Respiration-Driven Na⁺ Pump and Na⁺ Circulation in 

*Vibrio parahaemolyticus*

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Sodium circulation in *Vibrio parahaemolyticus* was investigated. We observed respiration-driven Na⁺ extrusion from cells by using a Na⁺ electrode. The Na⁺ extrusion was insensitive to a proton conductor, carbonyl cyanide *m*-chlorophenylhydrazone, and sensitive to a respiratory inhibitor, CN⁻. These results support the idea of the existence of a respiratory Na⁺ pump in *V. parahaemolyticus*. The respiration-driven Na⁺ extrusion was observed only under alkaline conditions.

The respiratory chain plays an important role in membrane energetics. It transfers electrons and extrudes H⁺, thus establishing an electrochemical potential difference of H⁺, proton motive force, across the membrane (10). The proton motive force is the driving force for ATP synthesis in H⁺-translocating ATPase, for the active transport of many substrates, and for other membrane processes (10). Thus, H⁺ circulation is crucial for membrane energetics in systems ranging from microbial cells to the mitochondria of animal, plant, and yeast cells. Another cation which is involved in membrane energetics is Na⁺. An electrochemical potential difference of Na⁺ is a driving force for some transport systems in several bacteria (9) and for flagellar movement in halophilic *Bacillus* spp. (7). In many bacterial cells, Na⁺ is extruded via the Na⁺-H⁺ antiporter (8). The driving force for the Na⁺ extrusion is the proton motive force in this system. Therefore, Na⁺ extrusion via the Na⁺-H⁺ antiporter is secondary transport. Recently, however, Tokuda and Unemoto have found a respiration-driven primary Na⁺ pump in a marine bacterium, *Vibrio alginolyticus* (16, 17). It has been reported that the Na⁺ pump functions at alkaline pH, and the pump is not inhibited by a proton conductor, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). Very recently, Tokuda and Unemoto determined the site of Na⁺ extrusion to be the NADH:quinone oxidoreductase segment of the respiratory chain (18). It should be noted that H⁺ is also extruded at other segments of the respiratory chain of this organism (17, 18). It seems that both Na⁺ and H⁺ circulation play important roles in energizing membrane processes in this organism. Since marine organisms live in a Na⁺-rich environment, Na⁺ circulation might be generally of great importance for their lives. If this is the case, the primary Na⁺ pump may be present in other marine organisms and halophilic organisms.

We have investigated Na⁺ transport in *Vibrio parahaemolyticus*, which lives in seawater and is frequently isolated from seawoods. This organism is a major cause of food poisoning in Japan (11). Therefore, investigation of this organism is also important from a clinical point of view. In this paper we describe a respiratory Na⁺ pump and Na⁺ circulation in *V. parahaemolyticus*. The respiratory Na⁺ pump was also observed in several other species of *Vibrio*.

**MATERIALS AND METHODS**

**Bacteria and growth.** The *Vibrio* species *V. parahaemolyticus* AQ 3334, *V. alginolyticus* ATCC 17749, *V. campbellii* ATCC 25920, *V. proteolyticus* ATCC 15338, and *V. cholerae* NCTC 4716 were used in this study. Cells were grown aerobically at 30°C (*V. campbellii*) or 37°C (others) in a basal medium (1) consisting of 50 mM Tris-hydrochloride (pH 7.4), 50 mM MgSO₄, 10 mM KCl, 10 mM CaCl₂, 0.33 mM K₂HPO₄, and 0.1 mM FeSO₄ supplemented with 1% polypeptide. NaCl was added to the medium at 0.2 M for *V. parahaemolyticus*, *V. alginolyticus*, *V. campbellii*, and *V. proteolyticus* or at 0.1 M for *V. cholerae*. Cells were harvested at late-exponential phase of growth.

**Preparation of membrane vesicles.** Cells were washed twice with 10 mM MOPS (4-morpholinepropanesulfonic acid)-Tris buffer, pH 7.0, containing 0.3 M choline chloride and 2 mM MgSO₄ and suspended in the same solution (5 mg/l of wet cells). The cell suspension was passed through a French pressure cell (1.500 kg/cm²), unbroken cells were removed by centrifugation, and membrane vesicles were pelleted by ultracentrifugation (13). The vesicles were suspended in the same buffer as described above (50 mg of protein per ml), an equal volume of glycerol was added, and the membranes were frozen in liquid nitrogen and stored at −80°C until used.

**Measurement of Na⁺ transport.** The cells were washed three times with 0.2 M MOPS, adjusted to pH 7.5 with tetramethylammonium hydroxide (TMAH), and suspended in the same buffer to ca. 50 mg of cell protein per ml. Transport of Na⁺ in cells was measured with a Na⁺ electrode (Radiometer, Copenhagen, Denmark) as described previously (20).

Na⁺-H⁺ antiport. The activity of the Na⁺-H⁺ antiporter was measured by fluorescence quenching (14) of quinacrine, with an excitation wavelength of 420 nm and an emission at 500 nm.

**NADH oxidase.** NADH oxidase was assayed with an oxygen electrode at 28°C. The assay mixture contained 10 mM *N*(2-hydroxy-1,1-bis(hydroxymethyl)-ethyl)-glycine (tricine)-TMAH buffer, pH 8.5 (or 10 mM MOPS-TMAH buffer, pH 7.0), 0.3 M choline chloride, 2 mM MgSO₄, and 0.25 mg of protein from membrane vesicles. NADH (disodium salt) was added at 0.5 mM. Thus, at least 1 mM Na⁺ was present in all assays.

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RESULTS

Effect of Na⁺ on respiration. NADH oxidation in membrane fractions of V. alginolyticus and Vibrio costicolus is stimulated by Na⁺ (21, 22). Similarly, 10 to 100 mM Na⁺ stimulated NADH oxidation (O₂ consumption induced by the addition of NADH) in the membrane vesicles of V. parahaemolyticus (Fig. 1). At pH 8.5, K⁺ slightly stimulated the respiration, and Li⁺ had no effect. Thus the stimulation seems to be fairly specific to Na⁺. Stronger stimulation of NADH oxidation by Na⁺ was observed at pH 7.0 than at pH 8.5. Both K⁺ and Li⁺ showed some stimulatory effect at pH 7.0. The stimulated NADH oxidase activity was higher at pH 7.0 than at pH 8.5 in the presence of all three cations, although the activity measured under a low salt concentration (1 mM Na⁺) was the same at pH 7.0 and 8.5.

Na⁺ extrusion driven by respiration. We tested Na⁺ extrusion driven by respiration in V. parahaemolyticus under anaerobic conditions. Respiration was started by adding H₂O₂ to the cell suspension. It should be noted that V. parahaemolyticus possesses catalase (23), and O₂ was immediately produced when H₂O₂ was supplied (data not shown). The addition of a small amount of H₂O₂ elicited Na⁺ extrusion (Fig. 2). After ca. 1.5 min, Na⁺ uptake by the cells occurred. The addition of a larger amount of H₂O₂ resulted in a longer period of Na⁺ extrusion, followed by Na⁺ influx (data not shown). These results indicate that Na⁺ extruded by respiration reentered the cells when respiration stopped, which suggests that Na⁺ was extruded against its concentration gradient and that it reentered cells down its concentration gradient. The respiration-driven Na⁺ extrusion was insensitive to a proton conductor, CCCP (Fig. 2). Therefore, a proton motive force is not involved in the Na⁺ extrusion. This fact eliminates the possibility of a Na⁺-H⁺ antiport mechanism for the Na⁺ extrusion observed in V. parahaemolyticus. We previously reported that Na⁺ extrusion was elicited by respiration in Escherichia coli (19). However, the Na⁺ extrusion was completely inhibited by CCCP in that case. The Na⁺ extrusion in E. coli is believed to occur via the Na⁺-H⁺ antiporter (2, 14, 19, 24). Thus, the mechanisms of Na⁺ extrusion driven by respiration in V. parahaemolyticus and E. coli are different. An inhibitor of the respiratory chain, CNO⁻, completely inhibited the Na⁺ extrusion, as expected. We also tried to supply O₂ by adding O₂-saturated water or ethanol, and we observed some Na⁺ extrusion (Fig. 2). Ethanol, however, had an unfavorable effect on the Na⁺ electrode; namely, ethanol changed the background reading. The addition of H₂O₂ to the assay mixture in the absence of cells did not have any effect on the Na⁺ electrode. Thus, the most convenient procedure of supplying O₂ was to add H₂O₂.

About 30 ng of Na⁺ were extruded from cells (15 mg of protein) at the maximal level. However, if intracellular Na⁺ was the same as that of the external medium (100 mM) under nonenergized conditions, the total amount of intracellular Na⁺ was calculated to be ca. 5 ng of ions. In this calculation, the intracellular water space was assumed to be 3.3 μl/mg of cell protein (17). Therefore, it is likely that a high concentration of Na⁺ was present in the cells, with the ions perhaps bound to cellular constituents.

The effect of the external Na⁺ concentration on the respiration-driven Na⁺ extrusion was examined. It should be noted that we can measure Na⁺ transport with a Na⁺ electrode over a certain concentration range of Na⁺ because of the sensitivity of the electrode and the extent of Na⁺ transport by cells. We observed Na⁺ extrusion at concentrations of Na⁺ between 1 μM and 1 mM, with increasing initial velocity (Fig. 3). The largest change in electromotive force (measured with an ion meter) was observed when the Na⁺ concentration was ca. 100 μM. Therefore, standard assays were performed at this Na⁺ concentration. An apparent Kᵣ for Na⁺ was calculated to be 0.3 mM, and Vₘₐₓ was calculated to be 22 ng of Na⁺ per min per mg of cell protein.
The effect of pH on Na⁺ extrusion was investigated (Fig. 4). No Na⁺ extrusion was detected at pH 7.0. Na⁺ extrusion driven by respiration was observed only at alkaline pHs, in agreement with the results with *V. alginolyticus* (16, 17). The pH optimum of the Na⁺ extrusion was 8.5.

Similar CCCP-insensitive, respiration-driven Na⁺ extrusion was detected with a Na⁺ electrode under alkaline conditions in *V. alginolyticus*, where the respiratory Na⁺ pump was first discovered with 22Na⁺ (16, 17). *V. campbellii*, *V. proteolyticus*, and *V. cholerae* were all tested, and each species exhibited similar Na⁺ extrusion (data not shown).

**Na⁺-H⁺ antiporter.** The Na⁺-H⁺ antiporter has been found in many biological membranes (8). This transport system is important for conversion of the proton motive force to a sodium motive force, which is the driving force for redundant processes such as Na⁺-substrate cotransport in cells not possessing primary Na⁺ pumps. Also, the antiporter is important for the removal of Na⁺ from cytoplasm and for the regulation of intracellular pH (12). Since *V. parahaemolyticus* possesses a respiratory Na⁺ pump, the Na⁺-H⁺ antiporter may not be necessary in this organism.

The antiporter activity was measured as a fluorescence change of quinacrine in a French pressure cell produced everted membrane vesicles (14). The addition of ATP resulted in fluorescence quenching, which indicated the formation of a pH gradient, with the interior acidic. The addition of Na⁺ to the assay mixture elicited a reversal of fluorescence intensity, which indicated a reduction of the pH gradient and supported the idea of the existence of a Na⁺-H⁺ antiporter (Fig. 5). The antiporter activity was higher at alkaline pHs than at neutral pH (data not shown). We also detected Ca⁺²-H⁺ antiport activity. It was not clear whether or not a K⁺-H⁺ antiporter existed (Fig. 5). The results also indicate that an H⁺-translocating ATPase is present in the cell membrane of *V. parahaemolyticus*. Although we tried to establish a pH gradient energized by NADH oxidation in everted membrane vesicles, we could not detect the gradient by fluorescence measurements. The reason for this failure is not clear at present.

**Na⁺-amino acid cotransport.** Recent studies have revealed Na⁺-substrate cotransport in several microorganisms (9). α-Aminoisobutyric acid transport is driven by a chemical gradient of Na⁺ in *V. alginolyticus* (15) and in *V. costicola* (4). If Na⁺-substrate cotransport exists, then Na⁺ uptake will be induced by the influx of the cotransport substrate. Uptake of Na⁺ was induced by serine (Fig. 6), but not by proline, α-aminoisobutyric acid (Fig. 6), glutamate, or alanine (data not shown). We conclude that at least Na⁺-serine cotransport is present in *V. parahaemolyticus*.
Na+ TRANSPORT IN V. PARATHAEMOLYTICUS

The results presented in this paper indicate the existence of a respiration-driven Na+ pump which extrudes Na+ from cells in V. parahaemolyticus. This fact means that the respiratory Na+ pump, which was originally found in V. alginolyticus (16, 17), is not exceptional for this organism. Furthermore, the respiration-driven, CCCP-insensitive Na+ extrusion was also detected in three other Vibrio species. Vibrio spp. may generally possess a respiratory Na+ pump. Furthermore, we have observed respiration-driven Na+ extrusion in the presence of CCCP in Aeromonas hydrophila (T. Tsuchiya and S. Shinoda, unpublished data), which is a member of the family Vibrionaceae (1). It is possible that other marine microorganisms or even some nonmarine microorganisms possess the respiratory Na+ pump. It seems important to check such activity in many organisms.

The properties of the Na+ pump present in V. parahaemolyticus seem to be similar to those of V. alginolyticus (16, 17). For example, the extrusion of Na+ was observed only under alkaline conditions with an optimum pH at 8.5. Although we observed no Na+ extrusion driven by respiration at pH 7.0, we cannot exclude the possibility that the pump may function even at neutral pH, because stimulation of respiration by Na+ was observed at pH 7.0. The apparent Km for Na+ of the respiratory Na+ pump was 0.3 mM, and the stimulation of respiration was seen at 10 to 100 mM Na+. Unfortunately, we could not measure Na+ extrusion at such high Na+ concentrations. It is possible that a higher Km value and perhaps a second system would be found if we could measure Na+ extrusion at a higher Na+ concentration. Similarly, although the Vmax was calculated to be 22 ng of Na+ per min per mg of cell protein, much stronger Na+ extrusion might occur at high Na+ concentration in marine Vibrio spp., which live in a Na+-rich environment.

We did not detect CCCP-sensitive Na+ extrusion driven by respiration at any pH tested in V. parahaemolyticus. It has been reported that the Na+-H+ antiporter seemed to be the only way to extrude Na+ at acidic pH in V. alginolyticus (18). Furthermore, in E. coli the CCCP-sensitive Na+ extrusion was clearly demonstrated, presumably via a Na+-H+ antiport (19). However, the Na+-H+ antiporter is obviously present in V. parahaemolyticus, as shown by the fluorescence assays. It seems that the Na+ extrusion activity of this organism via the antiporter is rather low. Therefore, the main pathway of Na+ extrusion seems to be the respiratory Na+ pump. We observed higher activity of the antiporter under alkaline conditions than under neutral conditions. This result seems reasonable from the viewpoint of pH regulation. Cells have to take up more H+ under alkaline conditions to maintain intracellular pH. If the Na+-H+ antiporter is involved in this process, it should function more strongly at more alkaline pHs.

The cotransport of Na+ and serine was observed in V. parahaemolyticus. Na+-amino acid cotransport has also been reported in other Vibrio species (4, 15). As described above, many Vibrio species possess a respiratory Na+ pump, which establishes an electrochemical potential difference of Na+ (18). Therefore, Na+ circulation will play a central role in the active transport of nutrients in Vibrio spp. similar to the case of Halobacterium halobium (9).

Furthermore, it seems possible that some other system(s) may be driven by Na+ in Vibrio spp. It has been reported that the motility of V. alginolyticus is supported by an artificially imposed Na+ gradient (3). In addition, a Na+-translocating ATPase has been reported in Streptococcus faecalis (5) and Propionigenium modestum (6). Thus, a search for a Na+-translocating ATPase in Vibrio spp. seems to be interesting. Moreover, generally, an investigation of the physiological role of Na+ circulation would be of value. In particular, there might be a relationship between the Na+ circulation and the halophilicity of Vibrio spp.

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LITERATURE CITED


