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Current-voltage characteristics of planar lipid membranes with attached *Halobacterium* cell-envelope vesicles

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We have studied the photoactivity of a system consisting of large, planar, essentially solvent free bilayers bearing adsorbed cell-envelope vesicles prepared from *Halobacterium halobium* (strain L 33). The system was made conductive by addition of a proton carrier (SF-6847). We observed photocurrents which were linearly dependent upon transmembrane voltage. Current-voltage curves were found to be well described by an equivalent circuit with the following significant parameters: planar bilayer conductance, planar bilayer-vesicle contact area conductance, cell-envelope vesicle conductance, and chloride pump equivalent voltage-generator potential. These parameters are uniquely obtained as a result of a few independent current measurements. The stationary photovoltage was dependent upon chloride concentration, and from this dependence an active transport (pump) affinity of the system for chloride was calculated to be about 50 mM.

Introduction

The retinal proteins of halobacteria constitute a unique set of light-energy transduction devices based on similar chemistry, but designed to perform different functions. Halorhodopsin is a retinal protein related to bacteriorhodopsin and sensory rhodopsin, but with many spectroscopic and photochemical properties quite different from those of the other halobacterial pigments [1–5]. Recent results indicate that halorhodopsin functions as an inward-directed chloride pump in the cytoplasmic membrane of the halobacteria [6] and chloride (as well as other specific anions) was indeed found to have profound effects on the chromophore and photochemical events in the pigment [3,7,8].

In cell-envelope vesicles containing halorhodopsin as the only light-dependent pump it was shown [6] that the development of potential during illumination was indifferent to the cation present but required chloride specifically on the vesicle exterior. During illumination chloride was accumulated in the vesicles, which consequently showed swelling (light scattering decrease) under these conditions. The light-dependent chloride transport was against an electrical (negative interior) potential, and against a concentration difference (higher in the interior), i.e., it was 'uphill' transport. Uncouplers had little or no effect, but other ionophores, such as valinomycin or gramicidin, accelerated chloride transport. Many of these observations have been confirmed and made more quantitative by following the volume changes with a spin probe [9].

Recently, Bamberg and coworkers used halorhodopsin-containing membrane fragments and halorhodopsin-liposomes attached to planar lipid films containing decane to study transport properties of this pigment [10,11]. Stationary photocurrents were shown in the case of halorhodopsin containing membrane fragments with a chloride

ionophore [10], and in the case of reconstituted vesicles with lipophilic ions [11]. They also showed that the presence of chloride is necessary for the halorhodopsin photoresponse and have studied chloride dependence of transient photocurrent [10]. Their observations confirmed that halorhodopsin must be a light-driven chloride pump.

Thus, a large amount of evidence is in favor of the proposed model of halorhodopsin as a chloride pump. The physical rationale for how a chloride pump might function is less clear.

Much important information concerning chloride translation is still missing. We have very limited information about the most important parameters of light-induced ion transport, i.e., relative permeabilities of the anion translocation pathway associated with the pump, specificities and number of anion-binding sites and ion transport voltage dependence.

Materials and Methods

We have grown *Halobacterium halobium* strain L-33 (containing halorhodopsin), and prepared cell-envelope vesicles as described elsewhere [12]. The vesicles were stored in 4 M NaCl. Phosphatidylethanolamine (PE) was purchased from Avanti Biochemicals Inc. (Birmingham, AL).

Our experimental set-up as shown in Fig. 1 consists of a hydrostatically closed Teflon chamber (1) with a 1 mm^2 round hole (2) on which the black lipid membrane is formed. The Teflon chamber is attached to a micromanipulator (not shown) to dip the chamber through the monolayer which is spread (hexane solution of PE, 0.3 mg/ml) on the surface of the buffer in the transparent Pyrex beaker (3). The membrane is formed by moving the Teflon chamber (filled with electrolyte and sealed), first up then down, through the monolayer which is formed prior to chamber withdrawal [13,14]. Electrical contact was provided by Ag-AgCl electrodes that were light-shielded and isolated from the solution bathing the membranes by agar bridges (4). A Burr-Brown 3523 low noise, low bias current FET operational amplifier (5) is used as a current to voltage transducer (voltage-clamp), with a microsize feedback resistor of 10^9 Ω (Eltec Instruments Inc, FL). Output current noise (amplifier load 5000 pF and $5 \cdot 10^9$ Ω) did not exceed 0.1 pA at 200 Hz, with a time constant of 5 s.

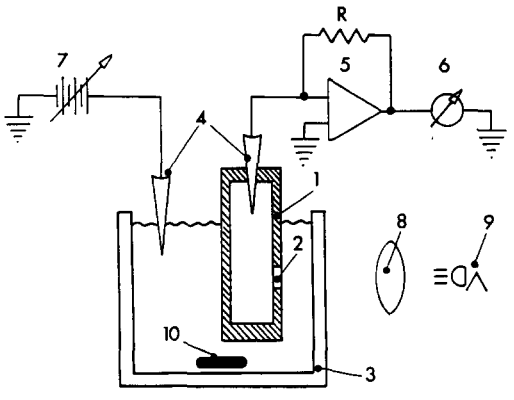

Fig. 1. Experimental apparatus used. Components: 1. Cylindrical Teflon chamber. 2. Round aperture of about 1 mm^2. 3. Glass (Pyrex) 10 ml beaker. 4. Ag-AgCl-agar (4 M NaCl) electrodes in black plastic enclosures. 5. Operational amplifier. 6. Recorder. 7. Voltage source. 8. Focusing lens. 9. Light source. 10. Magnetic stirring bar.

Current was recorded on a strip-chart recorder (6), and also monitored on a Tektronix 5110 oscilloscope and a digital voltmeter for measurement of membrane capacitance. A low impedance voltage source (7), and triangle-wave generator were used to measure dark (light) current voltage dependence and membrane capacitance, respectively. Membrane capacitance was 0.75 $\mu F/cm^2$. The membrane was illuminated by a 250 W projection lamp (9) through fiber optics and a 530 nm long pass filter, and the light was focussed on the membrane with a lens (8). Illumination was started and terminated with a mechanical shutter (Kodak) placed between the fiber optic and the focusing lens (not shown). The light intensity on the membrane, as measured with a calibrated thermopile detector (Oriel Corp. Stamford, CN), was 25 mW/cm^2.

After the membrane was formed and membrane conductance and capacitance were measured, the vesicles (halorhodopsin containing cell envelopes with protein concentration 30 mg/ml in either 4 M NaCl or 1.5 M Na_2SO_4 plus NaCl solution) were added to the outside compartment (10 ml volume), and the solution was magnetically stirred (10). Proton carrier SF-6847 (3,5-di-*tert*-butyl-4-hydroxybenzylide) (SF) was added to the salt solutions in both chamber compartments prior the membrane formation.

Results

Membrane conductance as a function of vesicle concentration

These measurements were done in order to obtain all initial electrical parameters of the system of planar black lipid membrane-attached vesicles. Some very important electrical parameters of the system using the dependence of membrane conductance on vesicle concentration can be determined: conductance of the membrane area which is covered by vesicles, G_v, and conductance of that part of the membrane which is free of vesicles, G_m (see Appendix).

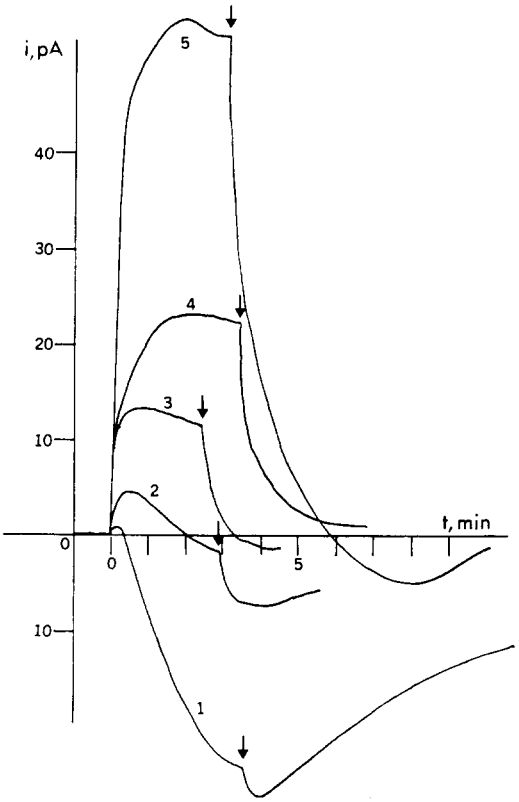

Fig. 2. Membrane current responses to continuous illumination. Halorhodopsin-containing vesicles were added to PE-containing black lipid membranes, as described in the text, and the excess removed after fusion. Salt solution: 4 M NaCl, 2 mM $CaCl_2$ (pH = 6.7). Light intensity was 25 mW/cm^2. The light was switched on at the time marked zero on the time axis and was switched off where indicated by a vertical arrows. Initial membrane conductance was $7 \cdot 10^{-9}$ S (0.01 μM SF was added) after vesicle fusion conductance was $3.8 \cdot 10^{-9}$ S. Numbers on the curves correspond to the following membrane voltages: 1, −40 mV; 2, −20 mV; 3, 0 mV; 4, +20 mV; 5, +30 mV.

Photocurrent measured during continuous illumination

Typical photocurrent traces from the system of planar bilayer-attached cell-envelope vesicles are shown in Fig. 2A, B. The curves were obtained with the following protocol: after the membrane was formed in 4 M NaCl, 2 mM calcium chloride in the presence of proton carrier, 75 μl of vesicle solution (30 mg/ml) were added to the outside compartment (10 ml) and the solution was stirred. After about 20 min the outside solution was exchanged with the original solution of salt and proton carrier (i.e., the unattached vesicles were removed), and a current-time as well as a current-voltage curve was obtained in the dark. The amplitudes of these dark currents (which are not voltage dependent) were subtracted when traces in Fig. 2 were drawn. Illumination was started at the time marked zero and ended where indicated by arrows. When zero voltage was applied to the membrane (short-circuit current measurement) the membrane current rose rather rapidly upon illumination, passed over a maximum, and decreased rapidly (but slower than the 'on' course) back to the dark current level, when the light was switched off. Positive membrane voltage (relative to the *cis* compartment of the chamber) increased the photocurrent amplitude proportionally to the voltage value, while negative voltage decreased the photocurrent amplitude and reversed the sign of the current, depending on the value of the voltage.

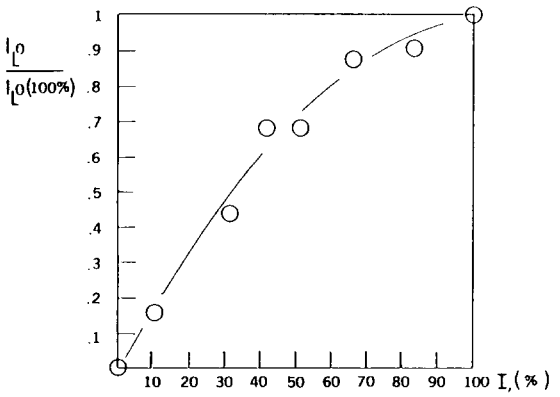

Fig. 3. Dependence of short-circuit current on light intensity.

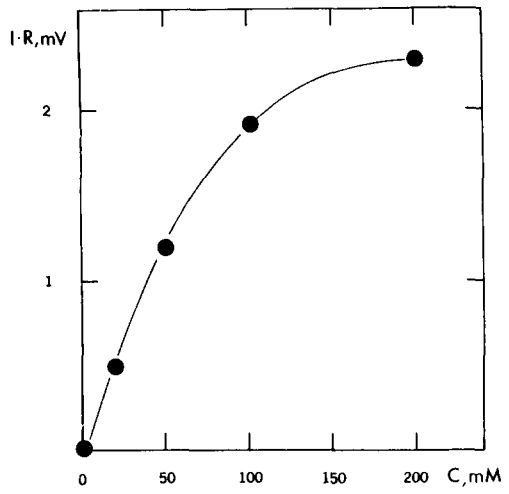

Fig. 4. Chloride effect on membrane photovoltage in the halorhodopsin system. Measurements were made on a few different membranes. Photovoltage was obtained as a product of corresponding short-circuit current and normalized membrane resistance. Calculated affinity is 54 ± 6 mM.

Fig. 3 shows relative steady-state short-circuit current ($I_{Lo}/I_{Lo}(100\%)$) in the system of planar black lipid membranes and attached halorhodopsin-containing vesicles as a function of the relative light intensity (expressed as percentages). The different light intensities were obtained using neutral density filters.

Dependence of the light-induced current on chloride concentration in the halorhodopsin system

Halorhodopsin-containing vesicles were dialyzed overnight against 1.5 M sodium phosphate plus NaCl at a concentration desired for each experiment. PE membrane was formed in the same salt solutions as used for dialysis. The same amount of proton carrier and vesicles was added in each experiment (0.01 μM SF and 75 μl of halorhodopsin-containing vesicles, 30 mg/ml). We followed the same protocol as described before. Only short-circuit steady-state currents were measured. The data were normalized by calculating photovoltages as products of the photocurrents and the membrane resistances for each experiment. The calculated photovoltages are shown in Fig. 4 as functions of the chloride concentration. No light-induced current was observed in the absence of chloride (photovoltage was zero). The photovoltages increased with increasing chloride concentration and neared saturation at about 200 mM chloride (calculated affinity is (54 ± 6 mM)).

Discussion

In the model proposed by Hermann and Rayfield [15], bacteriorhodopsin-containing vesicles are assumed to remain intact on the surface of black lipid membranes after fusion (adsorption). This model (parallel membrane R and C in series with parallel current generator, resistor and capacitor of the pump) explained the observed photoresponse on a time scale comparable to the relaxation time of the membrane. The steady-state photocurrent was modeled by choosing appropriate parameter values. The assumption was made that the equivalent current generator, I_p, is linearly dependent on bias voltage, and shows instantaneous on-off response. The time constant associated with the fast current transient under the 'long' time excitation by light is the membrane relaxation time (RC) constant and varied from 1 to 3 s. A similar circuit was used by Bamberg et al. [10,11,16] and Korenbrot and Hwang [17].

There are two possible extensions of this treatment. One was used by Rayfield [18] for measurements of transients faster than the membrane RC relaxation time, when the current leakage through resistors was small compared to the displacement currents through capacitors. The second extension is possible for steady-state current measurements on a time scale much longer than the membrane RC relaxation time.

This second approach is most suited for the study of light-induced steady-state ionic currents. We will consider a topological model which is similar to that established for bacteriorhodopsin and shown in detail in Fig. 5a. When light excites the chloride pump [6,7], chloride ions are transported from the outside into the cell-envelope vesicle and counterions will follow until a steady-state equilibrium is reached. If the membranes (both planar and vesicular) are permeable to protons, then the protons will be transported into the vesicle from outside, charging the left membrane side negatively. Therefore, the current through the membrane will be detected as a positive current (see Fig. 2, curve 3). Because the vesicle volume is

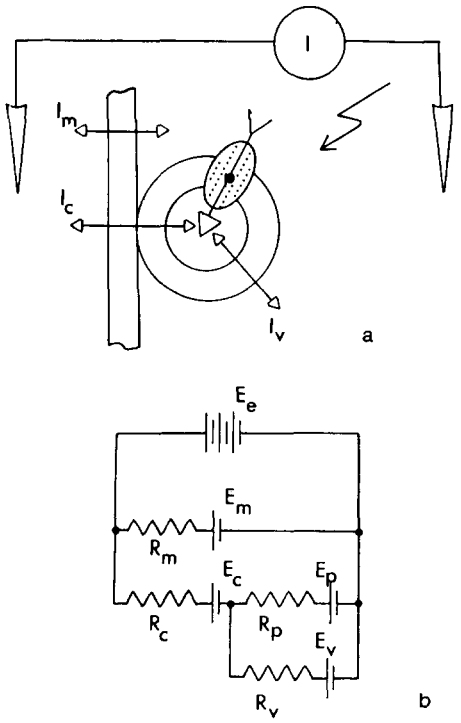

Fig. 5. Model of the halorhodopsin system. a. Schematic drawing of an halorhodopsin-containing vesicle fused with black lipid membranes. The chloride pump is shaded. Steady-state currents are measured (shown here as a measuring device and arrow). b. Equivalent circuit diagram of the halorhodopsin cell-envelope planar membrane reconstitution system. R_m is the resistance of that part of the planar membrane which is not occupied by vesicles. R_v is the vesicle resistance (passive leakage). R_c is the resistance of the vesicle-membrane contact area. E_e is the externally applied potential. E_p is the voltage of the chloride pump equivalent voltage generator. R_p is the chloride pump resistance.

very small, a proton gradient builds up, causing decrease of the current (this gradient being directed against the initial proton flux). If we apply an external voltage so that 'minus' is on the right side of the membrane (external chamber), then the proton current through the membrane will be increased (curves 1 and 2 in Fig. 2). 'Plus' outside voltage, on the other hand, will decrease the current (curves 4 and 5 in Fig. 2). The equivalent electrical circuit of such a model is shown in Fig. 5b. We have modified this circuit to analyze only the steady-state currents (capacitative transients are neglected), and we substituted the voltage-dependent current generator with the voltage-independent voltage generator, which has resistance in series (pump resistance). Analysis of this circuit gives the current-voltage relation as:

$$I = \frac{E_e}{R_m} + \frac{E_e R_p + (E_p + E_e) R_v}{R_v R_c + R_v R_p + R_p R_c} \tag{1}$$

where E_e is the externally applied potential, E_p is the chloride pump equivalent voltage generator potential, R_v is the vesicle equivalent resistance (proton leakage through the vesicle), R_m is the planar membrane equivalent resistance (except for the vesicle-membrane contact area), and R_c is the equivalent resistance of vesicle-membrane contact area.

In order to simplify the analysis of the above current-voltage relationship, we made the following assumption: pump resistance R_p in the dark is high ($R_p \gg R_m$, R_v, R_c) and R_p is low during the illumination ($R_p \ll R_m$, R_v, R_c). Now Eqn. (1) can be rewritten as follows: (a) in the dark

$$I_d = \frac{E_e}{R_m} + \frac{E_e}{R_v + R_c} \tag{1a}$$

where I_d is the membrane current in the dark with an external potential E_e applied; (b) during illumination

$$I_L = \frac{E_e}{R_m} + \frac{E_p + E_e}{R_c} \tag{1b}$$

where I_L is the membrane current during illumination and while the external potential E_e is applied.

Finally

$$I_{L_o} = E_p / R_c \tag{1c}$$

where I_{L_o} is the short-circuit current under light. Thus, we can determine all the parameters of our model, i.e., R_c, R_v, and E_p:

$$E_p = E_e I_{L_o} / (I_L + I_{L_o} - I_m) \tag{2}$$

$$R_c = E_p / I_{L_o} \tag{3}$$

$$R_v = E_e (I_d - I_m) - R_c \tag{4}$$

where $I_m = E_e / R_m$ current through the part of the membrane which is not occupied by vesicles.

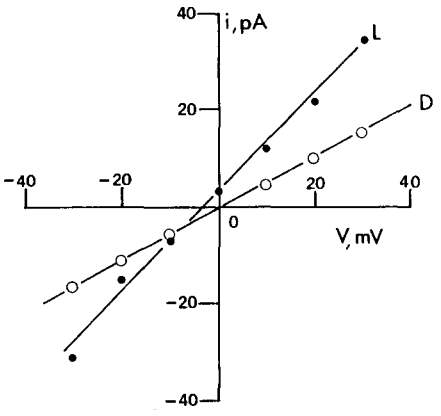

Fig. 6. Membrane current-voltage characteristics. Filled symbols are photocurrents, which are read as maximum steady-state light currents. Open symbols are dark currents. Conditions: 4 M NaCl, 2 mM $CaCl_2$, pH = 6.7, room temperature. 10 mM of sF present, 75 μl of halorhodopsin vesicles were added (30 mg/ml protein) after the membrane formation. Calculated parameters: $R_m = 2.33 \cdot 10^8$ Ω, $R_c = 5 \cdot 10^8$ Ω, $R_v = 1.4 \cdot 10^8$ Ω, $E_p = 6$ mV.

Fig. 6 shows membrane current-voltage curves where the circles are the experimentally obtained data (open circles are dark membrane currents, closed circles are the maximal light currents), and the lines were calculated from Eqns. 1a and b. Parameters were obtained from Eqns. 2–4.

Conclusions

Knowing initial membrane conductance, vesicle coverage, using the assumption that the pump resistance is small during the illumination, and using the results of three independent current-voltage measurements (short-circuit, 'dark' and 'light') we could derive the following electrical parameters of the system: resistance of the membrane-vesicle contact area, R_c, resistance of vesicular membranes, R_v, and the equivalent voltage generator potential E_p.

Our data on the chloride concentration dependence (see Fig. 4) of the photovoltage (as a product of steady-state photocurrent and membrane resistance) in halorhodopsin cell-envelope/planar black lipid membrane reconstitution system independently confirm studies with L-33 cell-envelope vesicles in the solution [3,6,7] which indicated that the apparent affinity of halorhodopsin for chloride in the transport is about 40 mM. As suggested by Bamberg et al. [10] only the chloride dependency of the stationary currents can prove that halorhodopsin acts as a light-driven chloride pump. We have shown such dependence in this work.

Appendix

After the membrane conductance and capacitance reached equilibrium (about 20 min), the first aliquot of vesicles was added. In 20–25 min the membrane reached a new equilibrium state, the conductance was recorded, and another aliquot of vesicles was added. This procedure was repeated 10–15 times until the membrane conductance decreased to about a tenth of its original value.

The results suggest that vesicles have adsorbed to the membrane. The isotherm most commonly used for such a process is that of Langmuir which can be written in its simplest form:

$$\theta/(1-\theta) = \alpha C_v \tag{1}$$

where α is the equilibrium constant of adsorption (i.e., α is the ratio of the rate of adsorption to the rate of desorption of adsorbed vesicles from the fully occupied membrane surface), θ is the ratio of the number of adsorbed vesicles per unit area of the membrane to the number of adsorbed vesicles per unit area under the conditions of the maximum adsorption (or it can be estimated as the fraction of the membrane surface covered with vesicles), and C_v is the concentration of free vesicles in the bathing medium.

If σ_m is the membrane conductance (per unit area) with no vesicles adsorbed and σ_v is the membrane conductance (per unit area) with the maximum number of vesicles adsorbed, the system conductance can be written as:

$$G/S = (1-\theta)\sigma_m + \sigma_v\theta \tag{2}$$

where S is the total membrane surface area. Therefore, if we know initial membrane conductance (conductance before vesicle addition), $G_i = \sigma_m S$, Eqn. 2 will give

$$\theta = (1 - G/G_i)/(1 - \sigma_v/\sigma_m) \tag{3}$$

Eqn. 3 has a very simple meaning: the ratio of

the relative change of membrane conductance to the relative change of partial conductance is equal to θ.

Combining Eqns. 1 and 3 we get

$$(1 - G/G_i)/C_v = \alpha(1 - \sigma_v/\sigma_m) - \alpha(1 - G/Gi) \quad (4)$$

Eqn. 4 shows that if we plot $(1 - G/G_i)/C_v$ against $(1 - G/G_i)$ we will get a straight line with the slope of α and intersection with the axis $(1 - G/G_i)/C_v$. Now, using the value of initial membrane conductance G_i (before vesicle addition) and θ (from Eqn. 1), one can estimate the following parameters: membrane conductances for that part which is covered by vesicles and for that part free of vesicles which are:

$$G_v = \theta G_i \sigma_v/\sigma_m$$

$$G_m = (1 - \theta)G_i$$

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