Na⁺(Li⁺)/H⁺ Antiporter in *Pseudomonas aeruginosa* and Effect of Li⁺ on Cell Growth

Kei INABA,^a Junko UTSUGI,^a Teruo KURODA,^a Masaaki TSUDA,^a and Tomofusa TSUCHIYA^{*,a,b}

Department of Microbiology, Faculty of Pharmaceutical Sciences,^a and Gene Research Center,^b Okayama University, Tsushima, Okayama 700, Japan. Received January 20, 1997; accepted February 24, 1997

Everted membrane vesicles were prepared from cells of *Pseudomonas aeruginosa*, and cation/H⁺ antiport was measured. We observed activities of Na⁺/H⁺ antiport, Li⁺/H⁺ antiport and K⁺/H⁺ antiport. Judging from the competition pattern, it seems that there are at least two types of antiporter, a Na⁺(Li⁺)/H⁺ antiporter and a K⁺/H⁺ antiporter. Na⁺ was a good substrate for the Na⁺(Li⁺)/H⁺ system, whereas Li⁺ was a poor substrate. Although the *K_m* value for Na⁺ (or Li⁺) was similar to those in *Escherichia coli* Na⁺/H⁺ antiporters, the *V_{max}* value for Na⁺ (or Li⁺) was much smaller in the *P. aeruginosa* antiporter than in the *E. coli* antiporters. Growth of *P. aeruginosa* was strongly inhibited by 0.4 M LiCl, but not by NaCl or KCl.

Key words antiporter; lithium; sodium; amiloride; *P. aeruginosa*

The Na⁺/H⁺ antiporter is widely distributed in membranes from microbial cells to animal cells. This system transports Na⁺ in exchange for H⁺. In all of the known Na⁺/H⁺ antiporters, Li⁺ is a good substrate for this system, and is transported in exchange for H⁺. In microbial cells, the antiporter has several roles such as, 1) establishment of an electrochemical potential of Na⁺ across membranes, which is the driving force for Na⁺-coupled active transport, Na⁺-coupled flagellar rotation, and so on; 2) extrusion of Na⁺ (or Li⁺) which is toxic to cells when present at high concentrations in cells; 3) intracellular pH regulation under alkaline conditions, and 4) regulation of cell volume.

Pseudomonas aeruginosa has active transport systems which are driven by an electrochemical potential of Na⁺. Previously, we reported that a branched-chain amino acid transport system LIV-II utilizes Na⁺ or Li⁺ as the coupling ion.¹⁾ This organism is able to survive under considerably high NaCl conditions in the growth medium. Thus, Na⁺ must be actively extruded from cells. Also, *P. aeruginosa* can grow under alkaline conditions (up to pH 10 or above). These facts suggest that this microorganism possesses Na⁺(Li⁺)/H⁺ antiporter(s).

MATERIALS AND METHODS

Materials Quinacrine hydrochloride was from Nakarai Chemical Co. Amiloride was from Sigma Chemical Co. (St. Louis, Mo. U.S.A.). Other chemicals were of reagent grade and obtained from commercial sources.

Bacterial Cells and Growth *P. aeruginosa* PAO1 was used. For the measurement of cell growth, cells were grown in L broth²⁾ (NaCl was replaced with KCl) supplemented with various concentrations of LiCl, NaCl or KCl at 37 °C under aerobic conditions. Cell growth was monitored turbidimetrically at 650 nm.

Preparation of Membrane Vesicles Cells of *P. aeruginosa* PAO1 were grown in a minimal medium³⁾ supplemented with 40 mM potassium lactate at 37 °C under aerobic conditions. Cells were harvested at the late exponential phase of growth. Everted membrane vesicles were prepared by passing the cells through a French press cell.⁴⁾

Measurement of Na⁺/H⁺ Antiporter Activity The activity of the Na⁺/H⁺ antiporter was measured by the quinacrine fluorescence quenching method.⁵⁾ pH of the assay mixture was 8.0 unless otherwise indicated.

RESULTS AND DISCUSSION

A convenient method for measuring activity of the Na⁺/H⁺ antiporter is to use everted membrane vesicles and the fluorescence quenching technique.^{6,7)} Thus, we prepared everted membrane vesicles by passing *P. aeruginosa* cells through a French press cell to disrupt them.⁴⁾ Since the pressure of the French press cell is an important factor in preparing suitable everted membrane vesicles from bacterial cells,⁴⁾ we prepared these vesicles by disrupting cells at various pressures. We investigated the relationship between the pressure of the French press cell and the degree of fluorescence quenching in the prepared vesicles caused by H⁺ transport into the vesicles due to respiration. At pressures between 1200 and 1500 kg/cm² we obtained vesicles that showed the largest quenching %, which represents formation of the largest pH gradient across the membrane (data not shown). Measurement of the Na⁺/H⁺ antiport is easier when the quenching is large. At lower pressures, the quenching % was lower. Therefore, membrane vesicles used in this study were prepared at 1500 kg/cm².

The decrease in the fluorescence intensity (quenching) caused by addition of the respiratory substrate (lactate) shown in Fig. 1 indicates acidification of the intravesicular space. This is strong evidence that the vesicles are everted.⁸⁾ Recovery of the fluorescence intensity (dequenching) after the addition of Na⁺, Li⁺ or K⁺ indicates H⁺ movement from the vesicles to the assay medium. Thus, the vesicles showed Na⁺/H⁺ antiport, Li⁺/H⁺ antiport and K⁺/H⁺ antiport. It is known in *Escherichia coli* that there are two major Na⁺/H⁺ antiporters (NhaA and NhaB) and one major K⁺/H⁺ antiporter (Kha or KefC).^{6,9–11)} Both Na⁺ and Li⁺ are substrates for all three antiporters in *E. coli*.⁶⁾ The data shown in Fig. 1 are consistent with the notion that *P. aeruginosa* also possesses both a Na⁺/H⁺ antiporter and a K⁺/H⁺ antiporter. From the competition pattern (Fig. 1), it seems that both Na⁺ and Li⁺ are

* To whom correspondence should be addressed.

Fig. 1. Cation/ H^+ Antiport Activities in Membrane Vesicles

Quinacrine fluorescence was measured in the assay mixture containing 10 mM Tricine-KOH (pH 8.0), 140 mM KCl, 1 μ M quinacrine and membrane vesicles (200 μ g protein) at 25°C. Quinacrine fluorescence quenching (downward deflection) caused by addition of lactate (L) (5 mM) and dequenching (upward deflection) caused by addition of NaCl (Na) (5 mM), LiCl (Li) (5 mM) or KCl (K) (5 mM) in membrane vesicles were recorded.

Fig. 2. pH Profiles of Na^+/H^+ Antiport Activity and Li^+/H^+ Antiport Activity

The Na^+/H^+ antiport activity (●, ▲) and Li^+/H^+ antiport activity (○, △) were measured as in the legend for Fig. 1 at indicated pHs, and the activities were evaluated by measuring dequenching caused by addition of NaCl (5 mM) or LiCl (5 mM). The buffers used were either Mops-KOH (triangles) or Tricine-KOH (circles).

substrates for the Na^+/H^+ antiporter in *P. aeruginosa*, and that Na^+ is the better of the two.

The Na^+/H^+ antiport activity in membrane vesicles of *P. aeruginosa* increased with increasing pH of the assay mixture between pH 7 and 8 (Fig. 2). When Na^+ was the substrate, the optimal pH was 8.0. When Li^+ was the substrate, however, the activity was very low between pH 7.0 and 8.0, and some activity was observed at pH 8.5. At higher pHs, the activity of H^+ transport due to the respiratory chain decreased, and the degree of quenching decreased. Therefore, it was difficult to evaluate the Na^+/H^+ antiporter activity at higher pH values such as 9.0.

Although it is not clear yet how many Na^+/H^+ antiporters are present in *P. aeruginosa* membranes, we determined the order of magnitude of kinetic parameters for the Na^+/H^+ (or Li^+/H^+) antiport. The K_m value and V_{max} value for Na^+/H^+ antiport measured by the

Fig. 3. Kinetic Analysis of the Na^+/H^+ and Li^+/H^+ Antiports

Apparent kinetic parameters of the Na^+/H^+ antiport (A) and the Li^+/H^+ antiport (B), respectively, were determined by the fluorescence quenching method. The results are presented as double reciprocal plots of the initial rate and NaCl (or LiCl) concentration. *a*) The initial rate is expressed as % dequenching/min per mg membrane protein.

Fig. 4. Inhibition of the Na^+/H^+ and Li^+/H^+ Antiports by Amiloride

Quenching and dequenching of quinacrine fluorescence were measured with membrane vesicles in the presence of various concentrations of amiloride. Na^+/H^+ antiport activity (●) and Li^+/H^+ antiport activity (○) were evaluated from dequenching.

quinacrine fluorescence method were roughly 1 mM and 5.8% dequenching/s per mg protein, respectively (Fig. 3); those for Li^+/H^+ antiport were roughly 2.7 mM and 0.6% dequenching/s per mg protein, respectively. The order of magnitude of the K_m value is the same as those of *E. coli* NhaA and NhaB Na^+/H^+ antiporters (data not shown). However, the V_{max} values of the Na^+/H^+ and Li^+/H^+ antiports in *P. aeruginosa* were two and three orders of magnitude, respectively, smaller than those in *E. coli* (data not shown). Thus, it seems that the Na^+/H^+ antiport, and especially the Li^+/H^+ antiport, in *P. aeruginosa* is very inefficient compared with that of *E. coli*. This makes it likely that Na^+ extrusion via the Na^+/H^+ antiporter is weak, and Li^+ extrusion via this system is very weak in *P. aeruginosa*.

Amiloride and its derivatives are known to inhibit Na^+/H^+ antiporters from various sources.¹²⁾ We tested the effect of amiloride on the Na^+/H^+ antiporter of *P. aeruginosa* and observed inhibition of Na^+/H^+ antiport activity and Li^+/H^+ antiport activity by amiloride (Fig. 4). At 1.5 mM, amiloride completely inhibited the activities, and 50% inhibition was observed at 0.3 to 0.5 mM

Fig. 5. Growth Inhibition of *P. aeruginosa* by Li^+

Growth of *P. aeruginosa* was measured in the presence of various concentrations of LiCl (▲), NaCl (■) or KCl (●) added to the L medium (NaCl was replaced with KCl; KCl concentration was 85 mM).

Fig. 6. Effects of Li^+ and Amiloride on the Growth of *P. aeruginosa*

Growth of *P. aeruginosa* was measured in the absence (A) or presence of 0.2 M LiCl (B). The growth in the absence (○) or presence of 2 mM amiloride (●) was measured in the L medium (NaCl was replaced with KCl; KCl concentration was 85 mM).

amiloride. This inhibition pattern is very similar to that observed with Na^+/H^+ antiporters of *E. coli* (data not shown).

Lithium ion has a toxic effect on the growth of *E. coli* when present at fairly high concentrations (0.5 M) in the growth medium (data not shown). We have shown previously that one of the targets of Li^+ inhibition is pyruvate kinase,¹³⁾ a key enzyme of glycolysis. It has also been reported that Li^+ inhibits *E. coli* DNA polymerase activity.¹⁴⁾ Because the Na^+/H^+ antiporters (NhaA and NhaB) are major systems for Li^+ extrusion, *E. coli* mutants lacking these two systems are unable to grow in the presence of 0.1 M LiCl¹⁵⁾ while wild type cells grow in the presence of 0.4 M LiCl (data not shown). Since Li^+/H^+ antiport activity in *P. aeruginosa* is very low, Li^+ may inhibit the growth of *P. aeruginosa* cells at lower concentrations. We tested the effect of Li^+ on the growth of these cells (Fig. 5), and found that growth was moderately inhibited at 0.1 M LiCl, strongly inhibited at 0.3 M, and completely inhibited at 0.4 M LiCl. In contrast, cells grew well at 0.4 M NaCl or KCl (Fig. 5).

As expected, amiloride (2 mM) significantly inhibited cell

growth in the presence of 0.2 M LiCl, but had no significant effect in the absence of Li^+ (Fig. 6). Thus, amiloride's inhibitory effect on growth would be due to its inhibition of the Li^+/H^+ antiport (Li^+ extrusion). Although 2 mM amiloride completely inhibited the Na^+/H^+ antiporter when measured in everted membrane vesicles, it did not completely inhibit growth in the presence of 0.2 M LiCl. Thus, amiloride may not be able to reach the binding site in the Na^+/H^+ antiporter from the exterior of the cytoplasmic membrane. Other possible explanations may be that the rather high concentration of LiCl (0.2 M) competed with amiloride, or that Li^+ at this concentration was not sufficient to inhibit cell growth even if the antiporter was inhibited by amiloride.

It has been reported that when a DNA fragment of *E. coli* NhaA was used as a probe in a Southern blot analysis of the chromosomal DNA of *P. aeruginosa*, no hybridized band was detected.¹⁵⁾ This suggests that the primary structure, and perhaps properties, of the Na^+/H^+ antiporter of *P. aeruginosa* are quite different from those of *E. coli* NhaA. It is likely that *P. aeruginosa* possesses a unique Na^+/H^+ antiporter, although its pH profile is similar to that of the NhaA Na^+/H^+ antiporter of *E. coli*. It is known in the *E. coli* NhaA system that the antiporter activity is enhanced when cells are grown in the presence of high NaCl.¹⁶⁾ However, no significant difference in the Na^+/H^+ antiporter activity in *P. aeruginosa* was observed between cells grown in the presence of 0.5 M NaCl and cells grown in its absence (data not shown).

Although the $V_{\max}$ value of the Na^+/H^+ antiport in *P. aeruginosa* is much smaller than that in *E. coli*, cells of *P. aeruginosa* have greater resistance to NaCl than *E. coli* cells. Therefore, cells of *P. aeruginosa* may possess another mechanism by which Na^+ is extruded from cells.

It is interesting to investigate the relationship between structure and function of proteins. Recently we have succeeded in cloning the gene encoding the Na^+/H^+ antiporter of *P. aeruginosa*. Sequencing of this gene is now under way.

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