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The transport activity of the light-driven chloride pump halorhodopsin is regulated by green and blue light

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Halorhodopsin-containing membrane fragments or purified halorhodopsin-containing asolectin vesicles were adsorbed to one side of a black lipid membrane, and the Cl^- transport activity was measured under different light conditions. Pump activity of halorhodopsin (HR_{578}) induced by green light decreased in time due to the formation of the inactive species HR^L_{410} and sodium azide accelerated this inactivation. Additional illumination of the system with blue light restored full activity. The regulatory function of blue light could also be demonstrated in suspensions of cell envelope vesicles and whole cells by the use of passive proton flow as a measure of the chloride transport activity. The antagonistic action of yellow and blue light suggests that halorhodopsin acts as a photochromic pigment in the intact cell.

Introduction

The retinal-binding protein halorhodopsin occurs in the cell membrane of *Halobacterium halobium* in addition to the predominant and well-characterized proton pump bacteriorhodopsin [1]. Halorhodopsin is a light-driven chloride pump [2,3] and the Cl^- transport activity can be demonstrated also with purified chromoprotein [4,5].

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; HR_{index}, halorhodopsin species which absorbs at the indicated wavelength; HR^L_{410}, HR_{410} produced in presence of light; HR^D_{410}, produced in the dark; Mops, 4-morpholinepropanesulphonic acid.

We use the term stationary for photocurrents which in contrast to capacitive currents decay more slowly than within 10 s.

Halorhodopsin shows a photocycle with distinct intermediates [8]. The scheme in Fig. 1 presents the model of the halorhodopsin photocycle which is based on the sum of available experimental information obtained with purified halorhodopsin in 1 M salt, 1% octylglucoside and at pH 7 [6,7]. Light excitation generates the photoproduct HR_{600} within 5 ps [9,10]. HR_{600} returns via the intermediates HR_{520}, HR_{640} and HR_{565} back to the ground-state HR_{578}. In the photocycle a branching occurs at the level of the intermediate HR_{520} (or alternatively at HR_{640}, see Ref. 7). A small proportion of the molecules release a proton and pass into HR^L_{410} [6,7]. Thermally HR^L_{410} returns to HR_{578} also via the HR_{520} species, i.e., HR^L_{410} is a side-product of the cycle [6,7]. Alternatively, HR^L_{410} can be photochemically reconverted to HR_{578}, a reaction which is also known for the M intermediate of bacteriorhodopsin. HR^L_{410} accumulates up to 100% under constant illumination with

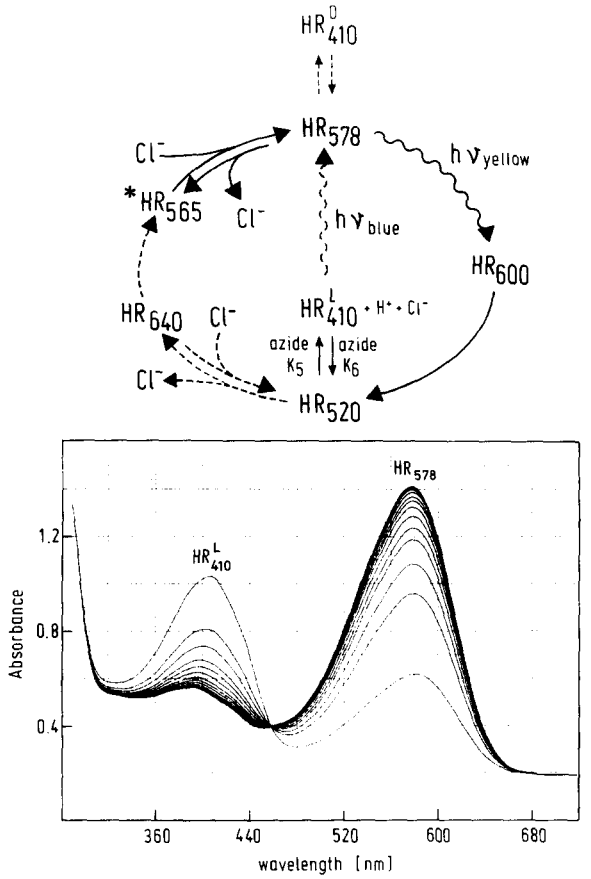

Fig. 1. Photochemical cycle of halorhodopsin (see also Refs. 1, 6 and 7). The spectra for HR^L_{410} are shown. Whether the photochemical reaction of HR^L_{410} leads directly to HR_{578} or passes intermediate stages is not known.

saturating intensities of yellow light * at pH 7 within several minutes [6,7]. Both the rate of formation and of decay of HR^L_{410} are drastically increased by the addition of azide [6], which facilitates the analysis of the physiological role of HR^L_{410}. In this communication we report the influence of blue light on the photosteady-state concentration of HR^L_{410} and its correlation with the chloride transport activity of halorhodopsin.

* The light from a halogen-tungsten lamp filtered through a cut-off filter $\lambda \geqslant 495$ nm appears yellow in colour because red light, not active on halorhodopsin, is also passing through. Light absorbed by halorhodopsin is green in colour. Therefore title and summary use the word green, but in the text the term yellow is preferred.

Material and Methods

Preparation of halorhodopsin-containing lipid vesicles (proteoliposomes)

Halobacterium halobium strains L-33 and OD 2 were grown and the Tween-washed membranes prepared from the cells as described [11,3]. Halorhodopsin was purified from Tween-washed membranes by the procedure described [11,4]. Liposomes were prepared [4] from 80 mg asolectin (Sigma) in 2 ml 1 M NaCl solution (pH 7.0) by sonication at 30°C in a sonifying bath (Branson) until turbidity no longer decreased (about 30 min). The liposomal suspension (50 µl) was mixed with 30 µl of a halorhodopsin-containing solution (80 nmol/ml, 1% octyl glucoside, 1 M NaCl, 10 mM Mops, pH 7.0) and 1 M NaCl solution was added to a final volume of 200 µl. After 2 h of incubation 1 M NaCl solution (2.8 ml) was added.

Cell envelope vesicles were prepared from cells as described [12]. Limited proteolysis with chymotrypsin was carried out in 1 M NaCl/0.1 M $MgSO_4$ solution at 37°C for 1 h with 0.04 mg chymotrypsin/mg vesicle protein.

Preparation of the planar lipid bilayers

Optically black lipid membranes with an area of about $7.5 \cdot 10^{-3}$ cm^2 were formed in a Teflon cell filled with an appropriate electrolyte solution (6 ml each compartment). The membrane-forming solution contained in the case of the Tween-washed membranes and the liposomes 1.5% (w/v) diphytanoyllecithin (Avanti Biochemicals, Birmingham, AL) and 0.025% (w/v) octadecylamine (Riedel de Haen, Hannover, F.R.G.) in decane (positively charged surface) [13]. In the case of the proteoliposomes also 1.5% (w/v) asolectin in *n*-decane (negatively charged) was used. For adsorption of the proteoliposomes to negatively charged membranes the presence of 1–20 µM Ca^{2+} was necessary [14]. The temperature was kept at 25°C. The membrane cell was connected to an external measuring circuit via Ag/AgCl electrodes. The time response of the measuring circuit was set to 10 ms rise time. In order to avoid artificial photoeffects the electrodes were separated from the aqueous compartments of the Teflon cell by salt bridges. The lipid membranes were illuminated with light of different colours: yellow, $\lambda \geqslant 495$

(OG 495, Schott. Mainz), blue, 410 ± 30 nm (Balzers, Liechtenstein) and white light (blue plus yellow), $\lambda \geqslant 395$ nm (OG 395, Schott, Mainz). Light was generated by two 250 W halogen-tungsten lamps equipped with heat-protection filters. The light beams were combined with the help of a semitransparent mirror and passed first the front compartment. Tween-washed membranes or proteoliposomes (100 μl) were added to the rear compartment. Light intensity was measured by a calibrated photodiode (Kontron). Maximal light intensity was 0.2 W/cm^2.

Results

Blue light regulation of halorhodopsin activity reconstituted in proteoliposomes

Halorhodopsin-containing asolectin vesicles (proteoliposomes) were added to the aqueous phase (1 M NaCl) of one side of the black lipid membrane. The tight association of the negatively charged proteoliposomes to the positively charged planar lipid bilayer led to electric (capacitive) coupling between the proteoliposomes and the underlying bilayer. As a consequence, the membrane system became photoelectrically active (Fig. 2a). The addition of the lipophilic anion tetraphenylborate yielded stationary photocurrents which only slowly decreased (Fig. 2b). The decay of the photocurrent in the time range of minutes indicates the formation of gradients of tetraphenylborate and chloride ions across the vesicle membrane as the result of the pump action of halorhodopsin [4].

Spectroscopic data had shown previously that continuous illumination with yellow light converts a fraction of the HR_{578} molecules into an HR^L_{410} species in the time range of minutes. In the dark HR^L_{410} reconverts to HR_{578} also within minutes. This reconversion can be photochemically accelerated by blue light [11] with a quantum yield (ϕ) of approx. 0.01 [7]. Both the formation and the decay of HR^L_{410} are drastically enhanced by the addition of azide. Experiments with stationary photocurrents should allow one to demonstrate that the accumulation of HR^L_{410} is connected to the inactivation of the pump. This, however, depends on the time course of inactivation being faster than the formation of the ion gradients, which also leads to a decay of the photocurrent

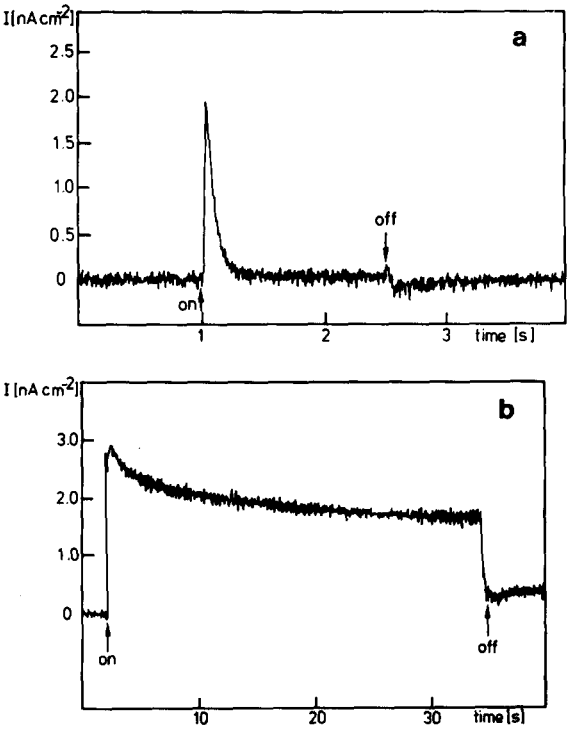

Fig. 2. Photocurrents induced by halorhodopsin-containing proteoliposomes (10 nM halorhodopsin) in the presence of 1 M NaCl and 10 mM Hepes (pH 7.5) ($\lambda \geqslant 495$ nm). (a) capacitive current in absence of tetraphenylborate; (b) stationary current in the presence of 1 μM tetraphenylborate. The light intensity was 0.2 W/cm^2.

(Fig. 2b and Ref. 4). The fast decay occurs under the conditions of the experiments with proteoliposomes only in the presence of azide. Fig. 3 (part a) shows an enhanced decay of the photocurrent in the presence of 2 mM azide (for comparison see Fig. 2b), which must be due to an inactivation of the pump. These experimental conditions also allow one to demonstrate the reactivation of the pump by blue light. After the decay of the stationary current to a low level, additional blue light was given (Fig. 3, b). As a result, not only was further decay prevented but an increase of the current reversed the effect of azide. As expected, simultaneous illumination of the membrane system with yellow ($\lambda \geqslant 495$ nm) and blue light ($\lambda = 410 \pm 30$ nm) prevented the inactivation of halorhodopsin in the presence of azide (2 mM) and turning off the blue light led again to an increased decay of the photocurrent (not shown).

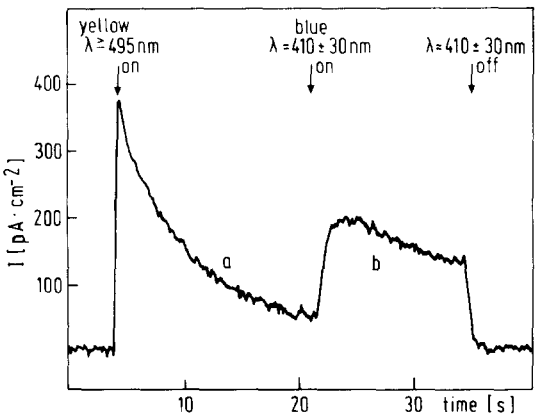

Fig. 3. Stationary photocurrent induced by halorhodopsin-containing proteoliposomes in the presence of 1 μM tetraphenylborate and 2 mM azide upon illumination with light (arrows). The conditions were as in Fig. 2. Yellow light intensity was 0.1 W/cm^2 and blue light intensity was 0.03 W/cm^2.

Blue light alone did not produce any photocurrent in a sample adapted to darkness for 20 min. At a higher azide concentration of 10 mM the peak current was reduced by at least 30–40% and the blue light effect was also diminished.

In another series of experiments cell envelope vesicles prepared from a bacteriorhodopsin-negative strain (OD2) were attached to the lipid bilayer membrane. The same effect of yellow and blue light on the photocurrents as with proteoliposomes was found with the cell envelope vesicles in the presence of azide. In contrast to the proteoliposomes, a blue light reactivation of halorhodopsin was found for the cell envelope vesicles also in the absence of azide, indicating the physiological relevance of this effect. The strain OD2, like L-33, contains besides halorhodopsin also the sensory rhodopsin (SR_{588}), which upon illumination is converted into a species absorbing at 373 nm and decaying into SR_{588} thermally within 800 ms. An interference of this chromoprotein in our measurements is not expected because sensory rhodopsin does not create a membrane potential upon light excitation [1,18].

Experiments with Tween-washed membrane

Recently it was shown that halorhodopsin-containing Tween-washed membranes adsorbed to one side of a black lipid membrane induce a capacitive photocurrent which depends on the presence of chloride [3]. Stationary photocurrents have been obtained by the addition of the Cl^-/OH^--exchanging agent triphenyltin in combination with the proton-carrier FCCP. The capacitive photocurrent is smaller by 70–80% in the presence of 200 mM azide during white light illumination (not shown). The observed inhibition could be caused by the formation of HR^L_{410} and was analyzed in more detail by measuring capacitive photocurrents. This type of analysis can be done in the absence of triphenyltin and FCCP and avoids photochemical artefacts by blue light possibly caused by the colour of FCCP.

Three experimentally verified relationships derived from a previously published equivalent circuit diagram hold for the transient photocurrent in white light and are important in this context [14,3]:

$$I_0 = I_0^s \frac{J}{J + J_{1/2}} \tag{1}$$

$$I_0 = I_{HR,0} \frac{C_m}{C_{HR} + C_m} \tag{2}$$

$$\tau = \frac{C_m + C_{HR}}{G_m + G_{HR} + I_{HR,0}/V^*} \tag{3}$$

J is the light intensity; $J_{1/2}$ is the half saturation light intensity; I_0 is the initial current which is observed after switching on the light at time zero; I_0^s is the initial current at saturating light intensities; $I_{HR,0}$ is the initial pump current in the absence of an ion motive force ($\Delta\mu_{Cl^-}$) which is proportional to the number of active molecules; G_{HR} and G_m are the conductances of the halorhodopsin-containing membrane and the underlying bilayer membrane (black lipid film); C_{HR} and C_m are the capacitances of these two systems and assumed to be constant; V^* is a constant.

The experiments were carried out at non-saturating (25%) and at saturating (100%) light intensities in the absence (Fig. 4a and b) and presence of 2 mM azide (Fig. 4c and d). Under non-saturating yellow light intensities (25%) in the absence of azide an additional signal upon additional yellow light illumination (75%) can be observed (Fig. 4a, Eqn. 1). If the same experiment was repeated, but with an additional 75% of white light intensity instead of yellow light, a larger second signal is obtained (Fig. 4b). The blue light

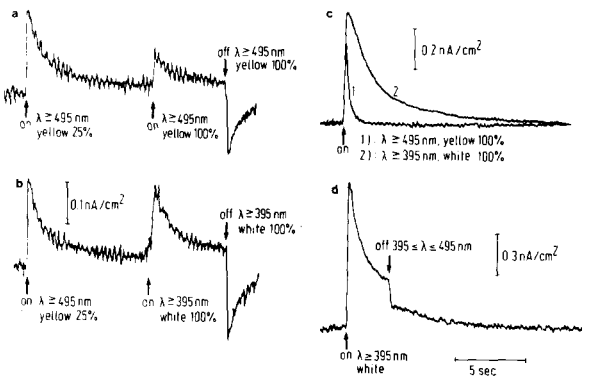

Fig. 4. Capacitive photocurrents induced by adsorbed Tween-washed membranes (1 mg/6 ml) from OD2 cells in the presence of 100 mM NaCl and 10 mM Hepes at pH 7.5 under different light conditions (a), and (b) in the absence of azide. (c and d) in the presence of 1 mM azide. 100% yellow light corresponds to 0.2 W/cm^2 and 100% blue light (λ = 395–495 nm) to 0.07 W/cm^2.

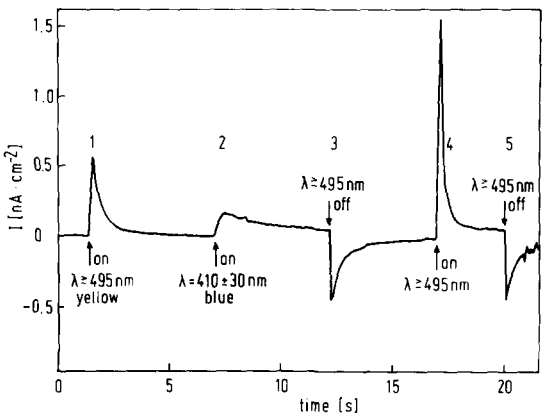

Fig. 5. Capacitive photocurrents induced by Tween-washed membranes (1 mg/6 ml) from OD2 cells in the presence of 100 mM NaCl, 10 mM Hepes and 2 mM azide at pH 7.5 under different light conditions as indicated by the arrows.

component of the white light does not cause an electric signal on its own (see also Fig. 5). Therefore the result of the experiments in Fig. 4a and b indicates that halorhodopsin is inactivated in the presence of yellow light, but only partially because a second signal can be observed (Fig. 4a). The white light pulse which contained blue light in addition to yellow light and which induced a larger second photocurrent (Fig. 4b) apparently reactivated halorhodopsin molecules.

The photoresponses in yellow light were also compared to that in white light ($\lambda \geq 395$ nm) at 100% light intensity (Table I). Without azide the initial current I_0 was equal under both light conditions. The decay, however, in yellow light is faster ($\tau = 1.9$ s) compared to that in white light ($\tau = 2.8$ s). Azide enhanced this effect, reducing the τ value from 2.9 s in white light to 0.8 s in yellow light. In addition, the initial current I_0 is reduced to 70% (Table I). The large decrease of the total current demonstrates the inactivation of halorhodopsin by yellow light during the response time of the system. The concomitant decrease of τ and I_0 upon a change from white to yellow light (Table I) is not predicted by Eqn. 3, which holds for a constant number of active pump molecules during the transient current. The decrease of both τ and I_0 therefore is explained by a decreasing number of active pump molecules in yellow light. This inactivation of halorhodopsin is in agreement with the photochemical cycle shown in Fig. 1 and caused by the transition of HR_{520} to HR^L_{410}. The reactivation of halorhodopsin by blue light, i.e., conversion of HR^L_{410} into HR_{578}, could be eliminated if the blue light component was removed from white light by insertion of a cut-off filter ($\lambda \geq 495$ nm) during the decay of the current (Fig. 4d). The decay of the transient current was accelerated. Upon addition of increasing amounts of azide (up to 100 mM) under constant yellow light intensities the initial current I_0 decreased concomitantly with τ. This not only confirms that yellow light inactivates and blue light reactivates the pump but also that HR^L_{410} is the inactive state.

The transport activities of halorhodopsin in Tween-washed membranes under different light conditions are summarized by the experiment

TABLE I

REPRESENTATION OF THE TIME CONSTANT τ OF THE CAPACITIVE PHOTOCURRENTS AND OF THE INITIAL CURRENTS I_0 UNDER DIFFERENT LIGHT CONDITIONS

	Without azide		With azide	
	$\geq$ 395 nm	$\geq$ 495 nm	$\geq$ 395 nm	$\geq$ 495 nm
τ (s)	2.8	1.9	2.9	0.8
I_0	1	1	1	0.7

shown in Fig. 5. In the presence of 2 mM sodium azide yellow light produced a transient photocurrent (Fig. 5,1) and additional yellow light a few seconds later (not indicated) did not produce a second signal, demonstrating that no active halorhodopsin molecules were left. If, however, additional blue light was projected onto the membrane system a second transient current was observed (Fig. 5,2). Turning off the yellow light with the blue light still on resulted in a transient current of opposite sign (off response, Fig. 5,3, see Ref. 3). By turning on the yellow light again a 2.5-times larger transient current (Fig. 5,4) was produced than by yellow light alone (Fig. 5,1). Turning off this additional yellow light produced an off response (Fig. 5,5) equal to the off response in Fig. 5,3. All these observations are explained by the formation of inactive HR^L_{410} during illumination (see Fig. 1). Its formation depends on light intensity and azide concentration and can reach the time range of the transient photosignal (see above). The blue light effect due to the photochemical reconversion of inactive HR^L_{410} into active HR_{578} was observable whenever an appreciable formation of HR^L_{410} occurred.

Lowering the chloride concentration decreased both the yellow and blue light-induced signal, but their ratio remained constant between 2 mM and 1 M chloride (not shown). This experimental result is explained by the fact that the relative ratios of all three dark forms, HR_{578}, HR_{565} and HR^D_{410}, are changed at different chloride concentrations (with significant HR^D_{410} concentrations under low chloride conditions, Ref. 15). At low chloride concentrations the amount of HR_{578} is low and only small photocurrents are measured [3,4]. During several seconds under saturating yellow light conditions, however, at high and at low chloride concentrations as well all three species are converted into HR^L_{410}. Blue light reconverts the HR^L_{410} back into HR_{578}, HR_{565} and HR^D_{410} so that the same amount of HR_{578} is available for a transport cycle as at the beginning of yellow light illumination. Thus, the blue light-induced signal also is proportional to the equilibrium concentration of HR_{578}.

From the experimental results in Figs. 4 and 5 it cannot be concluded whether the pathway from HR^L_{410} to HR_{578} involves an electrogenic event or not. This was answered by a separate experiment

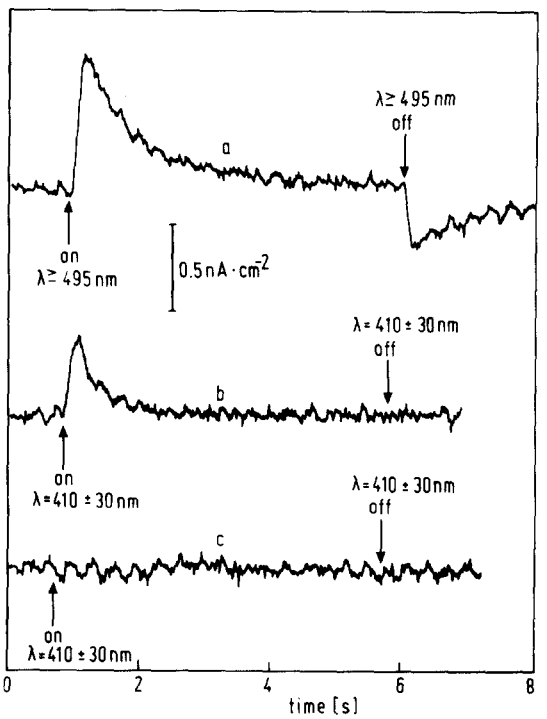

Fig. 6. Capacitive photocurrents induced by adsorbed Tween-washed membranes (1 mg/6 ml) from OD2 cells in the presence of 100 mM NaCl and 10 mM Hepes at pH 7.5 (a) in the presence of yellow light ($\lambda \geq 495$ nm), (b) in the presence of blue light ($\lambda = 410 \pm 30$ nm) 5 s after yellow light is turned off, and (c) 5 min after yellow light is turned off.

shown in Fig. 6. Fig. 6a represents the standard on and off response to yellow light in the absence of azide. During the time of yellow light illumination HR^L_{410} accumulated. After turning off the light HR^L_{410} had thermally decayed within minutes. 5 s after the end of yellow light illumination blue light was turned on (trace b). The electric response apparently represents a charge movement within the halorhodopsin molecules during the photochemical reconversion from HR^L_{410} into HR_{578} and is not linked to transport activity because the same response is obtained in the absence of chloride (see below). This electric response was not seen if the system was kept for 5 min after yellow light illumination in the dark before blue light was turned on (trace c) because HR^L_{410} had decayed already. An electric response to the thermal decay was not detectable because this process is slow. These results also explain the observation that the ratio of the yellow light- and blue light-induced

signals is not constant below 2 mM chloride. In the presence of 500 μM chloride at pH 7.5 halorhodopsin shows no detectable chloride transport activity but is converted into HR^L_{410} during yellow light illumination within seconds. An additional blue light pulse shows a small signal which represents the charge movement during reconversion from HR^L_{410} into HR_{578} and not a restored transport activity.

The results obtained from the experiments in Figs. 3–6 are in agreement with the spectroscopic data obtained in Refs. 6 and 7 for the purified protein, whereas the fast inactivation of the electrical response in the Tween-washed membrane is not yet clear. Isolated halorhodopsin in the absence of azide is converted into HR^L_{410} upon continuous illumination only within minutes. Although the initial velocity of HR^L_{410} formation is pH-independent, the time to reach equilibrium and the equilibrium concentration of halorhodopsin are strongly increased at alkaline pH. According to this, inactivation of halorhodopsin with a time course of minutes was observed with proteoliposomes under the conditions of the experiment in Fig. 2b, i.e., if stationary photocurrents were produced in the presence of tetraphenylborate.

Transport in cell envelope vesicles and whole cells

As shown in Fig. 2, the halorhodopsin-induced photocurrent decreased during illumination with yellow light due to the formation of ionic gradients [4] and the decrease was enhanced drastically in the presence of 2 mM azide due to the formation of HR^L_{410} (Fig. 3). Only in the presence of azide was a blue light effect observed. This effect of azide on the transport activity can also be investigated in cell envelope vesicles (OD2 cells for the experiments in Figs. 7–9) by measuring halorhodopsin activity as the light-induced passive proton flow in the presence of uncouplers (50 μM CCCP [19]). The pH-change which occurred as a response to the membrane potential created by the chloride transport was measured and found to be constant for the first 20 s (initial velocity, v_i). At low intensities of yellow light (100 W, $\lambda \geq 495$ nm) and in the presence of 4 M NaCl, v_i did not change significantly upon addition of azide up to 55 mM at pH 7. Higher concentrations of azide were not tested because of the strong buffer capacity effect of azide. At a lower concentration of chloride, however, the effect of azide became apparent. Fig. 7 shows the linear relationship between the inverse transport activity versus inverse chloride concentration with and without 50 mM azide. The plot in Fig. 7 seems to indicate that azide acts as a competitive inhibitor on the halorhodopsin-mediated chloride transport. The underlying mechanism, however, must not be a competition of azide for chloride binding sites in the halorhodopsin molecules. Rather, the catalytic action of azide on HR^L_{410} formation (Fig. 1) produces the effect.

In order also to see a deactivation of transport induced by azide at 4 M Cl^- concentrations, high light intensities (production of more HR_{520} in the photocycle) and low proton concentrations were applied. Fig. 8 shows original traces of pH changes at different azide concentrations uncorrected for the buffer capacity of azide. The total proton uptake was nearly equal for the experiments in a–d (up to 20 mM azide). Fig. 8a shows a halorhodopsin-induced pH change in an OD2 vesicle-containing suspension upon illumination with yellow light at pH 8.0 (yellow light = y_1: 900 W, 50%, $\lambda > 495$ nm, 2 ml suspension volume). The

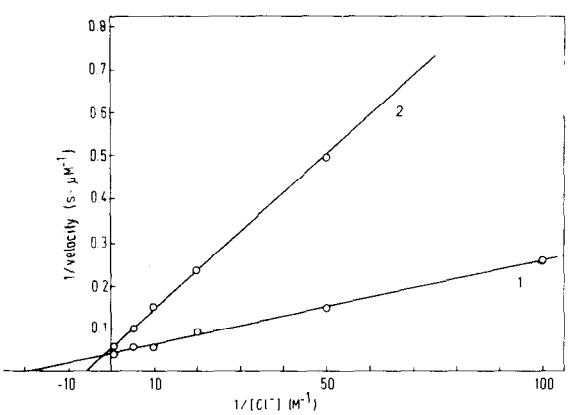

Fig. 7. Halorhodopsin transport activity in cell envelope vesicle-containing suspensions (8 ml, 0.2 mg/ml) measured as alkalinization of the medium at different chloride concentrations. Initial pH was 7.0, azide concentration 50 mM, CCCP concentration 50 μM. The reciprocal initial velocity is plotted against the reciprocal chloride concentration. The samples were prepared by dilution of a concentrated vesicle suspension (in 4 M NaCl) with different amounts of 1.5 M Na_2SO_4 and 4 M NaCl. Light ($\lambda \geq 495$ nm) from a 100 W projector was used (unfocussed). (1) Without azide, (2) with azide.

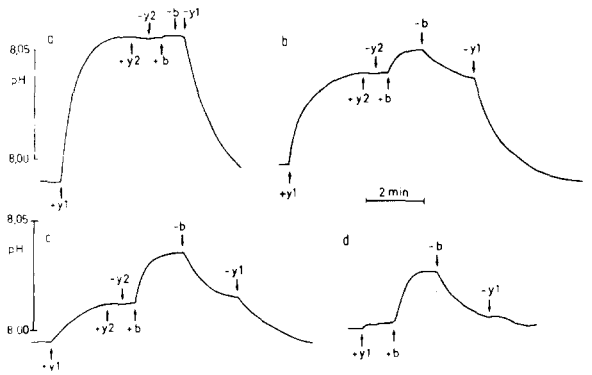

Fig. 8. Halorhodopsin transport activity in cell envelope vesicle-containing suspensions (2 ml, 0.1 mg/ml) measured as alkalinization of the medium in 4 M NaCl containing 50 μM CCCP at 0, 5, 10 and 20 mM azide (a–d). y_1, 50% yellow light intensity (900 W focussed on the sample, approx. 1.5 cm^2, $\lambda \geqslant 495$ nm); y_2, additional 50% yellow light; b: 100% blue light (900 W, $\lambda = 420 \pm 30$ nm).

alkalinization was enhanced neither by additional irradiation with yellow light (y_2) nor with blue light (b) within the time period of illumination. When the experiment was repeated in the presence of 5 mM azide (Fig. 8b) an effect of blue light on transport activity became obvious. After the steady state had been reached in yellow light additional blue light led to further alkalinization of the medium. This experiment demonstrates that during illumination HR^L_{410} was formed and thereby transport prevented, as already observed during the electrical measurements. In the presence of 10 mM azide (Fig. 8c) illumination with yellow light caused both a decreased initial velocity and a decreased extent of alkalinization (transport). Correspondingly, the blue light stimulation was increased. If 20 mM azide was added to the sample (Fig. 8d) halorhodopsin was inactivated in yellow light so fast by HR^L_{410} formation that no appreciable transport occurred. Blue light, on the other hand, fully restored the transport activity under these conditions.

In the absence of azide the HR^L_{410} formation proceeded only very slowly (5–30 min), seen as a slow decrease in the stationary pH of illuminated cell envelope vesicle suspensions. The physiological significance of HR^L_{410} formation leading to a blue light control of halorhodopsin transport activity therefore was demonstrated in an experiment with intact cells, which apparently respond faster

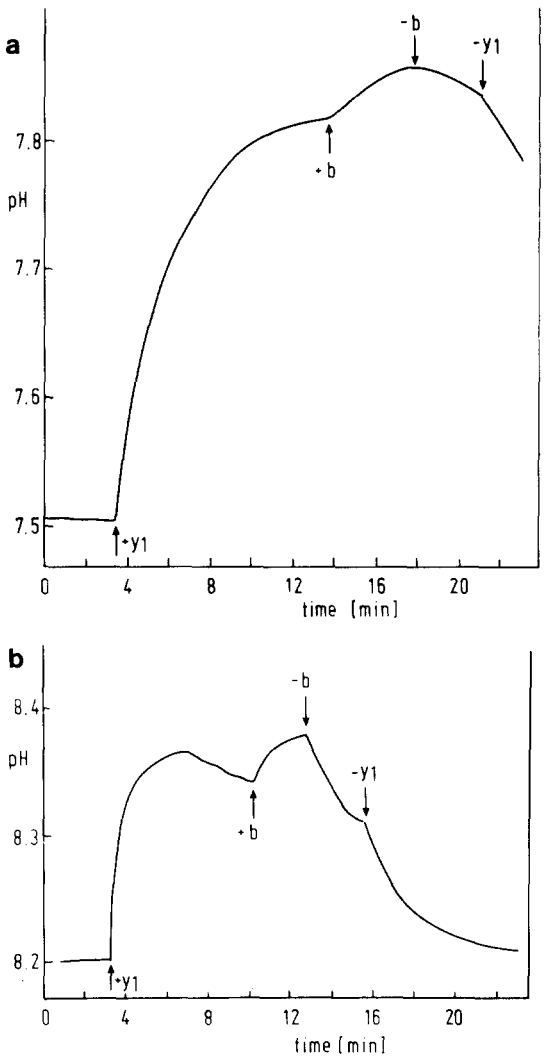

Fig. 9. Light-induced pH changes in a suspension containing OD2 cells in 4 M NaCl at pH 7.5 (a) and pH 8.2 (b) in the absence of azide during yellow light ($y_1 = 900$ W) and additional blue light (b, 900 W) irradiation.

to yellow light than do envelope vesicles (Fig. 9). A cell suspension in the absence of azide was illuminated at two different pH values (pH 7.5 and 8.2 in Fig. 9a and b). At pH 8.2 yellow light always induced alkalinization, which decreased after 3 min (Fig. 9b). At both pH values additional blue light increases transport activity after a period of 5 min of yellow light, seen as enhanced alkalinization.

Discussion

The analysis of the photochemical behaviour of halorhodopsin summarized in Fig. 1 predicted a regulatory influence of blue light on the halorhodopsin photocycle [6]. In this paper it is shown that halorhodopsin pump activity, which is dependent on yellow light, is increasingly inhibited by this light, but restored by additional blue light. This photochromic property of halorhodopsin is connected with the transport activity, as demonstrated with isolated halorhodopsin, halorhodopsin-containing Tween-washed membranes and halorhodopsin-containing cell envelope vesicles or intact cells.

Halorhodopsin-containing proteoliposomes or envelope vesicles were tested in the presence of 1–4 M NaCl either in suspension or on black lipid membranes. Both systems showed blue light effects, i.e., HR^L_{410} formation within minutes. Upon addition of azide, HR^L_{410} formation, and thus the blue light effect connected to it, appeared increasingly more quickly. The Tween-washed membrane, on the other hand, showed already in the absence of azide an almost immediate response to blue light, i.e., a response within the time period of the transient current (Fig. 4a, b). Since the conditions of illumination were similar for Tween-washed membranes and proteoliposomes, the faster formation of HR^L_{410} should be explained. The experiments with Tween-washed membranes were carried out at lower (100 mM) chloride concentrations compared to those with proteoliposomes (1 M). The HR^L_{410} formation is accelerated at low chloride concentrations and an influence on the decay of the photocurrent could be expected although the K_m value of halorhodopsin for chloride is near 10 mM [3]. Therefore the chloride dependence of the blue light effect was measured. Not only was the ratio of yellow and blue light response (see above) independent of Cl^- between 2 mM and 1 M but also the time to develop the blue light sensitivity remained within 5 s, i.e., 10–100-times faster than for proteoliposomes. Therefore the different chloride concentrations cannot be responsible for the different rate of HR^L_{410} formation.

A further difference between the short-circuit experiments with Tween-washed membranes and those with proteoliposomes lies in the fact that during the pump process electric potentials (V_p) are built up to a different extent, which may influence the photocycle and HR_{410} formation. During the pump process a potential V_p across the Tween-washed membranes and V_m across the underlying lipid bilayer is built up. In the case of a short-circuit experiment V_p is equal to $-V_m$ (the membrane system is clamped to $V = 0$). To prove that V_p accelerates the formation of HR_{410}, the Cl^-/OH^- exchanger triphenyltin, together with the uncoupler FCCP, yielding stationary photocurrents, was added in order to decrease the pump potential. No effect on the formation rate could be detected, so that it is still unclear whether the membrane potential influences the formation of HR^L_{410}. At present a final explanation for the fast inactivation of halorhodopsin in Tween-washed membranes cannot be given.

Besides the yellow and blue light intensities and the membrane potential, azide and pH also influence the photochromic behaviour of halorhodopsin. On the basis of the scheme in Fig. 1 the concentration ratio of HR_{578} and HR^L_{410} under conditions of non-saturating light intensities and a constant concentration of 1 M NaCl can be expressed as

$$\frac{[HR_{578}]}{[HR^L_{410}]} = \frac{k_6[H^+] + BJ_b}{k_5 \cdot AJ_y} \tag{4}$$

where k_5 and k_6 are the rate constants of formation and decay of HR^L_{410} and are azide-dependent [7]. A and B are constants describing the quantum efficiency of the two photoreactions, and J_y and J_b are the yellow and blue light intensities. The HR_{578}/HR^L_{410} ratio in the photosteady state represents the ratio of active and inactive pump molecules at high chloride concentrations where the HR_{565} species can be neglected. Two predictions for shifts in this ratio and therefore in the efficiency of activation of halorhodopsin by blue light can be made on the basis of Eqn. 4 and were borne out by experiments. (i) When the photosteady state is established, blue light should decrease in its efficiency with decreasing pH because the term BJ_b contributes decreasingly to the ratio HR_{578}/HR^L_{410}. (ii) Increasing concentrations of azide not only accelerate the formation of the

photosteady state but also diminish the blue light reactivation efficiency, as mentioned above. While under conditions of constant pH and light intensity, the term $k_6[H^+]/k_5\ AJ_y$ of Eqn. 4 remains constant due to the fact that k_5 and k_6 both increase in the same fashion with increased azide concentrations:

$$k_{5,6} = k^0_{5,6} = \frac{k_{5,6}}{1 + \frac{K_M}{\text{azide}}}$$

where k represents k_5 and k_6, $k^0_{5,6}$ are the respective rate constants at azide concentration zero (compare also Ref. 7), the second term $BJ_B/k_5\ AJ_y$ becomes smaller at higher azide concentrations, explaining the decreased blue light efficiency.

At higher pH values a second form of HR_{410}, i.e., HR^D_{410}, is formed in the dark and removes halorhodopsin molecules from the active state. In the presence of 1% octyl glucoside and 1 M NaCl this equilibrium establishes with a pK of 9.6 and therefore is not of practical importance at neutral pH. Formation of HR^D_{410}, however, might play a role in intact cells if the proton pump bacteriorhodopsin or respiration alkalinize the cytoplasm. Not only high internal pH but also a lowered pK value of 8.7 for the HR_{578}-to-HR^D_{410} transition measured in the absence of detergent might allow the formation of appreciable amounts of HR^D_{410} in the cell. This would lead to a decrease of active pump molecules and thereby to a regulation by pH in addition to that by blue light, membrane potential and chloride concentration.

In transport as well as in spectroscopic experiments azide is an important tool for the demonstration of blue light effects but it is without physiological relevance. Blue light regulation occurs without azide, as demonstrated in Figs. 4, 5 and 9. Azide cannot shift the ratio of HR_{578}/HR^L_{410} in the absence of blue light but accelerates the formation of the photosteady state and diminishes the influence of blue light (Eqn. 4). Considering the situation of living halobacterial cells under daylight conditions, the photosteady state will be reached within the time range of minutes. The halorhodopsin activity itself will therefore be regulated by light intensity and the wavelength of the incident light.

An interference of the reported photochromic behaviour of sensory rhodopsin with our results on halorhodopsin is rather unlikely. First, pure halorhodopsin preparations show qualitatively the same photochromism and reconstituted into liposomes the same transport activity as intact cells or cell envelope vesicles also containing sensory rhodopsin (see above and Refs. 4 and 5). Second, sensory rhodopsin was reported to cause no significant change in membrane potential upon illumination [18].

The M intermediate of the bacteriorhodopsin photocycle (BR_{412}) also contains a deprotonated retinylidene species and is blue-light-sensitive [16]. The photochemical back-reaction from BR_{412} to the ground state was analyzed in detail (for review see Ref. 20). The main difference between HR^L_{410} and BR_{412} is that BR_{412} is an intermediate of the transport cycle of bacteriorhodopsin whereas HR^L_{410} is a side-product of the halorhodopsin cycle (Fig. 1). This difference becomes obviously important for blue light regulation if the ratio of BR_{568}/BR_{412} is described by an equation analogous to Eqn. 4:

$$\frac{[BR_{568}]}{[BR_{412}]} = \frac{k_3[H^+] + BJ_b}{AJ_y} \qquad (5)$$

where k_3 is the rate constant for the decay of BR_{412} and is in the range of 10 ms. A proton motive force of the cell could slow down the decay of BR_{412} by a factor of not more than 10 [20], i.e., k_3 is still several hundred-fold faster than k_5 in Eqn. 4 describing halorhodopsin. With the assumption that BR_{412} and HR^L_{410} react with similar quantum efficiency the influence of blue light on the equilibrium in Eqns. 4 and 5 and therefore on the bacteriorhodopsin and halorhodopsin photocycles is very different. Under the same conditions of illumination (i.e., BJ_b/AJ_y is equal in both cases) $k_3[H^+]/AJ_y$ is much greater than $k_5[H^+]/AJ_y$, i.e., the contribution of blue light-induced regeneration is much less pronounced in bacteriorhodopsin than in halorhodopsin.

The BR_{412} decay is rate-limiting for the bacteriorhodopsin photocycle. A slower decay rate would facilitate blue light regulation but on the other hand it would also slow down the entire photocycle. Therefore only a side-reaction of a

photocycle as realized in halorhodopsin allows a slow thermal reconversion from the inactive state and thereby efficient blue light regulation.

At saturating light intensities, however, bacteriorhodopsin also reaches a state where BR_{412} dominates the photosteady state. Blue light under these conditions induces faster regeneration of BR_{568} [16]. The regeneration pathway seems not to be equivalent to the second half of the proton-translocating photocycle and therefore blue light does not activate but rather inactivates the pump [17]. Both halorhodopsin and bacteriorhodopsin are regulated by blue light but in a different fashion. The pump action of halorhodopsin at physiological light intensities is activated, whereas the action of bacteriorhodopsin at nearly saturating intensities is reduced by blue light.

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