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# A RESPIRATION-DEPENDENT PRIMARY SODIUM EXTRUSION SYSTEM FUNCTIONING AT ALKALINE PH IN THE MARINE BACTERIUM VIBRIO ALGINOLYTICUS

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SUMMARY

The membrane potential generated at pH 8.5 by $K^+$-depleted and $Na^+$-loaded Vibrio alginolyticus is not collapsed by proton conductors which, instead, induce the accumulation of protons in equilibrium with the membrane potential. The generation of such a membrane potential and the accumulation of protons are specific to $Na^+$-loaded cells at alkaline pH and are dependent on respiration. Extrusion of $Na^+$ at pH 8.5 occurs in the presence of proton conductors unless respiration is inhibited while it is abolished by proton conductors at acidic pH. The uptake of α-aminoisobutyric acid, which is driven by the $Na^+$-electrochemical gradient, is observed even in the presence of proton conductors at pH 8.5 but not at acidic pH. We conclude that a respiration-dependent primary electrogenic $Na^+$ extrusion system is functioning at alkaline pH to generate the proton conductor-insensitive membrane potential and $Na^+$ chemical gradient.

It became possible to prepare bacterial cells loaded with various monovalent cations by the treatment with diethanolamine-buffered salt solution (1). Using this method, we have shown the roles of $K^+$ and $Na^+$ in energetics of the marine bacterium Vibrio alginolyticus that requires $Na^+$ as well as $K^+$ for growth. $K^+$ was found to be necessary for the generation of pH-dependent $\Delta\Psi$ and $\Delta pH$ (1). $K^+$ was also required for the generation of $Na^+$ chemical gradient ($\Delta pNa^+$) which, together with $\Delta\Psi$, functions as a direct driving force for AIB uptake (manuscript submitted).

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Abbreviations: CCCP, carbonyl cyanide m-chlorophenylhydrazone; HQNO, 2-heptyl-4-hydroxyquinoline-N-oxide; $TPP^+$, tetraphenyl phosphonium ion; AIB, α-aminoisobutyric acid; Asc/TMPD, ascorbate plus N,N,N',N'-tetramethyl-p-phenylenediamine; MES, 2-(N-morpholino)ethanesulfonic acid; Tricine, Tris(hydroxymethyl)methylglycine; $\Delta\Psi$, membrane potential; $\Delta pNa^+$, the concentration gradient of $Na^+$ across the membrane; $\Delta pH$, the pH difference across the membrane; $\Delta p$, the electrochemical potential of $H^+$, defined as $\Delta\Psi - 59\Delta pH$; TCS, tetrachlorosalicyl anilide.

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Energetics in $Na^+$-loaded cells were further studied in this paper and it was revealed that V. alginolyticus extrudes $Na^+$ by two energetically different mechanisms. The one driven by the electrochemical potential of $H^+$ ($\Delta p$) is mediated by a $Na^+/H^+$ antiport system. On the other hand, we found another system which functions at alkaline pH and is independent of $\Delta p$. The examination of the generation of $\Delta\Psi$ and $\Delta pH$ and the extrusion of $Na^+$ at alkaline pH indicated that the latter system is a respiration-dependent primary electrogenic $Na^+$ extrusion mechanism.

## MATERIALS AND METHODS

Vibrio alginolyticus 138-2 grown aerobically on a defined medium (1) was harvested at the late logarithmic phase of growth.

$K^+$-depletion and $Na^+$-loading of cells were performed as described (1) with slight modifications; cells were treated twice at 25°C for 10 min with 50 mM diethanolamine-HCl, pH 8.5, containing 0.4 M NaCl and then washed twice with 0.4 M NaCl without buffer. $K^+$ and $Na^+$ were determined by atomic absorption after the extraction of cells with 5% TCA. For the determination of $Na^+$, cells were filtered (Millipore, type EH) and washed with 0.4 M choline chloride prior to the extraction. Intracellular concentrations of $K^+$ and $Na^+$ were found to be less than 3 mM and about 0.4 M, respectively. Protein was determined by the method of Lowry et al. (2) using bovine serum albumin as a standard.

Flow dialysis was performed as described (1) with a sample flow rate of 1 ml per min. Radioactivity was continuously monitored by Flow-One radioactivity monitor (Radiomatic Instruments and Chemical Co., Tampa, FL, USA) using liquid scintillator (flow rate; 4 ml per min) for $^3H$ or solid scintillator for $^{14}C$. Counts accumulated for one minute were printed out.

Extrusion of $Na^+$ and uptake of AIB were determined at 25°C by filtration (Millipore, type EH). Filters were washed twice with 2 ml of 0.4 M NaCl.

[$^3$H]$TPP^+$ was a generous gift from H.R.Kaback, Roche Institute of Molecular Biology. $^{22}NaCl$, [$^{14}$C]methylamine and [$^3$H]AIB were obtained from New England Nuclear. CCCP was purchased from Sigma Chemical Co. MES and Tricine were products of Nakarai Chemical Co.

## RESULTS

In previous paper (1), we have shown that $K^+$ is required for the generation of pH-dependent $\Delta\Psi$ (negative inside) and $\Delta pH$ (alkaline inside) using $K^+$-depleted and $Na^+$-loaded V. alginolyticus. $\Delta\Psi$ in such cells, measured by flow dialysis using $TPP^+$, was completely sensitive to 10 μM CCCP, a proton conductor, at pH 6.0 to 7.0 as shown in Fig. 1A (open circles) where $\Delta\Psi$ of -152 mV was detected in the absence of $K^+$ at pH 6.0. Strikingly, however, $\Delta\Psi$ generated at alkaline pH was only slightly and transiently collapsed by the addition of CCCP (Fig. 1A, closed circles). $\Delta\Psi$'s of -163 and -156 mV were calculated before and 15 min after the addition of CCCP, respectively. Neither higher concentrations of

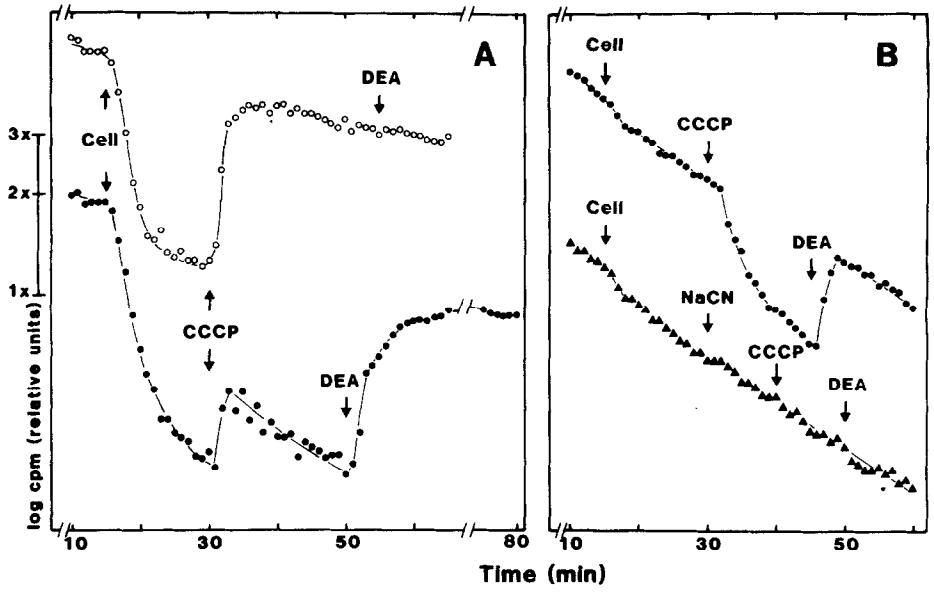


Fig. 1. Generation of ΔΨ (A) and ΔpH (B) by $K^+$-depleted and $Na^+$-loaded cells. ΔΨ (inside negative) and ΔpH (inside acidic) were determined from the distribution of [$^3$H]TPP$^+$ (19 μM) and [$^{14}$C]methylamine (20 μM), respectively, by flow dialysis in 50 mM of either MES-Na, pH 6.0, (open circles) or Tricine-Na, pH 8.5, (closed circles and triangles) containing 0.4 M NaCl and 20 mM glycerol. Experiments were started by the addition of radioactive probe at 0 time. Additions were made as specified in figures to give final concentrations of 1.76 mg/ml for cell protein, 10 μM for CCCP, 50 mM for diethanolamine (DEA)-HCl (pH was adjusted so as to cause little pH change) and 2 mM for NaCN. Diethanolamine was added at pH 6.0 after the collapse of ΔΨ for a control. Counts at 10 min in each curve were 2000 cpm in A and 6000 cpm in B.

CCCP nor TCS, another proton conductor, collapsed ΔΨ at pH 8.5. The CCCP-insensitive ΔΨ was collapsed by the subsequent addition of membrane permeable weak base diethanolamine (Fig. 1A, closed circles). In the absence of proton conductor, however, the addition of diethanolamine had no effect on ΔΨ. Although not shown, $Na^+$-loaded cells treated with NaCN or HQNO could not generate CCCP-insensitive ΔΨ. However, the addition of artificial electron donor system, Asc/TMPD, to the cells treated with HQNO led to the generation of CCCP-insensitive ΔΨ. These results and the observation that oxygen uptake due to Asc/TMPD was insensitive to HQNO indicate that the respiration is essential to the generation of CCCP-insensitive ΔΨ.

The generation of such a proton conductor-insensitive ΔΨ was specific to $Na^+$-loaded cells. ΔΨ in $K^+$- or $Li^+$-loaded cells determined in the absence of

$Na^+$ at pH 8.5 was considerably smaller than that in $Na^+$-loaded cells and the addition of $Na^+$ to such cells caused a significant increase in the magnitude of $\Delta\Psi$. When $K^+$ was present in the assay system, the extent of transient collapse of $\Delta\Psi$ in $Na^+$-loaded cells by the addition of CCCP at pH 8.5 was enhanced depending on the concentration of $K^+$. We have reported that CCCP caused a complete release of accumulated $TPP^+$ over the pH range of 6.5 to 8.5 in the presence of 10 mM $K^+$ (1). The effect of CCCP, however, was transient at alkaline pH (above 7.5) and considerable magnitude of $\Delta\Psi$ was again generated (unpublished results).

The generation of $\Delta$pH (inside acidic) was measured at pH 8.5 using a weak base methylamine (pK 10.7) by flow dialysis. Although the addition of $Na^+$-loaded cells caused a little decrease in the radioactivity in dialysate, the addition of CCCP (Fig. 1B, closed circles) or TCS (not shown) clearly induced the accumulation of methylamine. $\Delta$pH was calculated to be 2.2 units at 15 min after the addition of CCCP. This value, -135 mV as a chemical potential of protons, was in near equilibrium with the magnitude of $\Delta\Psi$ (-156 mV) obtained after the addition of CCCP (Fig. 1A, closed circles). Thus, $\Delta p$ in the presence of CCCP was only -21 mV. These results indicate that the membrane of $Na^+$-loaded cells at pH 8.5 was sensitive to the action of CCCP. The CCCP-induced $\Delta$pH was collapsed by the addition of high concentrations of permeable weak bases such as diethanolamine (Fig. 1B, closed circles) or ethanolamine (not shown). Once $\Delta$pH is collapsed by diethanolamine, a bulk influx of protons mediated by CCCP becomes possible. This may be the reason for the collapse of $\Delta\Psi$ by diethanolamine (Fig. 1A, closed circles). In the presence of 2 mM NaCN (Fig. 1B, triangles) or 10 $\mu$M HQNO (result not shown), the subsequent addition of CCCP did not induce the generation of $\Delta$pH. However, the addition of Asc/TMPD caused the generation of CCCP-dependent $\Delta$pH even in the presence of HQNO. Neither $Li^+$- nor $K^+$-loaded cells generated CCCP-dependent $\Delta$pH. Furthermore, under all conditions tested, no accumulation of acetylsalicylic acid, a probe for $\Delta$pH (alkaline inside), was detected. These results strongly

suggest that $\Delta\Psi$ observed in the presence of CCCP at pH 8.5 is generated by the extrusion of cations other than protons.

$Na^+$-loaded cells equilibrated with $^{22}Na^+$ on ice were transferred to 25°C and an active extrusion of $^{22}Na^+$ from cells was determined by filtration (Fig. 2A and B). The extrusion of $Na^+$ was observed over the pH range of 6.0 to 8.5 and required $K^+$ as a counter ion permitting overall electroneutrality. The extrusion of $Na^+$ at acidic pH (Fig. 2A) was almost completely inhibited by 10 µM CCCP suggesting that the driving force for $Na^+$ extrusion is $\Delta p$. On the other hand, CCCP (Fig. 2B) or TCS (not shown) had little effect on the generation of $\Delta pNa^+$ at alkaline pH. The addition of 2 mM NaCN (Fig. 2B, open triangles) or 10 µM HQNO (not shown) inhibited the extrusion of $Na^+$. These results indicate that

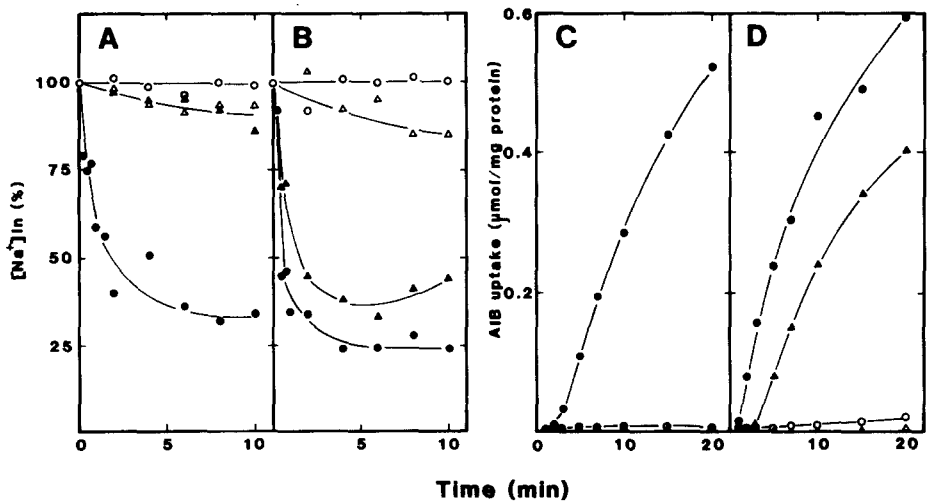


Fig. 2. Respiration-dependent extrusion of $^{22}Na^+$ (A and B) and uptake of AIB (C and D). The experiments were carried out in 50 mM of either MES-Na, pH 6.5, (A and C) or Tricine-Na, pH 8.5, (B and D) containing 0.4 M NaCl. Cells were preincubated for 3 min at 25°C in the presence of 20 mM glycerol and with or without 2 mM NaCN prior to the start of experiments. KCl and CCCP were added at 0 time as final concentrations of 10 mM and 10 µM, respectively. The level of $^{22}Na^+$ and the amount of AIB retained by cells were determined at given times by filtering and washing an aliquot (50 µl) of cell suspension. $^{22}Na^+$ extrusion; cells (3.02 mg protein per ml) equilibrated with $^{22}Na^+$ ($2.6 \times 10^4$ cpm/µl) on ice were used. Values shown are corrected for background radioactivity due to non-specific binding to filters and given in per cent of radioactivity at 0 time. AIB uptake; the experiments were started by the addition of [$^3$H]AIB (0.1 mM, 20 µCi/µmol) to cell suspension (0.11 mg protein per ml) at 0 time. In C, the amount of AIB uptake shown by symbols other than closed circles are all less than 0.01 µmoles per mg cell protein.
Symbols: ○ , No additions; ● , KCl; ▲ , KCl+CCCP; △ ,NaCN+KCl+CCCP.

the $Na^+$ extrusion observed at alkaline pH is driven by a primary electrogenic mechanism which is somehow coupled to respiration.

V. alginolyticus requires both $\Delta\Psi$ and $\Delta pNa^+$ for the active uptake of AIB. Therefore, from the results described above, the transport of AIB is expected to be insensitive to CCCP at alkaline pH but not at acidic pH. Fig. 2C and D clearly show that this is indeed the case. CCCP gave a complete inhibition at pH 6.5 (Fig. 2C), where neither $\Delta\Psi$ nor $\Delta pNa^+$ was present. On the other hand, CCCP did not abolish the uptake at pH 8.5 (Fig. 2D), where both $\Delta\Psi$ and $\Delta pNa^+$ existed. $K^+$ was required as a counter ion for the generation of $\Delta pNa^+$ and NaCN caused a complete inhibition of AIB uptake at both pH values. The lag observed in the uptake corresponded to the time required for the generation of critical magnitude of $\Delta pNa^+$ (manuscript submitted).

## DISCUSSION

The results presented in this paper strongly suggest that the possible existence of a primary electrogenic $Na^+$ extrusion system ($Na^+$ pump) in the marine bacterium V. alginolyticus. The mechanism functioning at alkaline pH is dependent on respiration and is specific to $Na^+$.

$\Delta\Psi$ generated by the $Na^+$ pump is not collapsed by proton conductors which make membrane completely permeable to protons. As a result, $\Delta pH$ (inside acidic) is formed which is just balanced by $\Delta\Psi$ and makes $\Delta p$ near zero. Therefore, the collapse of $Na^+$ pump-dependent $\Delta\Psi$ by an influx of protons becomes possible only in the presence of both proton conductor and permeable weak base which, dissipating $\Delta pH$, allows a bulk influx of protons. Although the activity of $Na^+$ pump is coupled to respiration, at present, the direct driving force for $Na^+$ pump is not known. The observation that Asc/TMPD induced the generation of CCCP-insensitive $\Delta\Psi$ and CCCP-dependent $\Delta pH$ in the presence of HQNO suggests the intimate coupling of $Na^+$ pump to the respiratory chain. In any event, it is obvious that V. alginolyticus possesses two different mechanism for the generation of $\Delta pNa^+$; one is driven by $\Delta p$, hence this is a secondary transport system and consistent with the concept of $Na^+/H^+$ antiport system (3,4), and another is the primary

$Na^+$ pump. Results in our hands suggest that considerable portions of $\Delta\Psi$ and $\Delta pNa^+$ at pH 8.5 are generated by the primary $Na^+$ extrusion mechanism.

Halobacterium halobium also extrudes $Na^+$ by either a $Na^+/H^+$ antiport system (5), which utilizes $\Delta p$ generated by bacteriorhodopsin, or a $Na^+$ pump, halorhodopsin (6), which has a pH optimum at alkaline region (7). The existence of such a primary $Na^+$ pump may give some advantages to cells living in salt-rich environments and especially at alkaline pH.

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