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Primary and Secondary Chloride Transport in *Halobacterium halobium*

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Chloride uptake in intact cells of *Halobacterium halobium* was characterized by rates of influx and efflux of $^{36}Cl^-$ under conditions of light, respiration, or both. Halobacterial mutant strains with and without retinal transport proteins allowed study of the effects of halorhodopsin and bacteriorhodopsin under illumination. Two structurally independent chloride transport systems could be distinguished: halorhodopsin, the already known light-driven chloride pump, and a newly described secondary uptake system, which was energized by respiration or by light via bacteriorhodopsin.

The identification of halorhodopsin (HR) as a light-driven chloride pump (18) focused new interest on anion transport in the halophilic archaebacterium *Halobacterium halobium* (1, 11, 20, 21). The cytoplasmic concentration of chloride in halobacterial cells is about equal to that in the external medium (3 to 4 M), and the halobacteria keep this level constant during the cell cycle (4). Although the transmembrane chemical gradient of chloride ($\Delta\mu_{Cl^-}$) is found to be close to zero, the chloride balance is far from the electrochemical equilibrium with respect to the membrane potential ($\Delta\psi$ = 90 to 150 mV, negative inside [3]). By using the Nernst criterion, active transport mechanisms therefore must be considered here.

Chloride transport in lower and higher organisms generally has to serve two functions: osmotic regulation and elimination of excess salt. Active chloride transport in plants is well documented by the chloride pumping ATPase of *Acetabularia* spp. (6) or by the H^+/Cl^- symport in *Chara corallina* (17). A Cl^-/OH^- antiport was described for *Chlorella fusca* (2). Several active chloride transport mechanisms have been investigated in absorptive and secretory epithelia of vertebrates and invertebrates (3, 26).

In the archaebacterium *H. halobium*, light-driven chloride transport via HR has been shown only in vesicles (18) and black lipid membranes (1). Here we determined uptake of $^{36}Cl^-$ into intact cells of *H. halobium* and demonstrated the action of HR in vivo.

Furthermore, we report here on a highly efficient, secondary uptake system for chloride in *H. halobium*. Mutant strains deficient in halobacterial retinal proteins allowed discrimination between the specific effects of HR, bacteriorhodopsin (BR), and the respiratory chain on active chloride transport.

MATERIALS AND METHODS

Strains and culture conditions. *H. halobium* was cultured and harvested as described before (7, 16). Mutant strains used were positive (+) or negative (−) for BR and HR as follows: Flx-3r, BR^- HR^- (19); L-33, BR^- HR^+ (24); M-18, BR^+ HR^- (G. Wagner, B. Traulich, K. M. Hartmann, and D. Oesterhelt, manuscript in preparation); M-1, BR^+ HR^+ (formerly named R_1M_1 [7]); and wild type, BR^+ HR^+ (23).

Influx measurements. Cells were centrifuged for 10 min at 8,000 × *g* and suspended in a Tris-buffered basal salt medium as described previously (7). If not otherwise indicated, the external pH was kept constant at 6.4. The cell density was adjusted to an absorbance of 7.0 at 700 nm for a layer of 1 cm with a Kontron Uvikon 810 (Eching, FRG) spectrophotometer. This corresponds to 3.35 mg of protein per ml of cell suspension as determined by the method of Goa (5) and is equivalent to a bacterial membrane surface of 77×10^{-3} m^2/ml of cell suspension (22). BR content was determined by difference spectroscopy of hydroxylamine-bleached and unbleached cells (14, 15). The BR concentration of wild-type cells was estimated by reference to the measured average values of 1.7 ± 0.06 (standard error of the mean) nmol/mg of protein for strain M-1 and 2.4 ± 0.1 nmol/mg of protein for strain M-18, respectively.

Cell suspensions were adapted to basal salt medium for at least 2 h in the dark and under nitrogen at room temperature. During the experiments, the suspension was stirred in a cylindrical glass vessel and kept at 25°C by a flowthrough water jacket.

Yellow or white light was provided by a 250-W slide projector with a tungsten-halogen lamp (Leitz, Wetzlar, FRG), occasionally equipped with a cut-on glass filter (GG 495; Schott, Mainz, FRG). Light intensities were adjusted by using neutral density filters (Balzers, Liechtenstein). Energy fluence rates were measured inside the glass vessel by a YSI-Kettering 65A radiometer (YSI, Yellow Springs, Ohio).

At the beginning of each experiment, $^{36}Cl^-$ as $Na^{36}Cl$ was added (4.44 kBq/ml of bacterial suspension) to reach a specific activity of 14 nmol/cpm. Samples (0.3 ml) were centrifuged for 5 min at 8,000 × *g* in a microcentrifuge (type B, Beckman Instruments, Palo Alto, Calif.) through an 80% silicon oil–20% brombenzene layer (vol/vol) (13). The tips of the 0.4-ml centrifuge tubes were cut off, and the silicon oil was removed by shaking with 0.3 ml of diethylether (22). The pellets were suspended in 0.5 ml of water and counted in a liquid scintillation counter.

Efflux measurements. Cells were loaded with $^{36}Cl^-$ under white light at 3,600 W/m^2 (strain M-1) or oxygen (strain L-33) for 1 h under the same conditions as described for influx measurements, except the specific activity of $^{36}Cl^-$ was 28 nmol/cpm. Ten milliliters of the suspension was centrifuged for 10 min at 6,000 × *g*, the supernatant was discarded, and the pellet was suspended in 10 ml of unlabeled basal salt medium. The entire process of centrifugation and suspension

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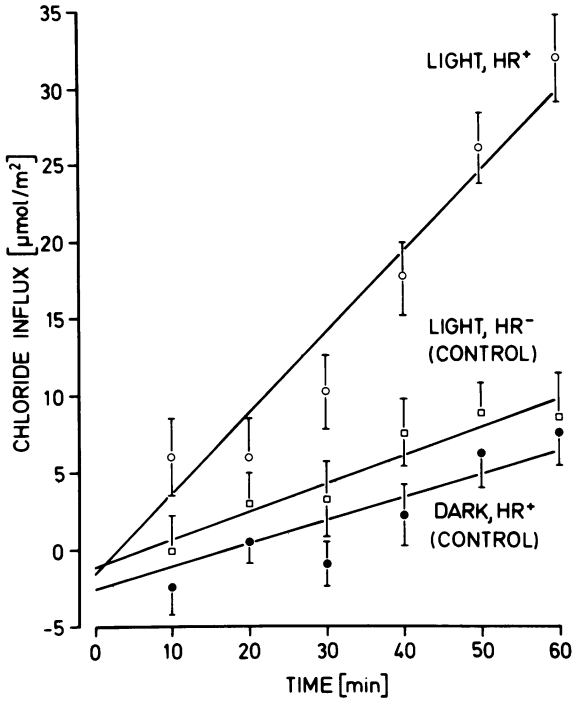

FIG. 1. HR as a light-driven chloride pump. Chloride influx was determined for L-33 (HR^+) cells in light (○) and dark (●) and for Flx-3 (HR^-) cells in light (□). Immediately after the addition of $^{36}Cl^-$, samples were pipetted and centrifuged to determine the radioactivity due to the extracellular space, which consists of the cell wall and medium dragged through the silicon oil layer during centrifugation. This value was subtracted from all the other values to determine the influx into the intracellular space. Irradiance was 2,000 W/m², with yellow light (GG 495). All experiments were done under nitrogen atmosphere. Error bars indicate the standard error of the mean for two to five independent experiments.

was standardized to take 25 min. Samples (0.3 ml) of the bacterial suspension were collected as described above at the beginning of each efflux experiment. The incorporated radioactivity was determined, and the process was repeated after 1 and 2 h.

Respiration measurements. Oxygen consumption was determined with a Clark-type polarographic oxygen electrode (Hansatech D.W.; Bachofer, Reutlingen, FRG). Experimental conditions were the same as in the influx experiments. Oxygen consumption of the cells was determined in nanomoles per second.

ATP levels under respiratory conditions were measured by lysing portions of cells by osmotic shock in 10 mM phosphate buffer and determination of ATP by bioluminescence (7).

Sources of chemicals. $Na^{36}Cl$ was obtained from Amersham Buchler (Braunschweig, FRG), silicon oil CR 500 was from Wacker (Munich, FRG), Tris from Fluka (Neu-Ulm, FRG), L-alanine from Serva (Heidelberg, FRG), and all other chemicals were from Merck (Darmstadt, FRG) and were of analytical grade.

RESULTS

Action of HR. Light-dependent chloride influx, constant in its rate for at least 60 min, was found in *H. halobium* mutant strain L-33, but far less in mutant strain Flx-3 (Fig. 1). This difference may reflect the presence of HR in L-33 and its absence in Flx-3.

Chloride influx in light as a function of energy fluence rate was determined for halobacterial mutant strains L-33 and M-1 (Table 1; Fig. 2). The chloride transport rate in M-1 was a close function of irradiance and showed no saturation in light up to energy fluence rates of 1,400 W/m² (Fig. 2). In contrast, $^{36}Cl^-$ influx in L-33 was easily light-saturated by about 100 W of yellow light per m². White light was slightly more effective in L-33 cells than yellow light at identical irradiances, but also seemed to be saturated at low intensities (Fig. 2).

Respiration-driven chloride transport. Under respiratory conditions in darkness, rates of chloride transport in the two BR-deficient mutant strains L-33 and Flx-3 were large (>18 nmol/m² per s), while the rates were significantly smaller (<8 nmol/m² per s) in the three strains which contained BR, i.e., wild-type cells and mutant strains M-1 and M-18 (Table 2). The low rates of respiration-driven Cl^- transport in the latter cells cannot be explained by low rates of respiration: oxygen consumption showed similar or even higher rates of respiration depending on the presence of BR (Table 2). Also, the ability for oxidative phosphorylation was not diminished in wild-type cells or mutant strains Flx-3, L-33, M-1, or M-18 (Table 2).

The average respiratory rate of 17.65 ± 1.95 nmol of O_2 per m² per s was nearly independent of the strain tested, and this was confirmed by manometric measurements (25; G. Krippahl, personal communication). The respiratory rate was enhanced 1.35 (±0.15)-fold by the addition of 20 mM L-alanine, which is actively accumulated by the bacteria (12) and may enhance the level of metabolism in the cells. Consistent with a factor apparently interfering with respiration-driven Cl^- influx in *H. halobium*, the BR-producing strain M-18 showed respiration-coupled Cl^- transport only when the rate of oxygen consumption was enhanced about twofold in the presence of L-alanine. Growth of strain M-18 lagged behind that of the other four strains tested.

BR-mediated Cl^- transport. Unlike Cl^- transport rates in darkness, BR-positive strains of *H. halobium* took up chloride very effectively in light, reaching rates of up to 43 nmol of Cl^- per m² per s (Fig. 3). Here again, the HR-deficient

TABLE 1. Influx and efflux of chloride in M-1 and L-33 cells of *H. halobium*[a]

Activity	Transport rate (nmol of Cl^-/m² per s)							
	Strain M-1				Strain L-33			
	N_2, 2,000 W/m²	N_2, 100 W/m²	N_2, dark	O_2, dark	N_2, 2,000 W/m²	N_2, 100 W/m²	N_2, dark	O_2, dark
Influx	43.2	7.0	0	6.2	8.8	5.9	1.8	24.4
Efflux	15.9	5.3	0	ND[b]	4.4	0.8	ND	12.9
Influx-efflux ratio	2.7	1.3	ND	ND	2.0	7.4	ND	1.9

[a] Strains were under the indicated conditions, with nitrogen or oxygen and with irradiation or in the dark.
[b] ND, Not determined.

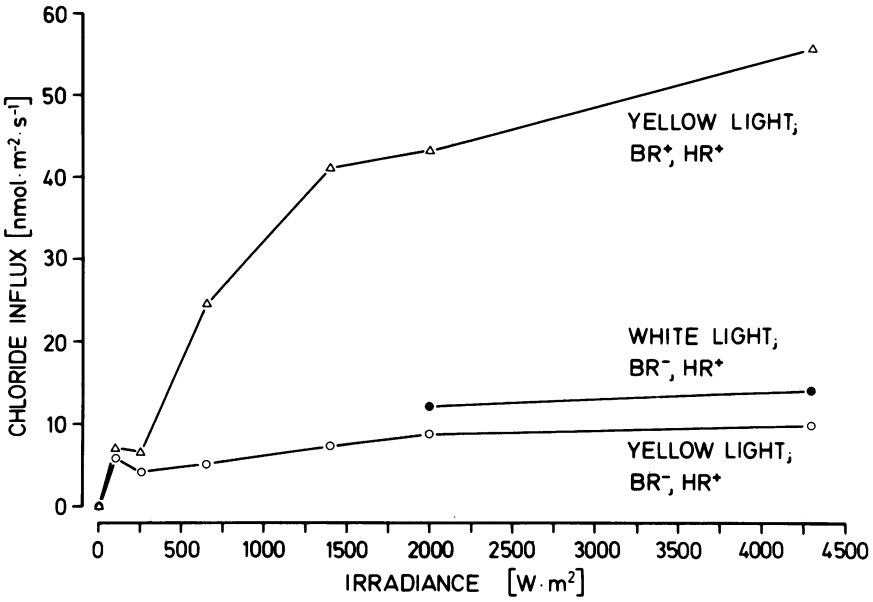

FIG. 2. Chloride influx in *H. halobium* as a function of irradiance with yellow light in M-1 (BR^+ HR^+) (△) and L-33 (BR^- HR^+) (○) cells and in response to white light in L-33 (BR^- HR^+) (●) cells. Experimental conditions are described in the legend to Fig. 1.

strain M-18 showed the lowest Cl^- influx despite having the highest concentration of BR (2.4 nmol of BR per mg of protein) of the strains tested (Fig. 3; see also Materials and Methods). Light had no additional or synergistic effect on respiration-driven chloride influx in the BR-deficient mutant strains L-33 and Flx-3. Similarly, oxygen had no measurable effect on light-driven chloride influx in wild-type cells and in the mutant strains M-1 and M-18, which contained BR. This does not appear to be a result of saturation: oxygen did not increase the rate of chloride influx at 2,000 W/m^2 in M-1 or L-33 cells (not shown), but an increased irradiance of 4,300 W/m^2 enhanced chloride transport rates by about 30 and 10% in M-1 and L-33 cells, respectively (Fig. 2). There is some kind of regulation in halobacterial chloride transport.

Cells in a state of enhanced chloride influx simultaneously released chloride (Table 1), as measured in efflux experiments of $^{36}Cl^-$-preloaded cells. Under low irradiance conditions of yellow light (100 W/m^2) in the HR-positive strain L-33, Cl^- influx exceeded Cl^- efflux by a factor of more than 7 (Table 1).

Driving force of secondary chloride uptake. Energy sources for the transport mechanism of chloride may be either ATP for a Cl^- ATPase, the proton motive force (pmf) for a proton-coupled symport mechanism, or $\Delta\psi$ for a symport mechanism coupled to one of the abundant cations in the salt

TABLE 2. Respiration-driven chloride influx, oxygen consumption, and ATP levels of five *H. halobium* strains tested under respiratory conditions in the dark

Strain	Pigmentation	Chloride influx (nmol/m^2 per s)	O_2 consumption (nmol/m^2 per s)	Cellular ATP concn (nmol/m^2)
L-33	BR^- HR^+	24.4	15.7	274.8
Flx-3	BR^- HR^-	18.1	16.1	258.7
Wild type	BR^+ HR^+	8.0	19.6	242.5
M-1	BR^+ HR^+	6.2	19.6	323.3
M-18	BR^+ HR^-	0	18.9	291.0

environment of this halophilic organism. As a first approach, the rate of chloride influx was compared with the cellular concentration of ATP (Table 2) and with pmf, ΔpH, and $\Delta\psi$ in intact cells of *H. halobium* (Fig. 4). The only correlation of chloride transport seen was with transmembrane electrical potential ($\Delta\psi$; Fig. 4).

DISCUSSION

HR-driven chloride uptake. Chloride accumulation in intact cells of mutant *H. halobium* strain L-33 (BR^- HR^+) under nonrespiratory conditions in light demonstrates the function of HR as a light-driven, inwardly directed chloride pump. The transport rates were found to be constant for more than an hour, in contrast to transient net proton uptake, which is correlated with a transient increase of pmf and evidently drives ATP synthesis (24). Only insignificant potassium influx (1.75 nmol/m^2 per s) could be shown in intact cells of mutant strain L-33 in light, but considerable sodium influx (10 nmol/m^2 per s) was found under the same conditions (A. Duschl, R Sawatzki, and G. Wagner, Eur. Bioenerg. Conf. [EBEC] Rep. 3:633–634, 1984). Net sodium import probably charge-balances HR-driven net chloride import in L-33 cells.

HR-mediated chloride transport was found to be easily light-saturated (Fig. 2). Under conditions of yellow light, strain L-33 showed transport rates of 5.9 nmol of Cl^- per m^2 per s at low light conditions (100 W/m^2), which were only moderately enhanced to 9.7 nmol of Cl^- per m^2 per s at high irradiance (4,300 W/m^2). White light was significantly more effective than yellow light, but showed similar kinetics of early light saturation (Fig. 2). This matches the recent findings of Hegemann and co-workers (8) that irradiating isolated HR with yellow light depletes the ground-state concentration of this pigment but enriches a long-living 410-nm-absorbing intermediate form. This intermediate, in turn, is photoelectrically inactive, but was backshifted to the ground state by blue or white light (8). Correspondingly, HR-mediated photophosphorylation in L-33 cells irradiated

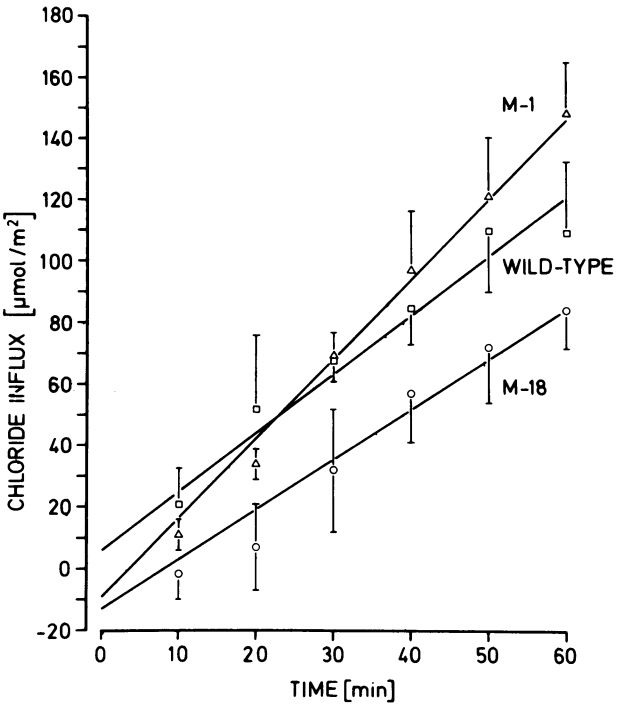

FIG. 3. BR-mediated chloride influx in strains M-1 (△), wild type (□), and M-18 (○) of *H. halobium*. Experimental conditions were the same as described in the legend to Fig. 1.

with white light compared with that in cells given the same irradiance of yellow light was more effective (G. Wagner, B. Traulich, K. M. Hartmann, and D. Oesterhelt, manuscript in preparation).

Sunlight intensity can be assumed to be at most 350 W/m^2 in the natural environment of the bacteria (7). HR thus appears to be a low-irradiance pigment, which acts effectively in the physiological range of light. Efflux experiments confirm this suggestion. Under high-irradiance conditions, about half of the apparent influx was due to exchange processes, while in the physiological light range there was almost no exchange of chloride (Table 1).

Respiration-driven chloride uptake. HR may be a supplementary system for an oxygen-driven chloride pump, like BR is supplementary to the respiratory chain. Therefore, *H. halobium* grows very well in the dark if provided with oxygen. We found respiration-driven chloride transport under suitable conditions in all strains tested. This effect was structurally independent of HR, as shown by the HR-deficient strains Flx-3 and M-18. The transport rates of chloride, however, differed widely. BR-containing strains showed a surprisingly small influx (0 to 8 nmol of Cl^- per m^2 per s) under respiratory conditions in darkness. This influx does not correlate with the rate of respiration determined by oxygen consumption or indirectly by the cellular level of ATP (Table 2). These results may indicate differences in the number or properties of the membrane translocation sites of chloride in *H. halobium*.

BR-coupled chloride influx. BR-mediated transport is clearly dependent on light intensity. Under the unphysiologically high irradiance of 4,300 W/m^2, we found extreme transport rates, up to 55.5 nmol of Cl^- per m^2 per s (Fig. 2). Under sunlight conditions, however, BR was rather ineffective relative to its high concentration (1.7 nmol/mg of protein) in the halobacterial membrane under our culture conditions.

A large part of the BR-mediated Cl^- transport is due to an exchange process, under both high- and low-irradiance conditions and under respiratory conditions. This exchange of chloride may be a side effect of BR or of another membrane transport protein. We conclude that under our experimental conditions, BR is not essential for net uptake. However, cells in the natural environment with low amounts of BR may show different behavior.

Driving force of secondary chloride uptake. The cellular ATP level under respiratory conditions was independent of the strain tested, in contrast to the chloride transport rates under these conditions (Table 2). Furthermore, the initial rate of photophosphorylation is saturated at light intensities

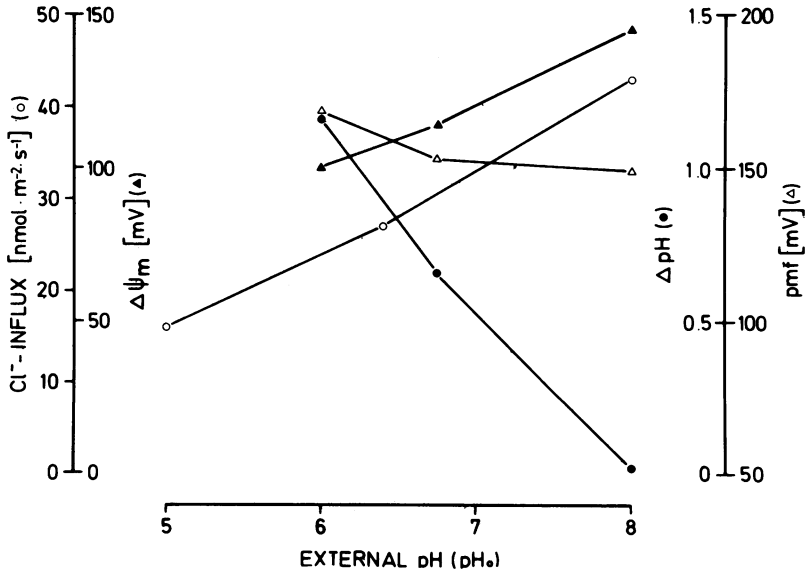

FIG. 4. Chloride influx in M-18 cells of *H. halobium* (○) as a function of the external pH (pH_0). The data are compared with Δψ (▲), ΔpH (●), and pmf (△) in M-1 cells (13).

of about 250 W/m^2 (7), while Cl^- uptake showed a steep increase in this range of irradiance (Fig. 2). These data do not suggest an ATP-dependent Cl^- transport.

A transport process coupled to an H^+ or OH^- gradient should correlate with ΔpH across the membrane. However, for example in *H. halobium* mutant strain M-18 in light, chloride influx increased only with decreasing ΔpH (Fig. 4). Our Cl^- transport measurements are also confirmed by results from G. Wagner and C. Kaiser (unpublished), showing that the Cl^- influx in strain M-1 under respiratory conditions in darkness increased with a decreasing proton gradient.

Evidently, chloride influx in *H. halobium* shows pH dependence similar to that of the membrane potential, $\Delta\psi$ (Fig. 4). The potassium ion is known to be transported in a uniport mechanism in response to $\Delta\psi$ (22). For unbound Mg^{2+} and SO_4^{2-}, no gradients have been reported so far, and other inorganic ions besides Na^+ are not components of the basal salt medium. We tentatively suggest that the membrane potential $\Delta\psi$ may energize secondary chloride uptake in *H. halobium* via a symport with Na^+, much as $\Delta\psi$ drives the uptake of amino acids (9, 12). The cellularly incorporated Na^+ is exported via the H^+/Na^+ antiport (10), and K^+ is accumulated by a uniport mechanism for Na^+ to charge-balance net chloride uptake. Further experiments will disprove or prove this hypothesis of the mechanism of secondary coupled chloride transport in *H. halobium*.

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