibits a reduced time constant that reflects the voltage dependence of the current generator. A similar circuit has been used by Bamberg et al. (4) and Korenbrot and Hwang (11) to describe other photoactive model membranes.

Because the gross features in Fig. 1 are described by assuming an instantaneous on-off response of the proton pump, little or no information is obtained regarding the transient response of the proton pump itself to a step function (of light).

The idea of the work presented here was to make measurements on a time scale that was short compared with the RC time constants of the model membrane, so that when a light pulse photoactivates the model membrane, only a negligible change in the potential at node b (Fig. 2) occurs, and leakage currents through R_1 and R_2 can be neglected as compared with the displacement currents through C_1 and C_2. Under these conditions of only capacitive coupling, the temporal response of the electrometer reflects that of the proton pump (10). That is, the ammeter in the external circuit shows the same time dependence as the proton pump: $I_A(t) = I_p(t) \times C_1/(C_1 + C_2)$.

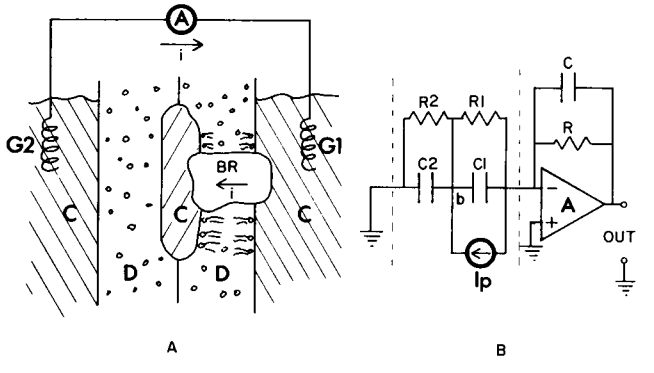

FIGURE 2 (*A*) A schematic drawing of BR incorporated into a photoactive membrane by vesicle fusion in shown. G_1 and G_2 are the Ag-AgCl electrodes and are connected to the external ammeter (*A*). The conducting regions (salt solutions) are labeled *C*, and the dielectric regions are labeled *D* and consist of lipids and/or Teflon. Charge movement within the protein (BR) gives rise to a small displacement current *i* in the external circuit due to capacitive coupling. (*B*) The equivalent electrical circuit for Fig. 2*A* is shown. *A* is a current-measuring electrometer (Analog Devices model 42L op amp) and provides a virtual ground for one chamber. *R* is the feedback resistance and *C* the shunt reactance required for critical damping of the electrometer (see text). Parameters associated with the photoactive membrane (R_1, R_2, C_1, C_2 and I_p) are contained between the dotted lines. I_p is the equivalent current generator (nonideal) for the proton pump.

METHODS

Fresh purple membrane was grown and purified (12) every 2 mo from strain R_1 of *H. halobium* provided by Dr. W. Stoeckenius, Dept. of Biochemistry, University of California at San Francisco, CA. Purple membrane sheets were stored in DH_2O with 0.1% sodium hydride in a refrigerator at 4°C. Azolectin (soyabean) and CCCP were obtained from Sigma Chemical Co., St. Louis, MO. Octadecylamine (ODA) was purchased from P-L Biochemicals, Inc. (Milwaukee, WI). Egg lecithin phosphatidylcholine (PC) and bovine phosphatidylserine (PS) were purchased from Avanti Biochemicals, Inc. (Birmingham, AL). Octyl-β-D-Glucopyranoside was purchased from Calbiochem-Behring Corp. (American Hoechst Corp., San Diego, CA). Chloroplast (spinach) was prepared following Tien (13).

Charged BR vesicles were formed by sonicating on ice for 10 min a lipid mixture of 10 mg/ml azolectin and PS (ratio 7:3) in DH_2O at pH 7 with purple membrane sheets (1 mg/ml). A probe-type sonicator was used (MSE Instrumentation Assoc., New York, NY) at a current setting of 1 A.

Vesicles containing BR were also formed by first solubilizing the purple membrane sheets in Octyl-β-D-Glucopyranoside, and then dialyzing away the detergent in the presence of excess lipids (Azolectin and PS) such that the monomeric form of bacteriorhodopsin would be preserved (14). The loss of exiton coupling effects in the visible circular dichroism spectrum was taken as an indication that the BR was in the monomeric form. These vesicles were then fused with the charged BLM described below.

Model planar membranes (BLM) containing BR were formed following procedures given earlier (1–5, 10). Charged BLM were formed from either a mixture of PC and ODA (5) or azolectin and PS (in the ratio 7:3). A 12-μm thick Teflon septum with a 0.45-mm diameter hole was used for support of the BLM. Either purple membrane sheets or charged vesicles containing purple membrane (above) were then fused to a "charged" BLM. The bathing solution was 100-mM NaCl buffered to pH 7 with 10-mM pipes. When PS was used to charge the membrane, the bathing solution also contained 10 mM $CaCl_2$.

Bacteriorhodopsin was plated onto a solid 6-μm septum separating the two chambers of the cell using the following procedure: the septum is first given a thin coat of charged lipids, either PS and azolectin or PC and ODA in decane. The vesicles or purple membrane sheets are then added to the bathing solution and allowed to interact or plate onto the septum.

BLM containing spinach chloroplast were formed following techniques described by Tien (13). Again, a 12-μm thick Teflon septum with a 0.45-mm diameter hole was used to support the BLM. Each chamber was filled with 100 mM sodium acetate buffer at pH 7. The front chamber (with a glass window) also contained 0.7 mM $FeCl_3$ while the back chamber contained 0.7 mM ascorbic acid in addition to sodium acetate buffer. Photoactive membranes on a solid 6-μm Teflon septum were

formed by completely coating the Teflon with the BLM-forming solution described above and then adding 0.7 mM $FeCl_3$ to the sodium acetate bathing solution.

The experimental apparatus is similar to that described in an earlier paper (10). Two Teflon chambers, each having a volume of 15 cm^3, are separated by the membrane to be studied. A glass window in one of the chambers allows illumination of the membrane. The light source is a 150 W projector lamp (model FCS; General Electric Co., Lamp Components Sales Operation, Cleveland, OH) which was chosen for its small filament size. Light from the lamp is passed through heat-absorbing glass, a water cell, (Corning Glass Works, Corning, NY) nos. 4-94 and 3-90 glass filters and an electric shutter model 26; (A. W. Vincent Assoc., Rochester, NY) before entering the chambers. Ag-AgCl electrodes are used for making electrical contact with the salt solutions that bathe the membrane. The Ag-AgCl electrode in the chamber with the window is connected to an electrometer (described below) that provides a virtual ground for this chamber. The other electrode in the opposite chamber is connected directly to ground. However, this so-called short-circuit arrangement does not necessarily ensure that there is zero-potential bias across the protein when the membrane is illuminated. This is because both sides of the protein are not in direct contact with the bathing solutions (see Fig. 2). Both electrodes are shielded from direct illumination.

The electrometer was constructed using an Analog Devices 42 L operational amplifier (Analog Devices, Inc., Norwood, MA) and a 10^9 feedback resistor (Victoreen, Inc., Sheller-Globe, Corp., Cleveland, OH) in a current-to-voltage converter configuration. This amplifier was chosen for its reasonable bandwidth (1 MHz), low-input bias current and low current noise. The input capacitance of the cell (mostly due to the membrane itself) ranges from ~300 pF for a solid Teflon septum to 3,000 pF for a 1-mm diameter BLM and limits the rise time of the electrometer (15). The electrometer is critically damped by shunting the feedback resistor with a 1–20 pF variable capacitor (15). Using a Hewlett-Packard (HP) PIN photodiode (Hewlett-Packard Co., Palo Alto, CA) (5,082-4,220) as the current source and a LED as the light source, the rise time of the electrometer was measured to be 200 μs. The rise (opening) time of the electric shutter was measured by replacing the LED with the shutter and found to be 0.7 ms. Current noise is dominated by the input circuit due to the relatively large capacitance-to-ground through the membrane and is, therefore, very frequency dependent. A tradeoff has to be made between frequency response and the signal-to-noise ratio desired.

The output from the electrometer is fed into a two-pole Butterworth filter (UAF 41 Burr-Brown Research Corp., Tuscon, AZ). This allows easy adjustment of the circuit bandwidth. Restricting the bandwidth to 1,000 Hz gives a current noise level (with the solid Teflon membrane) of 4 pA while at a bandwidth of 2,000 Hz the noise increases to 12 pA. Removing the membrane from the electrometer input reduces the noise by more than an order of magnitude.

The overall rise time of the shutter and electronics is determined by positioning the HP photodiode directly behind the transparent Teflon septum in the experimental cell and measuring its photoresponse with the electrometer. The rise time of the apparatus for two different bandwidths (1 and 2 kHz) is shown by the dotted curves in Fig. 3 (*inset*).

Light intensity at the membrane is determined by first calibrating the HP photodiode against a calibrated photovoltaic detector (model SEE 010; International Light Inc., Newbury Port, MA), which is traceable to a standard set by the National Bureau of Standards. It is then inserted behind the membrane as described above, the front chamber is filled with bathing solution and the photocurrent output from the diode determines the light intensity incident on the membrane. Intensity varied by <10% over the whole area of the larger solid Teflon membrane. A regulated DC power supply is used for the projector lamp to eliminate effects due to power line fluctuations.

The resistance between the Ag-AgCl electrodes with 100 mM NaCl pH 7 bathing solutions was 265 Ω (no membrane present). This decreased to 87 Ω with 1-M salt solutions. Typical photocurrents are <1 nA so that the voltage drop through the electrolyte bathing the membrane is negligible. The 100-mM solutions have a Debye-Huckel shielding length (13) of ~1 nm, which is short compared to the thickness of dielectric (Teflon and lipid bilayer) regions in the model membranes.

The output signal corresponding to the membranes photoresponse is sampled by an analog-to-digital converter (ADC) every 100 μs (1,000 data points) for short light pulses and every 10 ms for the long light pulses. The output of the ADC is fed to the computer (PDP-11/20 Digital Equipment Corp., Maynard, MA), which in turn controls the light shutter. Photoresponse curves are plotted on a digital plotter (HP 7225A). Signal to noise can be improved by using signal averaging but sufficient time between sweeps (~10 s) must be allowed to prevent charging the membrane. Digital averaging can also be used to reduce noise but care has to be taken to prevent degradation of the response time.

RESULTS

The photoresponse of a BR-BLM model membrane illuminated by a short (35 ms) light pulse is shown in Fig. 3 (solid curve) and, as expected, appears quite different from the long light-pulse photoresponse of the same membrane (Fig. 1). This photoresponse was taken with a light intensity of 18 mW/cm^2 and a bandwidth of 1 kHz. According to the arguments above, the time-course of the photocurrent shown in Fig. 3 follows that of the proton pump, however, the peak value depends on the relative displacement currents through C_1 and C_2 (Fig. 2 *B*). The peak value of the photocurrent also depends on the light intensity and shows evidence of saturation at the highest light intensity available, 30 mW/cm^2. Varying the length of the light pulse has no clear effect on the shape of the leading or trailing edge until the length of the light pulse exceeds ~60 ms. At this point charging of the membrane begins to become a significant factor and leakage currents through R_1 and R_2 begin to significantly contribute to the photoresponse. The small residual positive current at the end of the photoresponse may be due to leakage current (see below). The photocurrent is always observed to decay simultaneously with the closure of the shutter.

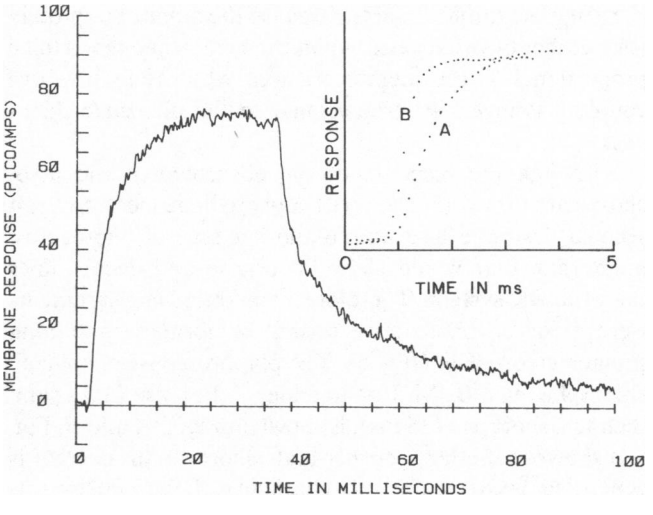

FIGURE 3 The short light-pulse photoresponse of the same membrane used for the data of Fig. 1 is shown (solid line). The pulse duration is 35 ms. The *inset* shows the rise time for two different bandwidths (1 and 2 kHz) of the experimental apparatus (electronics and shutter) discussed in the text.

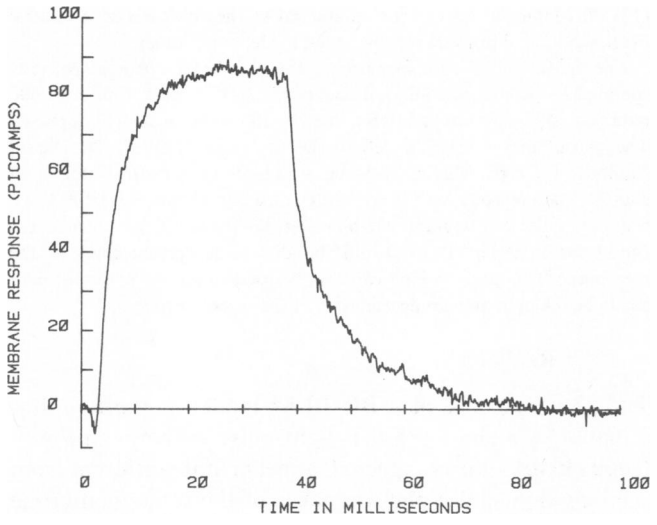

FIGURE 4 The short light-pulse photoresponse of a solid 6-μm thick Teflon septum coated with lipid and BR is shown.

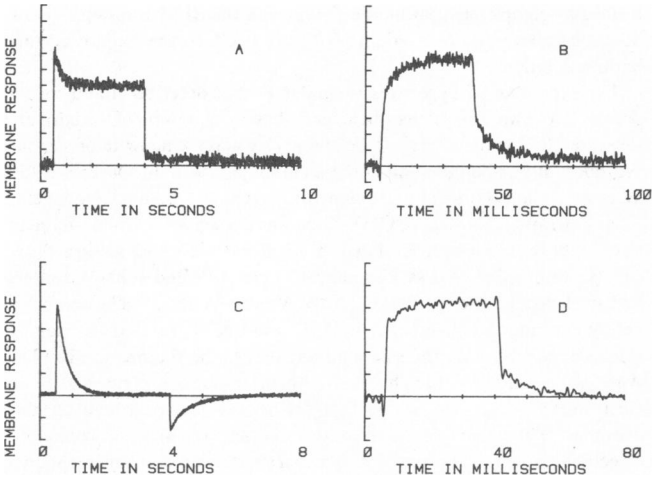

FIGURE 5 The photoresponse of spinach chloroplast in a BLM to long and short light pulses is shown in Fig. 5 *A* and *B*. Fig. 5 *C* and *D* shows the photoresponse of spinach chloroplast on a 6-μm thick solid Teflon septum, again first to a long light pulse and then to a short one.

Fig. 4 shows the short circuit photoresponse, due to a short light pulse (35 ms), of sonicated BR vesicles on a solid Teflon 6-μm septum (coated with ODA and PC). The total active area of the membrane is 0.68 cm^2 and the incident light intensity was 18 mW/cm^2 as in Fig. 3. The solid Teflon septum completely eliminates any leakage current between the two chambers of the cell, therefore, leakage currents should have a negative sign (charge movement from left to right in Fig. 2). This probably explains the small negative base line offset in the decay region of Fig. 4 and the positive offset in Fig. 3 (see above). Because in the data shown in Figs. 3 and 4 the electrometer is capacitively coupled to the protein, both curves should have the same shape, which indeed they have. However, at first glance, it seems surprising that they also have nearly the same magnitude. This is because the amount of protein (or number of fused vesicles) on the membrane tends to be proportional to the membrane area while the capacitive coupling is inversely proportional to the dielectric thickness.

Although the response of the electrometer and associated circuitry was tested with a photodiode (see above), it seemed desirable to examine another type of photoactive membrane that would show faster rise and decay times than the BR system. Therefore, some brief measurements were made on photoactive model membranes containing spinach chloroplast (Fig. 5). The photoresponse of spinach chloroplast in a BLM, first to a long light pulse (3.5 s) and then to a short one (35 ms), is shown in Fig. 5 *A* and *B*. Fig. 5 *A* shows that a large steady-state short-circuit current is generated between the two chambers of the cell by this photoactive membrane, in contrast to the photoactive membranes containing BR (Fig. 1). The faster response of the photoactivated current generator (spinach chloroplast) in this system is evident in Fig. 5 *B*. Again for comparison purposes, photoactive model membranes were made by coating solid 6-μm Teflon septa with spinach chloroplast, thereby preventing the direct transfer of charge between the chambers. The response of this membrane, first to a long light pulse and then to a short one, is shown in Fig. 5 *C* and *D*. The charging and discharging of the model membrane in Fig. 5 *C* qualitatively resembles that of model membranes containing BR (Fig. 1).

Fig. 6 *A* and *B* shows the photoresponse to long and short light pulses of BR dialysis vesicles fused to a BLM, and Fig. 6 *C* and *D* show the photoresponse of BR dialysis vesicles fused to a solid 6-μm septum. No evidence of complete incorporation of the protein into the BLM was obtained (Fig. 6 *A*). In fact, only negligible differences were observed in the photoresponse curves between this system and photoactive model membranes made using sonicated BR vesicles.

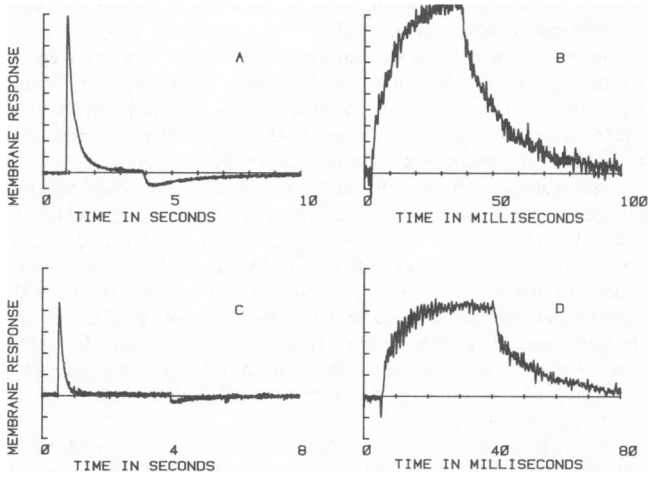

FIGURE 6 The photoresponse of a planar model membrane made by the fusion of dialysis vesicles with BR to a BLM (*A*,*B*) and a solid septum (*C*,*D*) is shown.

Photoactive model membranes formed with purple membrane sheets on either a BLM or solid Teflon septum were also studied. The response to long light pulses showed the same variabilities, presumably due to differences in the lipid conductivity, reported by Bamberg et al. (4). In fact, they report that the equivalent circuit of Fig. 2 does not describe the observed off-light response of purple membrane sheets fused to a BLM. However, in the work described here, the photoresponse due to a short light pulse was the same as that observed with sonicated or dialysis vesicles.

The addition of CCCP to the bathing solutions affects the long light-pulse photoresponse of BR-BLM as was reported earlier (10). However, CCCP does not affect the photoresponse due to a short light pulse providing that [CCCP] $< 0.6\ \mu$M.

The variation in the peak short circuit photocurrent, I_0^M was measured over the range 5.1 mW/cm^2 $< J <$ 30 mW/cm^2. Evidence of light saturation was observed (see the discussion section below); however, a linear relation was found between $1/J$ and $1/I_0^M$.

Varying the salt concentration of the bathing solutions from 100 mM to 1 M NaCl had no noticeable effect on the photoresponse.

DISCUSSION

The use of short light pulses to photo excite model membranes containing bacteriorhodopsin offers a convenient method for studying the kinetic photoresponse of the proton pump. The length of the light pulse must be short enough so that the total charge transferred during the photoresponse (proportional to the area under the photoresponse curve) is small. This means in turn that the potentials induced in the model membrane due to capacitive charging will be small and that leakage currents through the membrane can be neglected. Under these conditions, the photoresponse of a variety of different photoactive membranes shows the same temporal response and reflects that of the proton pump.

A log plot of a photoresponse curve is shown in Fig. 7. Data for Fig. 7 were obtained from the same membrane as that used for Fig. 3 (but with a higher light intensity, 30 mW/cm^2). The peak photocurrent was used as the base line for calculating the log of the leading edge, and the small residual current at the end of the photoresponse was used as the base line for the trailing edge. Fig. 7 shows that the photoresponse of the pump to a step function is not a simple exponential as was suggested earlier (10), but rather a fast initial response followed by a slow response. Extensive studies of the shape of the photoresponse curves have not yet been made. However, preliminary measurements show that the trailing edge is independent of light intensity, whereas the slope of the leading edge is not.

The total maximum energy delivered to a membrane during a single 35 ms light pulse is ~1 mJ/cm^2 in the present experiments. This is enough to begin to show the observed light saturation effects in the photoresponse (9).

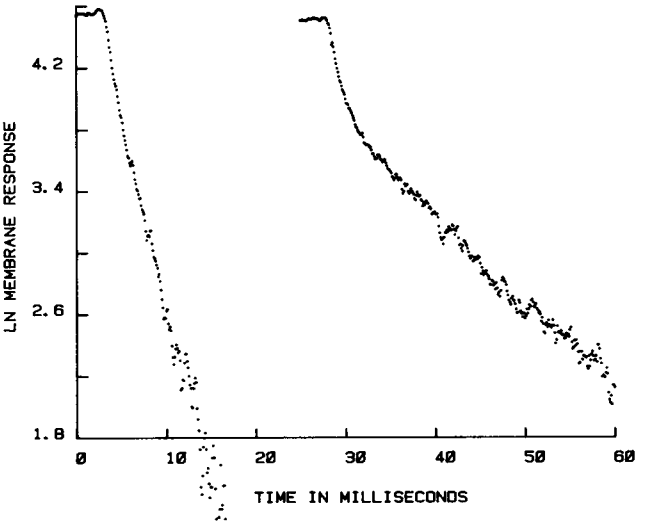

FIGURE 7 A log plot of the leading and trailing edges of the photoresponse curve is shown for the same membrane as in Figs. 1 and 3, but with a light intensity of 30 mW/cm^2. The lines in the figure are to denote deviations from exponential behavior and are not the result of any theory.

The small negative transient seen in Fig. 4 but barely visible in Fig. 3 was found to be due to a microphonic pickup from the shutter. No evidence of an early negative current transient reported by other investigators (8, 9) was observed, perhaps due to the limited bandwidth of the present measurements.

The model membrane formed by plating BR onto a solid 6-μm Teflon septum offers a very simple and durable photoactive membrane for studying the kinetic response of the pump under a variety of conditions not possible with a fragile BLM. This membrane is clearly not useful for experiments that require low resistance rather than low reactance between the electrodes and the protein.

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