FEBS 15128 FEBS Letters 359 (1995) 255–258

Electrogenic Na^+ transport by *Enterococcus hirae* Na^+-ATPase

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Received 28 December 1995

Abstract **Energy-dependent generation of a membrane potential ($\Delta\psi$) (−45 mV, interior negative) was observed in the F_0F_1, H^+-ATPase-defective mutant of *Enterococcus hirae*. The generation of $\Delta\psi$ was found at high pH (but not at low pH), for which intracellular Na^+ was required but not extracellular K^+. The $\Delta\psi$-generating activity was induced in cells cultured in media containing high concentrations of Na^+, and was not observed in the Na^+-ATPase mutants. These results suggest that *E. hirae* Na^+-ATPase is responsible for the electrogenic sodium pump.**

Key words: Na^+-ATPase; Membrane potential; *Enterococcus hirae*

1. Introduction

The Gram-positive bacterium *Enterococcus hirae*, which lacks the respiratory chain, has two sodium extrusion systems: Na^+/H^+ antiporter [1,2] and Na^+-translocating ATPase [3]. Na^+/H^+ antiporter functions to extrude Na^+ from the cytoplasm at low pH where the proton potential is generated by F_0F_1, H^+-ATPase, and Na^+-ATPase functions to extrude Na^+ at high pH where generation of the proton potential is minimal [4]. In the early work on Na^+-ATPase, it was speculated that Na^+-ATPase functions electroneutrally and may exchange Na^+ for H^+ [3]. Later Kakinuma and Harold reported the Na^+-ATPase-dependent K^+ uptake (KtrII) activity in whole cells; an apparently equimolar exchange of internal Na^+ for external K^+ took place in the absence of proton potential [5]. This Na^+/K^+ exchange could be explained in two ways: (i) a direct Na^+/K^+ exchange by the Na^+-ATPase or (ii) the combined effect of two separate pumps: the Na^+-ATPase and the K^+ pump [4,6].

Our recent biochemical and molecular biological studies have shown that the expected structure of *E. hirae* Na^+-ATPase resembles those of vacuolar (V)-type H^+-ATPases distributed in various organisms [7–9]; the two major subunits (NtpA of 69 kDa and NtpB of 52 kDa) of *E. hirae* Na^+-ATPase are homologous counterparts of V-ATPases [8]. Very recently we found a 16 kDa proteolipid subunit in the *E. hirae* Na^+-ATPase complex, the amino acid sequence of which is similar to those of various 17 kDa proteolipid subunits of eukaryotic V-type H^+-ATPases [10]. The V-type H^+-ATPases so far reported in various organisms are all electrogenic proton pumps [11]; the 17 kDa proteolipid subunits of eukaryotic V-ATPases are considered as the electrogenic proton pathway.

In this report, we examined whether the Na^+-ATPase works as the electroneutral Na^+/K^+ exchanger or the electrogenic Na^+ pump. In a *E. hirae* H^+-ATPase mutant, we observed the generation of a membrane potential (interior negative) which was totally dependent on both the internal Na^+ and the activity of Na^+-ATPase, but not on the external K^+. *E. hirae* Na^+-ATPase may be an electrogenic Na^+ pump.

2. Materials and methods

2.1. Strains andMedia

E. hirae ATCC 9790 or mutants derived from this strain were used: these were strain 7683, a mutant defective in sodium extrusion, and its revertants R-I and R-II [3], and mutant AS25, which is defective in F_0F_1-ATPase and in proton extrusion [12]. Cells were grown on complex media [5], NaTY (1% tryptone, 0.5% yeast extract, 1% glucose and 0.85% Na_2HPO_4) or KTY (tryptone, yeast extract and glucose as above, and 1% K_2HPO_4 in place of Na_2HPO_4). In some experiments, sodium chloride was added to the culture in KTY medium to induce the Na^+-ATPase activity [4]. In order to induce the arginine deiminase pathway [13], cells were grown on NaTY medium containing 1% arginine and 0.1% galactose instead of 1% glucose.

2.2. Preparation of the cation-loaded cells

The Na^+-loaded cells were prepared by the monactin method as described previously [14]. The choline-loaded cells were prepared by incubating the Na^+-loaded cells in a buffer containing 50 mM Tris-chloride, 400 mM choline chloride, and 10 mM glucose at pH 8.5 for more than 70 min at room temperature [5].

2.3. Measurement of the membrane potential

The membrane potential was calculated from the accumulation of [^{14}C]tetraphenylphosphonium ion (TPP^+). The Na^+-loaded cells were suspended in 50 mM Na^+-*N*-tris(hydroxymethyl)methylglycine (Tricine) buffer (pH 8.6) at a density of 1 mg (dry weight) per ml, and [^{14}C]TPP^+ (10 μM, 18.5 MBq/mmol) was added to the cell suspension. At intervals, aliquots were collected by filtration on Millipore filters (pore size 0.45 μm) and were washed twice with 2 mM $MgSO_4$. The radioactivity of the filters were counted by a liquid scintillation counter. The cellular water space was considered to be 1.75 μl/mg dry weight.

2.4. Others

Preparation of membrane vesicles and assay of sodium-stimulated ATPase activity in the presence of 0.5 mM *N,N'*-dicyclohexylcarbodiimide (DCCD) were performed as described previously [5]. [^{14}C]TPP^+ was purchased from NEN Research Products; other reagents were of analytical grade.

3. Results

3.1. Generation of membrane potential by a H^+-ATPase mutant at high pH

As this bacterium lacks the respiratory chain, the $\Delta\psi$ (interior negative) is usually generated by electrogenic proton extrusion via the H^+-ATPase, the activity of which is maximal at around pH 7 [15]. It has been reported that a $\Delta\psi$ of about −120 mV is generated at pH 7.5 in the wild-type strain 9790 [16]; with Na^+-loaded 9790 cells suspended in a buffer containing 50 mM Na^+, a $\Delta\psi$ of about −60 mV was generated in the absence of glucose, and $\Delta\psi$ was further increased to −120 mV by addition of glucose. Fig. 1 shows the generation of $\Delta\psi$ in strain AS25, which is defective in H^+-ATPase activity and generation of the

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SSDI 0014-5793(95)00056-9

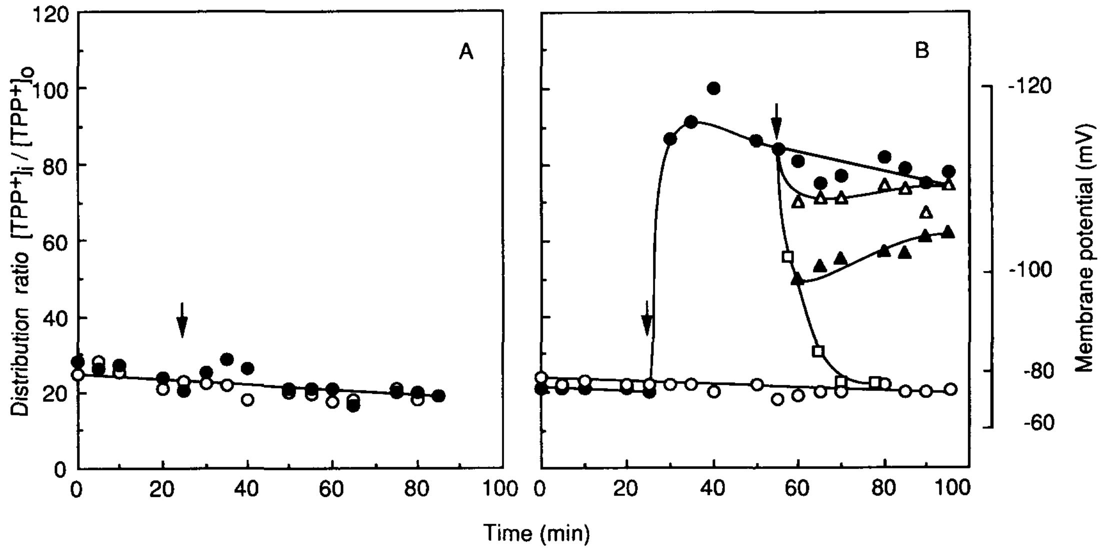

Fig. 1. Generation of a membrane potential in a F_0F_1-ATPase mutant. Mutant AS25 was grown on NaTY medium, loaded with Na^+ by the monactin method [14], and suspended in 50 mM Na^+-Tricine buffer at pH 7.0 (A) or at pH 8.6 (B). After the addition of 0.5 mM DCCD at 0 min, [^{14}C]TPP^+ uptake was measured as described in section 2. As indicated by the arrows, 10 mM glucose (●), 10 μM TCS (△), 0.5 mM KCl (▲) or 5 mM KCl (□) was added at 25, 55, 55 or 55 min, respectively.

proton potential [12]. Cells were cultured in NaTY medium, and the assays were performed with Na^+-loaded cells suspended in 50 mM Na^+-Tricine buffer. At pH 7.0, glucose-dependent generation of $\Delta\psi$ was negligible in this H^+-ATPase mutant (Fig. 1A). At pH 8.6, on the other hand, a $\Delta\psi$ of −45 mV (interior negative) was generated by addition of glucose (Fig. 1B), although the H^+-ATPase is genetically defective and DCCD, an inhibitor of H^+-ATPase, was included in the assay buffer. With arginine-adapted AS25 cells in which the arginine deiminase pathway was induced (see section 2) a $\Delta\psi$ of about −50 mV was also generated by addition of 10 mM arginine (data not shown), suggesting that ATP is probably the common energy donor of the $\Delta\psi$ generation. The generation of $\Delta\psi$ was only slightly inhibited by addition of a protonophore such as tetrachlorosalicylanide (TCS), but K^+ collapsed the $\Delta\psi$ at high concentrations (Fig. 1B). These results suggested that ATP-driven generation of $\Delta\psi$, which is not coupled with proton movement, occurred at high pH.

3.2. *Intracellular Na^+ is required for generation of membrane potential*

When the experiment was performed with the choline-loaded AS25 cells suspended in Tris-Tricine buffer (pH 8.6) (Fig. 2A), glucose-dependent generation of $\Delta\psi$ was small. However, the generation of $\Delta\psi$ by the choline-loaded cells was increased to about −40 mV by addition of 25 mM Na^+ to the reaction buffer (Fig. 2B). The generation of $\Delta\psi$ (about −45 mV) was also observed in Na^+-loaded AS25 cells suspended in a buffer free of Na^+ (50 mM Tris-Tricine buffer, pH 8.6) (Fig. 2C), suggesting that internal Na^+ is required for ATP-driven generation of

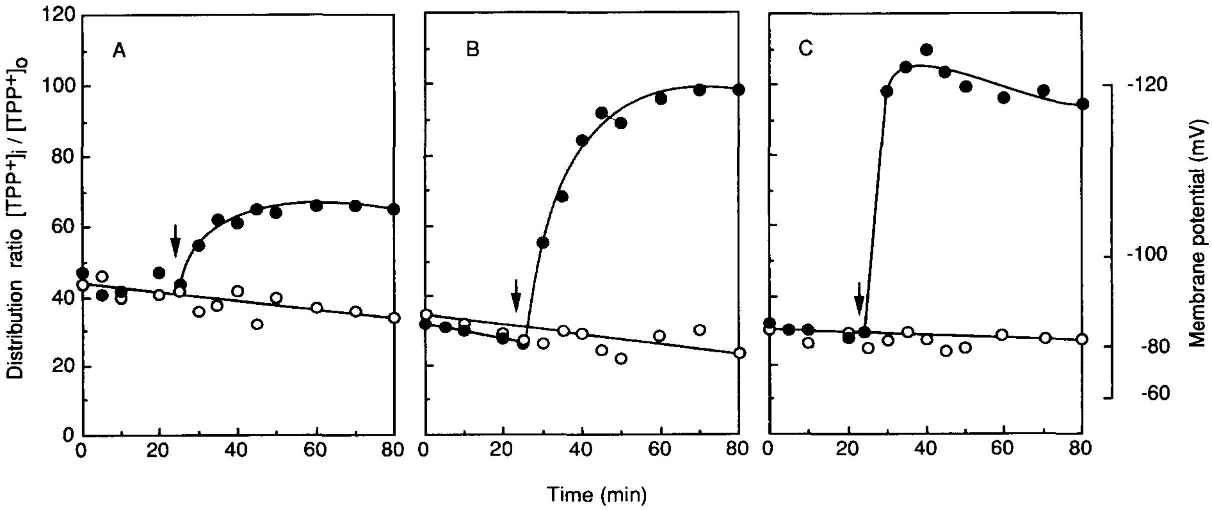

Fig. 2. Sodium dependence of generation of a membrane potential. Mutant AS25 was grown on NaTY medium, and loaded with Na^+ or choline as described in section 2. [^{14}C]TPP^+ accumulation was measured in choline-loaded cells suspended in 50 mM Tris-Tricine buffer (pH 8.6) (A), choline-loaded cells suspended in Tris-Tricine buffer containing 25 mM Na^+-Tricine (B), and Na^+-loaded cells suspended in 50 mM Tris-Tricine buffer (C). Glucose (10 mM) was added at 25 min.

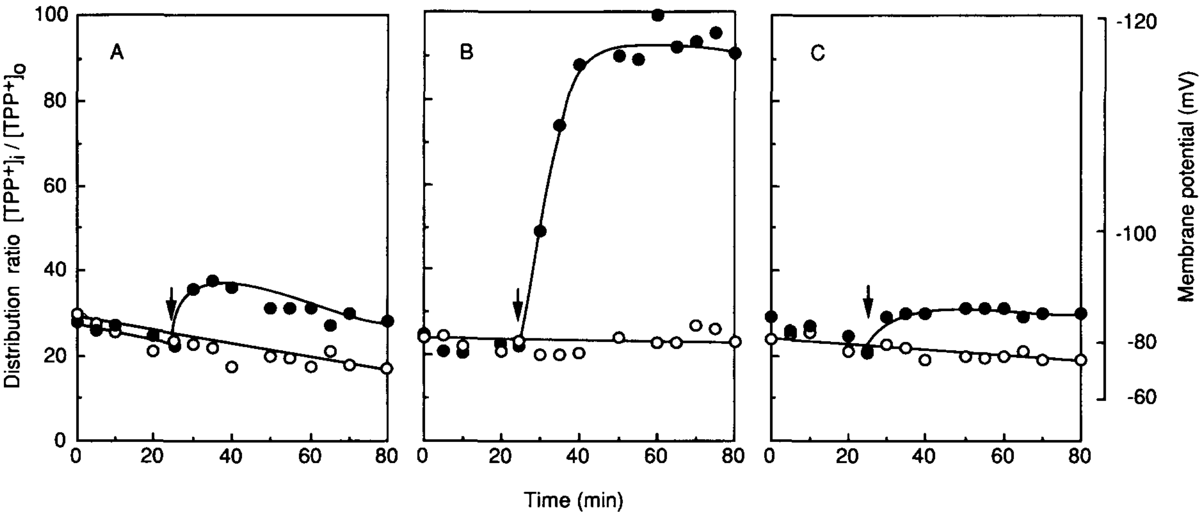

Fig. 3 Inducibility of generation of a membrane potential. Mutant AS25 was grown on KTY medium (A) or KTY medium containing 0.2 M NaCl (B). Chloramphenicol (100 μg/ml) was added together with 0.2 M NaCl at A_{600} = 0.1 to the culture, and cells were harvested at A_{600} = 0.6 (C). The Na^+-loaded cells were prepared by the monactin method and [^{14}C]TPP^+ accumulation was assayed as described in the legend of Fig. 1. Glucose (10 mM) was added at 25 min.

$\Delta\psi$ at high pH. The generation of $\Delta\psi$ was minimal by the K^+-loaded AS25 cells suspended in 50 mM K^+-Tricine buffer (pH 8.6) (data not shown).

3.3. Generation of membrane potential by Na^+-ATPase

E. hirae Na^+-ATPase functions to extrude Na^+ from the cytoplasm at alkaline pH [4]. It is most probable that the ATP-driven $\Delta\psi$ generation observed in the above sections results from extrusion of sodium ions via the Na^+-ATPase, since the $\Delta\psi$ generation was observed at high pH (Fig. 1) and depended on the internal Na^+ (Fig. 2). Glucose-dependent generation of $\Delta\psi$ was examined with several Na^+-ATPase mutants in the presence of 0.5 mM DCCD (Table 1). Mutant 7683 lacks Na^+ extrusion activity; that is, both the Na^+-ATPase and Na^+/H^+ antiporter are defective [17]. R-I is a revertant of 7683, which had recovered only the activity of the Na^+/H^+ antiporter, while R-II is another revertant that had recovered both Na^+ extrusion activities [13]. Glucose-dependent $\Delta\psi$ generation of −41 mV, which was resistant to DCCD, was generated in the parent strain 9790, but it was not observed in 7683. Generation of $\Delta\psi$ was not recovered in R-I but was in R-II. These results indicate that the Na^+-ATPase is essential for the generation of $\Delta\psi$ under our conditions.

As *E. hirae* Na^+-ATPase is induced in high-Na^+ medium [4,6], the effect of culture conditions on the generation of $\Delta\psi$ was also examined (Fig. 3). Generation of $\Delta\psi$ at pH 8.6 was minimal in cells grown on KTY medium which contained about 15 mM Na^+ (Fig. 3A), but it increased remarkably in cells grown on high Na^+ medium (KTY medium containing 0.2 M NaCl) (Fig. 3B). However, when chloramphenicol (100 μg/ml) was added to KTY medium together with NaCl, the generation of $\Delta\psi$ was limited (Fig. 3C). The Na^+-stimulated ATPase activities of the membranes prepared from these cells were 0.01 (KTY medium), 0.15 (KTY medium containing 0.2 M NaCl) and 0.02 (the same medium in the presence of chloramphenicol) μmol/min/mg protein, respectively. From these results we propose here that the inducible *E. hirae* Na^+-ATPase transports sodium ions electrogenically.

Table 1
Generation of a membrane potential by Na^+-ATPase mutants

Strain	Generation of the membrane potential (mV)		
	− Glucose	+ Glucose	Glucose-dependent
9790	78 ± 5	119 ± 7	41
7683	78 ± 4	78 ± 8	0
R-I	80 ± 8	84 ± 9	4
R-II	81 ± 11	121 ± 9	40

Cells were grown on NaTY medium, loaded with Na^+ by the monactin procedure, and suspended in 50 mM Na^+-tricine buffer (pH 8.6). After the preincubation for 10 min with 0.5 mM DCCD, the membrane potential was assayed by [^{14}C]TPP^+ accumulation as shown in Fig. 1. The values are in means ± S.D.

4. Discussion

We observed here the Na^+-ATPase-dependent generation of $\Delta\psi$ in intact *E. hirae* cells. As Na^+-ATPase activity is optimal at pH 8.5–9.0 [4], the $\Delta\psi$ was generated at pH 8.6 but not at pH 7.0 (Fig. 1). One decade ago, the direct Na^+/K^+ exchange model was considered the simplest hypothesis for Na^+-ATPase-dependent K^+ uptake (KtrII) [5]. However, we should reconsider this model of *E. hirae* Na^+-ATPase, since K^+ is probably not essential for the Na^+-ATPase reaction. First, the Na^+-ATPase-dependent generation of $\Delta\psi$ was observed under the experimental conditions where potassium ions are free; less than 0.1 mM K^+ may be contaminated. Second, the generation of $\Delta\psi$ was not stimulated by the external K^+ (Fig. 1B). At the high concentration, K^+ dissipated the $\Delta\psi$ (Fig. 1B). Thus K^+ is not obligatorily coupled with Na^+-ATPase. This ATPase may uniport Na^+; analogous to other V-type ATPases [11], the NtpK proteolipid subunit of the Na^+-ATPase is the candidate for the electrogenic Na^+-penetrating pathway. Although the molecular mechanism of the KtrII system is still unknown, the direct exchange model of the Na^+-ATPase should be withdrawn. It is noteworthy that $\Delta\psi$, although small, was generated by choline-loaded AS25 cells suspended in a buffer free from

Na^+ (Fig. 2A). This $\Delta\psi$ was not observed in the presence of protonophore (data not shown), suggesting that it results from proton transport. It may be possible that the Na^+-ATPase also transports H^+ under non-physiological conditions [18,19]. Purification of reconstitutively active enzyme is important for further investigation of the mechanism of Na^+-ATPase and it is now in progress.

Skulachev has pointed out the importance of Na^+ circulation for bacterial physiology [20]. We have observed the generation of a Na^+ electrochemical potential of about −100 mV in growing cells in NaTY medium at high pH [4]. Although only the KtrII activity has been reported to be Na^+-dependent [5], the generation of the Na^+ potential by means of the Na^+-ATPase should be utilized for the survival of *E. hirae* at high pH. In order to understand the significance of Na^+ circulation in *E. hirae* it is important to investigate the interplay of many Na^+-coupled systems.

Acknowledgements: We thank I. Yamato for critical reading of the manuscript. This work was supported in part by grant-in-aids for Scientific research (to Y.K.) from the Ministry of Education, Science, and Culture of Japan.

References

[1] Kakinuma, Y. (1987) J. Bacteriol. 169, 3886–3890.
[2] Waser, M., Hess-Bienz, D., Davies, K. and Solioz, M. (1992) J. Biol. Chem. 267, 5396–5400.
[3] Heefner, D.L. and Harold, F.M. (1982) Proc. Natl. Acad. Sci. USA 79, 2798–2802.
[4] Kakinuma, Y. and Igarashi, K. (1988) J. Bioenerg. Biomemb. 21, 679–692.
[5] Kakinuma, Y. and Harold, F.M. (1984) J. Biol. Chem. 260, 2086–2091.
[6] Kakinuma, Y. (1993) in: Alkali Cation Transport Systems in Prokaryotes (Bakker, E.P. ed.) pp. 277–290, CRC Press, Boca Raton, FL.
[7] Kakinuma, Y. and Igarashi, K. (1990) FEBS Lett. 271, 97–101.
[8] Takase, K., Yamato, I. and Kakinuma, Y. (1993) J. Biol. Chem. 268, 11610–11616.
[9] Takase, K., Kakinuma, S., Yamato, I., Konishi, K., Igarashi, K. and Kakinuma, Y. (1994) J. Biol. Chem. 269, 11037–11044.
[10] Kakinuma, Y., Kakinuma, S., Takase, K., Konishi, K., Igarashi, K. and Yamato, I. (1993) Biochem. Biophys. Res. Commun. 195, 1063–1069.
[11] Nelson, N. (1992) Biochim. Biophys. Acta 1100, 109–124.
[12] Kobayashi, H. and Unemoto, T. (1980) J. Bacteriol. 143, 1187–1193.
[13] Heefner, D.L. and Harold, F.M. (1980) J. Biol. Chem. 255, 11396–11402.
[14] Harold, F.M. and Baarda, J.R. (1968) J. Bacteriol. 95, 816–823.
[15] Kobayashi, H., Murakami, N. and Unamoto, T. (1982) J. Biol. Chem. 257, 13246–13252.
[16] Bakker, E.P. and Harold, F.M. (1980) J. Biol. Chem. 255, 433–440.
[17] Harold, F.M., Baarda, J.R. and Pavlasova, E. (1970) J. Bacteriol. 101, 152–159.
[18] Laubinger, W. and Dimroth, P. (1989) Biochemistry 28, 7194–7198.
[19] Dibrov, P.A., Skulachev, V.P., Sokolov, M.V. and Verkhovskaya, M.L. (1988) FEBS Lett. 233, 355–358.
[20] Skulachev, V.P. (1985) Eur. J. Biochem. 151, 199–208.