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RECONSTITUTION OF THE LIGHT-DRIVEN ELECTROGENIC ION PUMP HALORHODOPSIN IN BLACK LIPID MEMBRANES

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Halorhodopsin-containing membrane fragments were isolated from *Halobacterium halobium* strain L-33, a bacteriorhodopsin-deficient mutant. When these so called Tween-washed membrane fragments are added to one side of a positively charged planar lipid bilayer the system becomes photoelectrically active. Under stationary light conditions photocurrent transients are obtained while the steady-state photocurrent is extremely low. The steady-state photocurrent, however, is considerably increased when the lipid bilayer is doped with the Cl^-/OH^- exchanging carrier triphenyltin together with a proton carrier. These results suggest that halorhodopsin is associated with the underlying black lipid membrane in a sandwich-like structure. The photoresponse occurs only in the presence of Cl^-, Br^- and I^- with half-saturation concentrations in the range of 1–10 mM and is virtually independent on the type of the cation (Na^+, K^+, Mg^{2+}) present. With other anions, such as SO_4^{-2}, F^- and NO_3^- no photoresponse was obtained. The results provide direct evidence that halorhodopsin is a light-driven electrogenic pump with a high specificity for halides.

Introduction

Retinal-containing chromoproteins have both photosynthetic and phototactic functions in the halobacterial branch of Archaebacteria [1,2]. Photosynthesis is mediated by bacteriorhodopsin (BR) on the basis of its function as a light-driven proton pump [3,4]. The molecular basis of phototaxis mediated by another retinal protein, proposed as 'slowly cycling rhodopsin' (SR), still awaits elucidation [5]. A third retinal protein, halorhodopsin (HR), was identified spectroscopically in halobacterial cells. In contrast to bacteriorhodopsin, which forms a two-dimensional array in the purple membrane patches of the halobacterial cell membrane, halorhodopsin seems to be uniformly distributed in the cell membrane at a concentration of approx. 5% of that of bacteriorhodopsin in the cytoplasmic membrane of *Halobacterium halobium* strain S9. At first halorhodopsin was believed to act as an outwardly directed light-driven sodium pump [6]. It was later claimed to function as an inwardly directed chloride pump on the basis of light scattering and chloride uptake measurements with suspensions of cell envelope vesicles [7]. Here, we report on experiments with halorhodopsin associated with black lipid membranes. The results demonstrated directly the function of halorhodopsin as a light-driven electrogenic chloride pump.

Abbreviations: TTFB, tetrachloro-2-trifluoromethylbenzimidazole; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-ethanesulphonic acid; Mops, 3-(*N*-morpholino)propanesulphonic acid.

Materials and Methods

Halobacterium halobium was grown under limited aeration as described previously [8,9] and purple membranes were isolated from S9 cells as described [4]. The strain S9 is bacteriorhodopsin - and halorhodopsin-positive but lacks the C_{50}-carotinoid bacteriorhuberin. The strains L-33 [8] and OD2 [10] are bacteriorhodopsin negative. In contrast to L-33 strain OD2 contains bacteriorhuberin and twice the amount of halorhodopsin. Flux3 cells [10] are both bacteriorhodopsin- and halorhodopsin-negative. All strains contain comparable amounts of 'slowly cycling rhodopsin'.

Preparation of the halorhodopsin-containing Tween-washed membrane. 10 litres L-33 or OD2 cells were spun down at room temperature (3000 $\times g$ for 60 min), resuspended in 200 ml 4 M NaCl and centrifuged at 14000 $\times g$ for 15 min. After resuspension in 100 ml 4 M NaCl and addition of 2 mg DNAase I, the sample was dialyzed overnight against 3 liters of water at 4°C. After centrifugation at 200000 $\times g$ for 45 min the pellet was washed twice with 50 ml 0.1 M NaCl and resuspended in 20 ml 0.1 M NaCl (total membrane fraction, 20 mg protein/ml).

To obtain the Tween-washed membrane the total membrane fraction was mixed with 40 ml 50 mM Mops buffer (pH 7) containing 5% Tween 20 (protein/detergent ratio of 1 : 5, w/w). The suspension was stirred for 60 min at room temperature in the dark until the solution cleared. Aliquots of 25 ml were added to tubes of a Kontron TST 28 swingout rotor and underlayed successively with 6 ml 25% sucrose and 3 ml 45% sucrose. After centrifugation at 80000 $\times g$ for 13 h at 10°C, the red material in the lower part of the gradient was collected and diluted with 10 mM Mops (pH 7) to a final volume of 50 ml. After centrifugation (200000 $\times g$, 60 min, 10°C) the membranes were washed twice with 10 mM Mops (pH 7) and finally adjusted to a volume of 10 ml with the same buffer (protein concentration: 26 mg/ml). This preparation is a modification of the method described in Ref. 11. The protein concentrations are based on the determination by the method of Warburg and Christian (1942) [12].

Membrane setup. Optically black lipid membranes with an area of about $5 \cdot 10^{-3}$ cm^2 were formed in a teflon cell [13] filled with an appropriate electrolyte solution. The temperature was kept at 25°C. The membrane forming solution contained 1.5% (w/v) diphytanoyl phosphatidylcholine (Avanti Biochemicals, Birmingham, AL) and 0.025% (w/v) octadecylamine dissolved in *n*-decane. The latter agent was added to obtain a positive surface charge, so that the adsorption of membrane fragments was optimized [14]. The membrane cell was connected to an external measuring circuit via Ag/AgCl electrodes. In order to avoid artificial photoeffects, the electrodes were separated from the membrane cell by salt bridges. Light from a 250 W halogen tungsten lamp equipped with a heat protection filter (Balzers, Liechtenstein) was focussed onto the membrane. The light beam passed first the front compartment, whereas the Tween-washed membrane was added to the rear compartment.

The action spectrum of the photocurrent was determined using a series of narrow band interference filters (Balzers B 40, 10 nm half-width). The light intensity in the plane of the membrane was determined by a calibrated thermopile (Kipp and Zonen, Model CA 1). Further details are described by Bamberg et al. (1979) [13].

Results

Development of the photocurrent

Addition of Tween-washed membrane prepared from L-33 cells to one side of the black lipid film does not create any photosensitivity in a short circuit experiment if only 100 mM sodium sulfate is present in the electrolyte solution (Fig. 1A). Immediately after the addition of small amounts of chloride, however, a transient photocurrent develops (Fig. 1B). Control experiments were carried out with two other membrane fractions. First, cell membranes of the same strain which were not treated with detergent showed the same chloride-dependent photoresponse as the Tween-washed membrane but with half the maximal photocurrents. Secondly, bacteriorhodopsin in the form of purple membrane was added to the black lipid membrane. Upon illumination in the absence of chloride bacteriorhodopsin produced the well characterized photoresponse [13] shown in Fig. 1C, which did not change significantly upon addi-

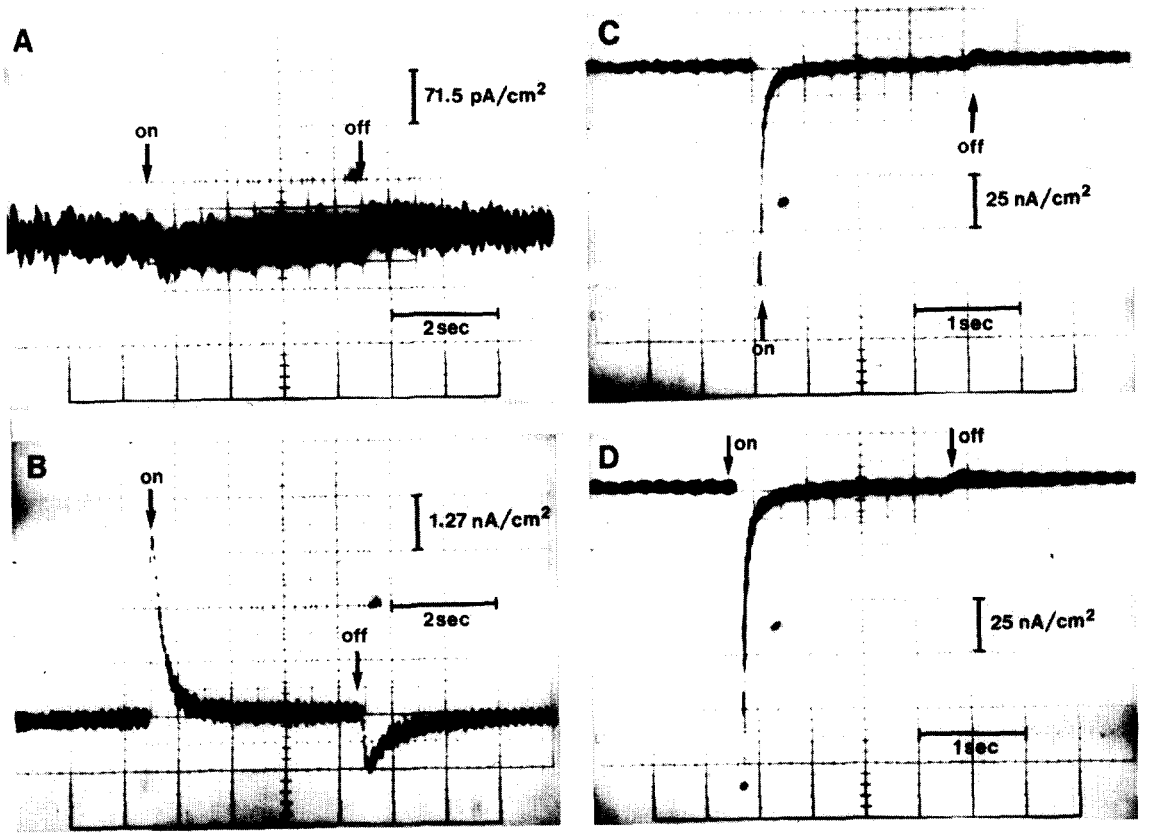

Fig. 1. Short circuit photocurrents after addition of halorhodopsin-containing membranes (1 mg) from L-33 cells to one aqueous compartment (6 ml) in 0.1 M Na_2SO_4 (A) and 0.1 M Na_2SO_4/6 mM NaCl (B). (C) and (D) show the corresponding experiments with bacteriorhodopsin-containing purple membranes (1 mg) in the presence of 0.1 M Na_2SO_4 (C) and 0.1 M Na_2SO_4/6 mM NaCl (D). Saturating light intensity (white light) was 5 mW/cm^2.

tion of chloride (Fig. 1D). The maximal transient current was more than 10-times greater than that obtained with halorhodopsin at saturating chloride concentrations, but of opposite sign. The difference observed results presumably only from the lower concentration of halorhodopsin in Tween-washed membranes compared to bacteriorhodopsin in purple membranes considering the similar frequency of their photocycles [15,16].

The action spectrum of the peak current and the absorption spectrum of halorhodopsin are compared in Fig. 2. The agreement of both spectra unambiguously identifies a retinal protein chromophore as the photochemically active species. However, the Tween-washed membrane contains two retinal proteins, halorhodopsin and slow cycling rhodopsin [11]. Halorhodopsin-negative strains are available and membranes prepared from these

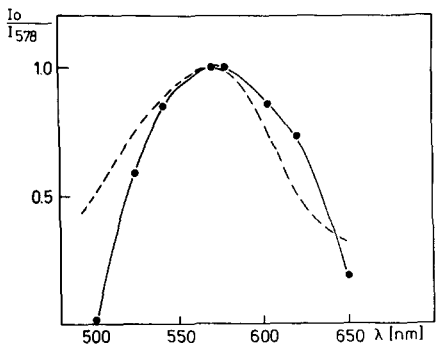

Fig. 2. Comparison of the action spectrum of the halorhodopsin transient photocurrent I_0 with the difference absorption spectrum of halorhodopsin in Tween-washed membranes carried out with bleached and unbleached membranes (dashed line). The experimental set up was as in Fig. 1. I_{578} and the absorption maximum at 578 were normalized to 1. The action spectrum was normalized to equal quantum flux density.

strains can be used as proper controls. Such a strain (Flx3) [10] was tested and no photoresponse was obtained with halorhodopsin-deficient membranes prepared from these cells. This proves that slow cycling rhodopsin is photoelectrically inactive and halorhodopsin mediates the electrogenic event.

The photocurrent satures with increasing light intensity. This is shown in Fig. 3, where the reciprocal values of I_0 (that is the current at time zero) are plotted as function of the the reciprocal light intensity. This behaviour can be described by the following equation:

$$I_0 = I_0^s \frac{J}{J + J_{1/2}} \qquad (1)$$

where I_0^s is the saturation current and $J_{1/2}$ the half saturation intensity, which is approx. 2.5 mW/cm^2.

The observed photocurrent transients are due to the fact that the halorhodopsin-containing Tween-washed membrane forms a sandwich-like structure by adsorption to the underlying black lipid film but is not integrated into it [17]. The occurrence of photocurrent transients, however, suggests the arrangement shown in Fig. 4A. The sandwich-like structure can be described by an

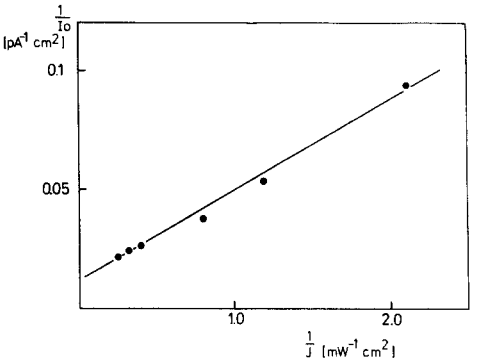

Fig. 3. Reciprocal values of the initial photocurrent I_0 were plotted as a function of the reciprocal light intensity J. White light intensity was varied with a series of calibrated grey filters. All measurements were performed on the same black lipid film.

equivalent circuit diagram as shown previously by [13,18]. The photoelectrical phenomena of the baceriorhodopsin-containing purple membrane have been described satisfactorily by a simple circuit analysis [13] and should be applicable to the halorhodopsin-containing Tween-washed membrane. The photocurrent transient in Fig. 1B is due to the pump current I_{HR} which loads the two membrane capacitors C_{HR} and C_m according to

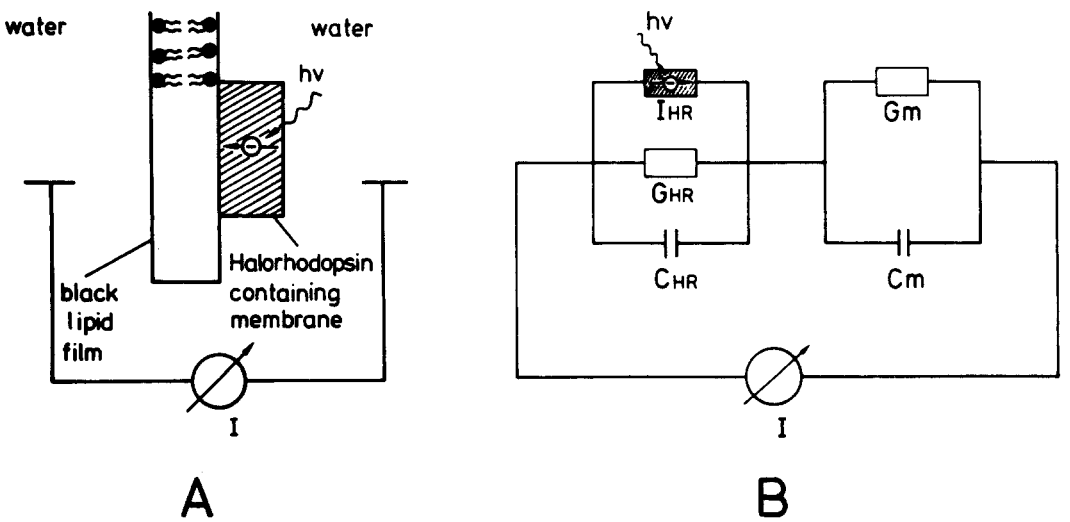

Fig. 4. (A) Schematic representation of the photocurrent generator. (B) Equivalent circuit diagram of the compound membrane system shown in (A), G_{HR} and C_{HR} are the conductance and capacitance of the halorhodopsin-containing membrane (G_{HR} includes contributions from leakage pathways in the space between the membrane and black lipid film). G_m and C_m are the conductance and capacitance of the underlying bilayer membrane. I_{HR} is the photocurrent generated in the membrane by halorhodopsin. I is the externally measured short circuit current.

the equation [13]:

$$I_0 = I_{HR,0}\frac{C_m}{C_{HR}+C_m} \quad (2)$$

where $I_{HR,0}$ is the initial pump current.

The decay time of the photocurrent depends at constant chloride concentration (8 mM) on the intensity of the exciting light. The decay time is 5 s at low light intensity (1 mW/cm^2) and decreases to a constant value of about 0.1 s at saturating light intensity (6 mW/cm^2). These results are in agreement with the equivalent circuit diagram (Fig. 4B), where the reciprocal decay time of the photocurrent should be proportional to the pump current I_{HR} and therefore to the light intensity [13].

The evidence that halorhodopsin acts as an electrogenic pump cannot be derived from the photocurrent transients alone because an alternative explanation exists: these transient currents can also be interpreted as a chloride activated charge movement during illumination. The pump action, however, can be proved unambiguously by appearance of stationary photocurrents upon continuous illumination. This can be achieved by making the underlying black lipid membrane permeable for the ion species which is transported by halorhodopsin. Fig. 5 shows an experiment where stationary photocurrents have been obtained in the presence of the Cl^-/OH^- exchanging carrier triphenyltin (10^{-8} M) [19] in combination with the proton carrier FCCP (10^{-6}M). The result in Fig. 5 gives definite proof of halorhodopsin's function as a chloride pump. It is noteworthy that compounds such as nystatin and amphotericin B which form anion conducting channels and gramicidin A which forms cation conducting channels, did not yield measurable steady-state photocurrents. In the case of nystatin and amphotericin B, the fluctuation of dark current induced by small electrode asymmetry was too high to detect the expected photocurrents.

Ion specificity

As shown in Fig. 1 the presence of chloride is necessary for the halorhodopsin photoresponse while the bacteriorhodopsin is indifferent to chloride. The dependence of the photocurrent I_0 on the concentration of chloride (Fig. 6A) at constant

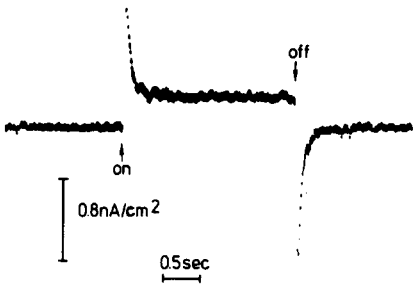

Fig. 5. Generation of steady-state photocurrents in the presence of halorhodpsin. 10^{-8} M triphenyltin and 10^{-6} M FCCP were added to both sides of the compound membrane. The dark conductance of the membrane after addition of the ionophores was 5 μS/cm^2. 10 mM Na_2SO_4/40 mM NaCl/0.5 mM Tris-Hepes (pH 6.0), light intensity 5 mW/cm^2 white light.

light intensities (5 mW/cm^2) can be described by a Michaelis-Menten formalism

$$\frac{1}{I_0} = \frac{1}{I_{0,max}}\cdot\left(1+\frac{K_m}{C_{Cl}}\right) \quad (3)$$

A K_m value of 8 mM for chloride (Fig. 6A) was determined with halorhodopsin from strain L-33. Chloride is not the only anion which allowed a halorhodopsin induced photocurrent. Iodide and bromide are slightly more efficient than chloride with half saturating concentrations of 5 mM for iodide and 3 mM for bromide (Fig. 6B). The $I_{0,max}$ for bromide and iodide is approximately the same (5 nA/cm^2), but decreases at higher concentrations for unknown reasons. No photoresponse, however, was obtained upon addition of fluoride, nitrate, azide and sulfate (0–200 mM). No effect on the photoresponse has been detected by the varying cations (Na^+, K^+, Mg^{2+}) in the absence and presence of chloride. A similar ion specificity has been obtained with cell envelope vesicles [7,20] with light scattering experiments and flash photolysis. Nitrate (100 mM) and azide (200 mM) inhibit the chloride-dependent photoresponse by 70–80% at 8 mM chloride and therefore will be of great value for studies of halide-binding sites.

All experiments described above have been carried out at a sulfate concentration of 100–150 mM. Below that concentration the chloride-dependent photoresponse decreased and in fact disappeared at Na_2SO_4 concentrations below 10 mM

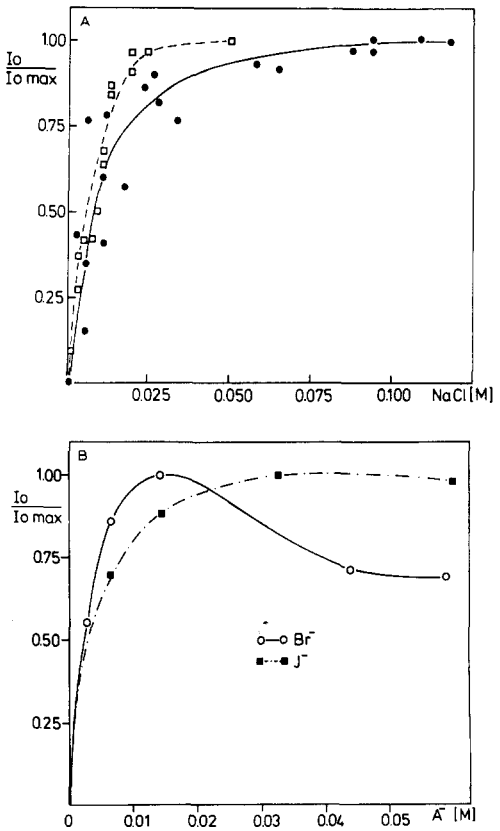

Fig. 6. (A) Halorhodopsin induced photocurrent as a function of the chloride concentration. The photocurrent was measured at a light intensity of 5 mW/cm^2. Chloride was added to both compartments. Silver/silver chloride electrodes were kept in 10 mM chloride and connected using chlorides free bridges to the compartments of the cuvette. ●———●, represents the results from three different experiments with strain L-33 mutants and □— — —□ shows the results of one experiment with strain OD2 membranes. The maximal photocurrent at saturating chloride concentration $I_{0,max}$ was normalized to 1 (100 mM Na_2SO_4/0.5 mM Tris-Hepes (pH 7.0)). (B) Halorhodopsin induced photocurrents as a function of the bromide (solid line) and iodide (dotted line) concentration. Each point represents the average of five measurements.

even in presence of 8 mM chloride. This effect can be interpreted as a direct influence of ionic strength on the activity of the protein because adding extra NaCl again turns on the photoresponse. Not only very low but also concentrations higher than 200 mM sulfate reduced the photoresponse. This can be explained by a lower degree of binding of the Tween-washed membrane to the positively charged lipid bilayer at high ionic strength.

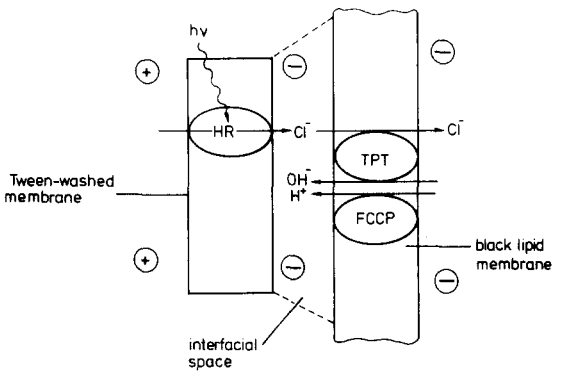

Fig. 7. Schematic representation of the ion movement on the compound membrane (sandwich-like structure) in the presence of the uncoupler FCCP and the Cl^-/OH^- exchanging carrier triphenyltin (TPT).

Discussion

Presently, the purest and most concentrated form of membrane bound halorhodopsin is the 'Tween-washed membrane' obtained from the bacteriorhodopsin-deficient strains L-33 or OD2 [8,10]. Therefore these membranes were used in our experiments which demonstrate for the first time light-induced electrical transport properties of halorhodopsin in an artificial system.

The concentration of halorhodopsin in the Tween-washed membrane from L-33 cells is about 5% that of bacteriorhodopsin in the purple membrane of S9-cells and, as expected, the corresponding maximal photocurrents show a similar ratio (Fig. 1B, D). Furthermore, Tween-washed membranes prepared from the mutant strain OD2 which contains about twice as much halorhodopsin as the mutant L-33 [5] produce about twice the maximal photocurrent. The decay time (τ) of the photocurrent is 10 ms for bacteriorhodopsin and 100 ms for halorhodopsin (L-33). The reciprocal value of the decay time is proportional to the measured photocurrent in bacteriorhodopsin [13] or halorhodopsin according to:

$$\tau = \frac{C_m + C_{HR}}{G_m + G_{HR} + I_{HR,0}/V^*} \quad (4)$$

where V^* is constant and I_{HR} is proportional to the measured photocurrent I_0. Therefore the 10-

fold larger value found for halorhodopsin agrees well with its 10–20-fold lower concentration and the 10-fold photocurrent (Fig. 1B + C). Under normal experimental conditions C_m, C_{HR}, G_m and G_{HR} are constant, whereas I_{HR} varies with light intensity and must be greater than $G_{HR} + G_m$ (see light dependence of I_0 on the light intensity J, Fig. 3).

In intact cells both chromoproteins produce, upon light excitation, an increase in membrane potential (interior negative) [21,22], which is due to bacteriorhodopsin pumping protons out of the cell and halorhodopsin pumping chloride into the cell. For bacteriorhodopsin the sign of the steady-state photocurrent in the black lipid film experiment corresponds to a proton transfer towards the purple membrane free side and for halorhodopsin to a chloride transfer towards the Tween-washed membrane free side [13] (Figs. 1B and 4A). This indicates a preferential binding of the extracellular side of purple membranes and a binding of the intracellular surface of the Tween-washed membrane to the bilayer surface. It is obvious from Figs. 1B and 1D that the return to dark results in a transient current of opposite sign (off-response) for halorhodopsin as expected on the basis of the equivalent circuit diagram in Fig. 4B. A much smaller off-response was observed for bacteriorhodopsin (Figs. 1C and 1D). A calculation of the number of protons released from bacteriorhodopsin into the interface between the purple membrane and the black film allows the conclusion that a large pH drop changes the interaction of the two surfaces, diminishing the electrical capacitive connection. This explains a small off-response for bacteriorhodopsin. Halorhodopsin, on the other hand, releases chloride or other halide ions into the interface. This causes less perturbation of ionic interaction of the two membranes during the light period and shows a relatively greater discharge of the capacitance seen as an off-response.

The development of stationary photocurrents as shown in Fig. 5 can be explained by the scheme in Fig. 7. (1) Halorhodopsin pumps cloride ions into the interfacial space between the Tween-washed membrane and the lipid bilayer and causes an electrochemical gradient (inside negative). (2) The electrogenic proton carrier (FCCP) transports protons into the interfacial space. Stationary photocurrents can not be observed because proton and chloride gradients are produced by these ion movements. (3) Upon the addition of the Cl^-/OH^- exchanger triphenyltin, however, these ion gradients are abolished and a permanent chloride flow, seen as a stationary photocurrent, occurs.

An additional explanation for the net ion translocation is that the pumped chloride ions accumulate in the small volume of the interface so that a concentration gradient is formed large enough to drive electroneutral Cl^-/OH^- exchange, converting the Cl^- gradient into a pH gradient which then pulls protons via the uncoupler into the interface. It should be mentioned that electron micrographs of Tween-washed membranes showed only sheets but no vesicles.

Previous work had demonstrated that chloride has a number of effects on halorhodopsin in vesicles [23–25] and on the purified chromoprotein [11,26]. Chloride not only influences the absorption maximum of its chromophore but also stabilizes the chromophore and changes its photochemical behaviour. In the absence of chloride a rapid photocycle (1 ms half-time) with the longest living intermediate absorbing near 640 nm was found. This so called low-chloride cycle did not show transport activity in cell envelope vesicles. Upon addition of saturating amounts of chloride, a slower photocycle (10 ms half-time) with an intermediate absorbing at 520 nm was observed, which mediates chloride transport. The Tween-washed membranes prepared under low salt conditions and in the absence of halide ions, have the properties expected from the results shown above. Our electrical measurements on black lipid films demonstrate clearly that at low salt concentrations (Cl^-, Br^-, I^-) the cycle of halorhodopsin does not exhibit photoelectrical activity.

Considering the lack of specific cation effects, complex cation coupled mechanisms for example a cation anion symport are not likely mechanisms of the transport phenomena exhibited by halorhodopsin.

In conclusion, we have demontrated the electrogenic action of halorhodopsin as a light-driven anion pump specific for halides in an artificial system. Chloride is the predominant halide ion

under most physiological conditions and therefore it seems appropriate to call halorhodopsin a light-driven chloride pump, the first so far described. Furthermore, the advantage of the black film method over vesicular or liposomal systems is clearly demonstrated by the direct and sensitive measurement of the primary electrical event in the pump molecule. The question whether halorhodopsin's function is mediated by a single protein species or a protein complex cannot be answered by our experiments with an intact membrane. However, the halorhodopsin chromoprotein has been isolated in pure form recently [11,21]. In preliminary experiments we were able to demonstrate in black lipid membrane experiments that this chromoprotein itself is active as a chloride pump.

Acknowledgements

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