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A SECOND MECHANISM FOR SODIUM EXTRUSION IN HALOBACTERIUM HALOBIUM: A LIGHT-DRIVEN SODIUM PUMP

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SUMMARY

Membrane vesicles from a red mutant of Halobacterium halobium R_1 accumulate protons when illuminated causing the pH of the suspension to rise. Sodium is extruded from the vesicles and a membrane potential is formed. This potential and the proton uptake are abolished by valinomycin if K^+ is present. In contrast, Na^+-efflux is uninhibited by valinomycin even though no membrane potential is detectable and H^+ influx does not occur. Bis (hexafluoracetonyl)acetone (1799) stimulates proton uptake but does not abolish membrane potential. We propose that a light-dependent sodium pump is present. Passive proton uptake occurs in response to the electrical gradient created by this light-driven Na^+ pump in contrast to the active proton, and passive Na^+ flux that occurs in response to the light-driven proton pump described in vesicles of the parent strain of H. halobium R_1.

Cells and membrane vesicles of the salt-dependent organism Halobacterium halobium have been very useful in the elucidation of details on the mechanism of the conversion of light energy into ionic gradients and the role of these gradients in the transport of metabolites. An electrochemical gradient of Na^+ appears to be the driving force for the uptake of amino acids in the halophiles (1,2,3,4) and has been implicated in the transport of amino acids and sugars in many other organisms (5).

The role of the purple membrane protein, bacteriorhodopsin, in the formation of a proton gradient in whole cells of H. halobium membrane vesicles and reconstituted phospholipid vesicles has been documented (6,7). Lanyi and MacDonald (8) have described a H^+-Na^+ antiporter that exchanges protons for Na^+ in an electrogenic fashion. This exchange converts the light-derived

Abbreviations: $[^3H]TPMP^+$, $[^3H]$ triphenylmethylphosphonium bromide; DCCD, N,N'-dicyclohexyl-carbodiimide; 1799, bis(hexafluoracetonyl)acetone; Hepes, N-2-hydroxy-ethyl piperazine-N'-2-ethanesulfonic acid; $\Delta\Psi$, the electrical potential difference, CCCP, m-chlorocarbonylcyanide; SF6847, 3,5-di-tert-butyl-4-hydroxybenxylidenemalononitrile.

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proton gradient into a Na^+ gradient that can effectively drive the symport of a wide range of amino acids (4). Thus the circulation of protons and sodium in H. halobium appears to be linked: light providing the energy for the creation of an electrochemical proton gradient as the primary event.

In a recent report Matsuno-Yagi and Mukohata (9) described a red mutant of H. halobium which contains much less bacteriorhodopsin than the wild type strain. Illumination of cells of this mutant stimulates ATP synthesis but acidification of the medium does not occur as it does in the wild type. Instead there is a pH increase on illumination of suspensions of these cells. The action spectrum for this increase appears to be different from that for bacteriorhodopsin and the authors suggest that a bacteriorhodopsin different from that in the purple membrane may be present.

We have prepared membrane vesicles of this mutant and find that they also accumulate protons on illumination. Our data suggest that this uptake occurs in response to the negative membrane potential which is formed as the result of the light-driven efflux of Na^+.

Materials and Methods

Envelope membrane vesicles were prepared from cells grown on a defined medium as previously described (10). The strain of Halobacterium halobium used was described by Matsuno-Yagi and Mukohata (9) and was a generous gift of Dr. A. Matsuno-Yagi, Osaka University. All vesicle preparations and reaction solutions contained 1 mM Hepes, pH 6.2. Sodium and potassium content of vesicles and solvents was determined by atomic absorption: samples of vesicles were collected on membrane filters (Type EG, Millipore Corp) washed with 3.3 M KCl or 3.0 M NaCl and then extracted by soaking the filters in distilled water containing 40 $\mu g\ ml^{-1}$ Triton X-100. Protein concentrations were determined by the method of Lowry et al. (11) using crystalline eggwhite lysozyme (Sigma Chemical Corp.) as the standard.

Reaction mixtures were illuminated with Sylvania projector lamps (300W) as described previously (10). Light intensity was varied with neutral density filters and calibrated with a YSI-Kettering, Type 65A radiometer. All experiments were conducted at 30° C. Room light intensity was uniformly low. [^{3}H]TPMP$^+$, ^{22}NaCl, and H^{36}Cl were obtained from New England Nuclear Corp. [^{14}C]N-methylmorpholine was a gift from E. Berry, Cornell University. DCCD was obtained from Mann Research Laboratories and valinomycin from Sigma Chemical Corp. 1799 was a gift from Dr. P. Hytler of Dupont. NaCl and KCl were obtained from Mallinckrodt, Inc (reagent grade) or from Ventron Corp (ultra pure).

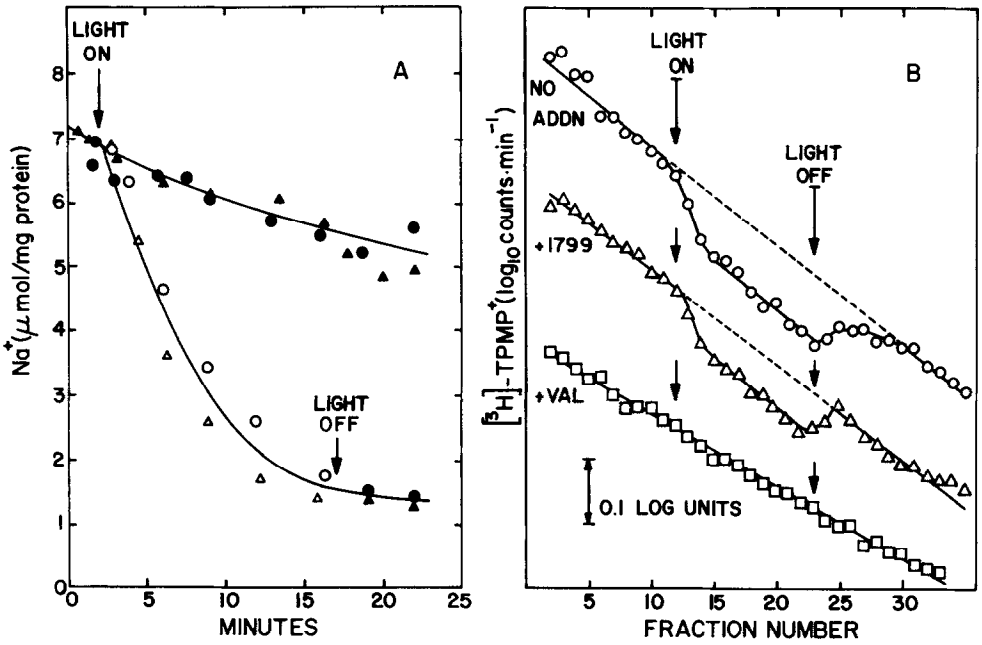

Fig. 1A. Light-stimulated efflux of $^{22}Na^+$. Vesicles loaded with $^{22}NaCl$ (10 mg ml^{-1} protein, 12.5 μCi ml^{-1}) were diluted 33-fold into 3 M NaCl 5 min prior to start. Na^+ efflux calculated from $^{22}Na^+$ remaining on membrane filter (Gelman GA-8) after filtering and washing a 100 μl aliquot. Light intensity 38 x 10^5 erg cm^{-2} sec^{-1}. Symbols: No additions, dark —●— , light —○— ; 10 μM 1799, dark —▲— light —△— .

Fig. 1B. Light-induced formation of ΔΨ measured by flow dialysis. Vesicles loaded with 3 M NaCl suspended in 1.5 M NaCl, 1.5 M KCl, 3 mg ml^{-1} protein. [^{3}H]TPMP$^+$ concentration 2.2 μM (2 μCi ml^{-1}). Flow buffer same as suspension buffer in cell similar to that described by Feldman (12) with Spectrapor 2 (Spectra Physics) dialysis membrane. 1.3 ml samples collected by drop counting. Flow rate 3 ml min^{-1}. 1799 (to 50 μM, final concentration) and valinomycin (to 100 μM) were added 1 min before [^{3}H]TPMP$^+$. Light intensity 25 x 10^5 ergs cm^{-2} sec^{-1}. Fraction 2 in each curve was 5000 cpm. Calculated membrane potentials from TPMP$^+$ distribution: No additions, -88 mV; with 1799, -79 mV, with valinomycin <-5 mV.

Results

When membrane vesicles derived from the "red mutant" strain of *H. halobium* (R-vesicles) are loaded with $^{22}Na^+$ and diluted into label-free 3 M NaCl, efflux of $^{22}Na^+$ occurs. This efflux is markedly stimulated by illumination as shown in Fig. 1A, and is not inhibited by 10 μM 1799 (or by 25 μM CCCP or 1 μM SF 6847, data not shown). This contrasts with the Na^+ efflux found in envelope membrane vesicles from the parent R_1 strain which is abolished by these uncouplers (1,3). In vesicles of this latter type, sodium efflux

occurs via a H^+-Na^+ antiporter (8) which exchanges internal Na^+ for external protons and is driven by the electrochemical proton gradient (protonmotive force) created by the bacteriorhodopsin proton pump. Thus, Na^+ efflux occurs whenever an electrochemical proton gradient is present which permits the "downhill" entry of protons. Conditions which abolish this gradient will abolish Na^+ efflux.

Illumination of R-vesicles generates a membrane potential (interior negative) as shown by the light-dependent uptake of $[^3H]TPMP^+$ (Fig. 1B), in addition to Na^+ efflux. This potential ($\Delta\Psi$), can result from the transmembrane movement of charges due either to sodium efflux as the primary light-driven event, or to the movement of some other ion as the primary event. In the former case the rate of Na^+ efflux would be driven directly by light energy and onl secondarily dependent on other ion movements. In the latter case Na^+ flux would be coupled electrically via an antiporter, as for example, the H^+-Na^+ antiporter previously described (8). Conditions which abolished electrochemical gradients of the coupling species would abolish Na^+ efflux.

As shown in Fig. 1A Na^+ efflux in R-vesicles is not inhibited by 1799, an uncoupler which abolishes electrochemical proton gradients. Membrane potential, as indicated by the ability of vesicles to accumulate the membrane permeant cation $TPMP^+$, is reduced but not abolished by 50 μM 1799 (Fig. 1B), but is abolished when external K^+ and valinomycin are present or when illumination is terminated. Sodium efflux is not inhibited by valinomycin even in the presence of K^+ and 1799 (data not shown). These observations indicate that the membrane potential developed by illuminated R-vesicles is not created by proton efflux alone via a proton pump.

Since these experiments can be carried out entirely in NaCl the membrane potential must be created by the transmembrane flux of either H^+, Na^+ or Cl^-. (0.01 mM potassium is present in 3 M NaCl, but it could not generate a $\Delta\Psi$ since illumination causes K^+ accumulation in these vesicles (data not shown)). Chloride uptake followed with $^{36}Cl^-$ in illuminated vesicles was not detected

under conditions in which extensive $^{22}Na^+$ efflux occurred and thus is unlikely to be responsible for formation of the $\Delta\Psi$. Proton efflux could generate a membrane potential of the magnitude observed particularly if proton return were coupled to Na^+ efflux, but as we have seen in Fig. 1A, Na^+ efflux is not abolished by uncouplers nor is the membrane potential (Fig. 1B). Sodium efflux rate and membrane potential both increase in proportion to light intensity (data not shown). Since it is possible to abolish this potential with valinomycin and K^+ without abolishing Na^+ efflux, Na^+ efflux cannot be occurring as a result of this potential. On the other hand $\Delta\Psi$ could be created by sodium efflux.

From these data we must conclude, either that these vesicles are not sensitive to uncouplers, or that the efflux of sodium in these vesicles is not energized by an electrochemical proton gradient.

The former possibility can be tested in the following way: if vesicles loaded with 3 M KCl are suspended 2.95 M NaCl, 0.05 M KCl in the dark, a diffusion potential will be created if the vesicles are permeable to potassium and should cause protons to be taken up if the membranes are also permeable to protons. As shown in Fig. 2A very little pH change occurs when vesicles loaded with KCl are added to 2.95 M NaCl, 0.05 M KCl because the membranes are relatively impermeable to K^+ and to H^+ (the slight change observed on addition of the vesicles can be attributed to small pH differences between the 3 M salt solutions). If 1799 (to 2 μM final concentration) is added, a small pH increase occurs since the membranes are now permeable to H^+ and respond to the small diffusion potential resulting from the limited K^+ permeability. The addition of valinomycin (to 1 μM) causes a large pH increase because now both K^+ and H^+ are freely permeable. This experiment repeated with the order of addition of uncouplers reversed has the same overall result. Increasing the concentrations of the uncouplers has no effect on the pH changes observed. This shows that both uncouplers are effective at concentrations well below those used in the experiments described in Fig. 1. It is clear

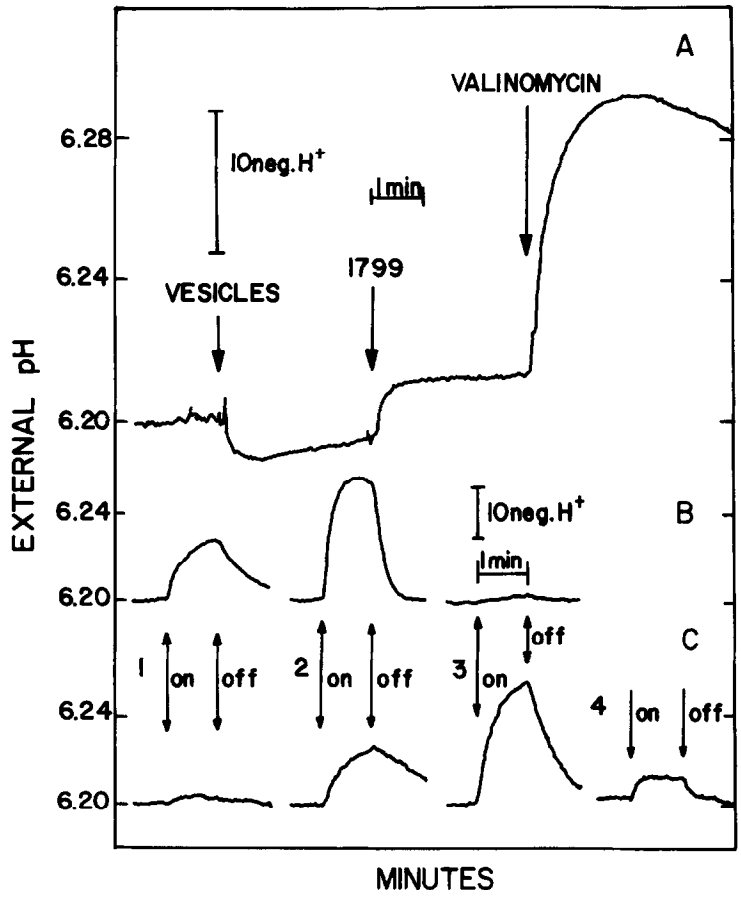

Fig. 2A. pH changes in R-vesicles in response to K^+-diffusion potential in the dark. Vesicles were loaded with 3 M KCl and added to 2.95 M NaCl, 0.05 M KCl pH 6.2. 1799 (to 2 µM) was added after 3 min and valinomycin (to 1 µM) after 6 min. Vesicle protein concentration 65 µg ml^{-1}. Traces represent pH electrode response.

Fig. 2B. Light-induced pH response. Vesicles were loaded with 3 M KCl and added to 1.5 M NaCl, 1.5 M KCl, protein concentration 80 µg ml^{-1}. >500 mM Na^+ inside of vesicles at time of first illumination (calculated with atomic absorption). Curve 1, no addition; Curve 2, 20 µM 1799; Curve 3, 10 µM valinomycin.

Fig. 2C. Light-induced pH response in presence and absence of sodium. Vesicles loaded with and suspended in ultrapure 3 M KCl, 80 µg ml^{-1} protein. 0.05 mM Na^+ inside vesicles at time of first illumination. Curve 1,no addition; Curve 2, 310 mM Na^+ added 20 min prior to illumination (internal Na^+ calculated to be 230 mM); Curve 3, as in curve 2, with 2 µM 1799, Curve 4, as in curve 2 with 100 µM DCCD and 2 µM 1799. Light intensity 25 x 10^3 ergs cm^{-2} sec^{-1} in B and C.

that 10 µM 1799 (Fig. 1A) does indeed make these membranes very permeable to protons, and therefore Na^+ efflux cannot be driven by an electrochemical proton gradient.

We conclude that the primary light response is sodium efflux and that the membrane potential observed is created by this flux. Proton uptake will occur passively in response to this potential particularly if an uncoupler such as 1799 is present to make the membranes more permeable to protons. Thus as shown in (Fig. 2B), when R-vesicles are illuminated, protons are taken up. If 1799 is present the proton uptake is greatly enhanced, but uptake is abolished if valinomycin is added since the membrane potential can not develop due to the free entry of K^+. We have determined the pH gradient developed on illumination in the presence of 50 μM 1799 (with 3 M NaCl inside and outside of the vesicles) to be 0.95 pH units, using [^{14}C]N-methylmorpholine as the probe for intravesicle pH (data not shown). Thus the pH increase observed in the medium may be accounted for by a pH decrease inside of the vesicles.

As shown in Fig. 2C, proton uptake does not occur if Na^+ is absent from the reaction mixture. (The small pH increase seen in the absence of any added sodium is presumably due to the small amount of sodium present even in the ultra pure KCl used). Addition of Na^+ to these vesicles causes a great stimulation in proton uptake after a period of incubation to allow Na^+ entry (>200 mM Na^+ internal, determined by atomic absorption at time of illumination). 1799 increases the Na^+-dependent uptake of protons but 100 μM DCCD inhibits it. The mechanism of inhibition by DCCD is not understood but also occurs in vesicles containing only 3 M NaCl (data not shown).

Discussion

The evidence presented suggests that the mutant strain of H. halobium R_1 described by Matsuno-Yagi and Mukohata (9) creates an electrochemical sodium gradient by a means that has been undetected previously: a light-dependent Na^+ pump.

Light-driven efflux of Na^+ has two immediate consequences: 1) a substantial membrane potential is generated, and 2) protons are taken into the vesicles in passive response to the $\Delta\Psi$. Sodium efflux can be separated from the movement of H^+, since when free entry of H^+ is faciliated by the

uncoupler, 1799, an electrochemical gradient of protons cannot exist to serve as a driving force for other ion movements, but Na^+ efflux is not inhibited. A membrane potential is not required for the extrusion of Na^+ as demonstrated by the substantial Na^+ efflux that still takes place after the addition of valinomycin which in the presence of K^+ abolishes $\Delta\Psi$.

In agreement with the observations of Matsuno-Yagi and Mukohata in whole cells (9) we also observe that the action spectrum for the alkaline response in vesicles is red-shifted some 20 nm from the bacteriorhodopsin peak at 570 nm. It is obvious that a chromophore is involved in this function and could be similar to the retinal of the purple membrane but further speculation is unwarranted.

The phenomenon of Na^+ circulation in H. halobium is clearly more complex than previously thought. The red mutant strain provides a simple system for the demonstration of the light-dependent Na^+ pump although we have recently prepared a fraction of membrane vesicles from H. halobium R_1 that is enriched in a light-dependent Na^+ pump that is remarkably similar to the pump described here (manuscript in preparation).

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