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Mutational Analysis of Amiloride Sensitivity of the NhaA Na^+/H^+ Antiporter from *Vibrio parahaemolyticus*

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The activity of the NhaA Na^+/H^+ antiporter of *Vibrio parahaemolyticus* is inhibited by amiloride. We found an amino acid sequence in the NhaA that was identical to a putative amiloride binding domain of the Na^+/H^+ exchanger in mammalian cells. We constructed mutant NhaAs that had amino acid substitutions in the putative amiloride binding domain by site-directed mutagenesis. These include V62L (Val62 replaced by Leu), F63Y, F64Y, and L65F. Most mutant NhaAs showed decreased sensitivity for amiloride. Among these, the F64Y mutant NhaA showed the least amiloride sensitivity, with a K_i value 7 to 10 times greater than that in the wild type. Thus, the sequence between residues V62 and L65 in NhaA, especially F64, is very important for the inhibitory effect of amiloride on the antiporter.

The Na^+/H^+ antiporter is a membrane transport system which mediates the antiport (exchange) of Na^+ and H^+ across cell membranes and is widely distributed from bacterial cells to mammalian cells. The driving force for the Na^+/H^+ antiporter in bacterial cells is an electrochemical potential of H^+ across the membranes which is established mainly by the function of the respiratory chain. The driving force for the antiporter in mammalian cells (usually called exchanger) is an electrochemical potential of Na^+ which is established by the function of Na^+,K^+-ATPase. In *Escherichia coli*, in which the Na^+/H^+ antiporter is best characterized, the antiporter is known to have various biological roles, such as (i) the establishment of an electrochemical potential of Na^+ across the cell membrane, which is the driving force for Na^+/solute symporters (3, 7, 10, 24, 25); (ii) the extrusion of toxic Na^+ (when it is present at a high concentration) and Li^+ (8, 16); and (iii) intracellular pH regulation under alkaline conditions (20). Thus, this antiporter plays a central role in Na^+ circulation in bacterial cell membranes (20). So far, three Na^+/H^+ antiport systems (NhaA, NhaB, and ChaA) (9, 11, 17, 21) are known to exist in *E. coli* cell membranes. Of these three systems, the NhaA system has the strongest activity (8, 20).

It has been reported that the diuretic drug amiloride inhibits the Na^+/H^+ exchanger in mammalian cells by competing with Na^+ (1, 23). However, the inhibitory action of amiloride on the Na^+/H^+ antiporter in *E. coli* was controversial (15, 20). Recently it has been reported that amiloride gave no significant inhibitory effect on purified *E. coli* NhaA (21) and that amiloride was a potent inhibitor of purified (or highly enriched) *E. coli* NhaB (18). We reported previously that amiloride inhibited an Na^+/H^+ antiporter (NhaA) in the marine bacterium *Vibrio parahaemolyticus* (14). Kinetic analysis of the NhaA of *V. parahaemolyticus* produced in *E. coli* cells indicated that amiloride inhibited the Na^+/H^+ antiporter by competing with Na^+ (13). In mammalian cells, amiloride-resistant Na^+/H^+ exchangers have been reported, and the amiloride binding domain in the exchanger has been deduced (4, 6, 27). We have cloned and sequenced the *nhaA* gene encoding the NhaA antiporter in *V. parahaemolyticus* (13). We found in the NhaA of *V. parahaemolyticus* a sequence (VFFL) which was identical to the sequence of the putative amiloride binding domain in the mammalian Na^+/H^+ exchanger (13). The amino acid sequence around this region showed high homology with the Na^+/H^+ antiporters of other organisms such as *E. coli* and *Haemophilus influenzae*, etc. (5, 11, 21). To evaluate the involvement of this domain (the VFFL region) in amiloride action and in antiport activity of the *V. parahaemolyticus* NhaA, we constructed mutant NhaA antiporters and characterized them.

The *E. coli* mutant KNabc lacks all of the three major Na^+/H^+ antiporters (NhaA, NhaB, and ChaA) (16). Thus, with this mutant it was easier to analyze properties of the Na^+/H^+ antiporter produced from the cloned antiporter gene. Plasmid pTIS216 carries the wild-type *nhaA* gene of *V. parahaemolyticus* (13). Cells of KNabc/pTIS216 were grown in L(K) medium (1% polypeptone, 0.5% yeast extract, 85 mM KCl [pH 7.0]). Membrane vesicles were prepared from the cells as described previously (13), and Na^+/H^+ (Li^+/H^+) antiport activity was measured by the quinacrine fluorescence quenching method (13). As we reported previously, amiloride strongly inhibited activity of the *V. parahaemolyticus* NhaA Na^+/H^+ antiporter (13). We observed strong inhibition by amiloride of the NhaA Na^+/H^+ antiporter produced in *E. coli* KNabc (Fig. 1). Amiloride inhibited the activity in a manner that elevated the K_m value for Na^+, with little effect on the V_{max} of the transport, i.e., demonstrating competitive inhibition (Fig. 1A). A similar effect of amiloride was observed when Li^+ instead of Na^+ was added as the transport substrate (Fig. 1B). Judging from these results, it seems that amiloride competes with Na^+ (and Li^+) for the binding site in the antiporter.

Several site-directed mutants of the NhaA (V62L, i.e., V62 replaced with L; F63Y; F64Y; and L65F) were constructed with synthetic oligonucleotides by the method of Tomic et al. (22). The nucleotide sequence of each of the mutated *nhaA* genes was confirmed by sequencing (19). We measured both the Na^+/H^+ antiport activity and the Li^+/H^+ antiport activity of each of the mutant antiporters (V62L, F63Y, F64Y, and L65F) in addition to that of the wild-type antiporter. The wild-type NhaA antiporter of *V. parahaemolyticus* showed pH-dependent activity; i.e., activity was much higher at pH 8.5 than at pH 7.0 (13, 14). All of the mutant antiporters showed pH

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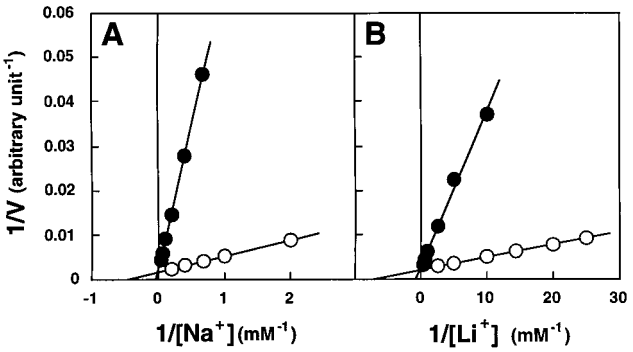

FIG. 1. Kinetic analysis of inhibition of the Na^+/H^+ antiporter by amiloride. Na^+/H^+ (A) and Li^+/H^+ (B) antiport activities were measured with membrane vesicles prepared from cells of KNabc/pTIS216 (wild-type NhaA) at pH 8.5 either in the absence (○) or in the presence (●) of 1 mM amiloride. Arbitrary unit, percentage of fluorescence dequenching per minute per milligram of protein.

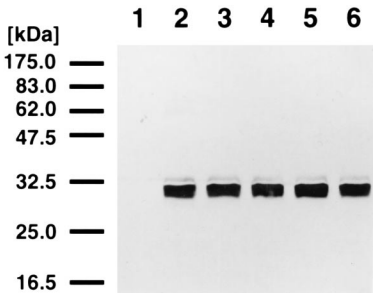

FIG. 2. Western blot analysis of fusion proteins of wild-type and mutant NhaA-MelB. Membrane proteins were subjected to polyacrylamide gel electrophoresis and analyzed by Western blotting. Lane 1, KNabc; lanes 2 to 6, KNabc/pTIS216 with the following NhaA-MelB decapeptide: wild type (lane 2) V62L (lane 3), F63Y (lane 4), F64Y (lane 5), and L65F (lane 6).

profiles that were similar to that of the wild type (data not shown). Thus, it seems that these residues (V62, F63, F64, and L65) are not involved in pH sensing. By kinetic analysis we obtained roughly comparable values for both K_m and V_{max}, using Na^+ or Li^+ with both the wild-type and the mutant antiporters, although the F64Y antiporter showed a significantly higher K_m value and a lower V_{max} value than did the others when Li^+ was used as the substrate (Table 1). Thus, amino acid substitutions in the putative amiloride binding domain did not significantly affect the Na^+/H^+ antiport activity as measured by the fluorescence quenching method. However, the F64Y mutant antiporter showed a K_i value seven times higher than that of the wild-type antiporter when Na^+ was used as the substrate, and the F63Y mutant antiporter showed a K_i value two times higher (Table 1), indicating that affinity for amiloride decreased in these mutant antiporters. When Li^+ was used as the substrate, we observed a K_i value for amiloride with the F64Y antiporter that was 10 times higher. Thus, there was an extensive decrease in the affinity of amiloride in the F64Y mutant when Li^+ was used as the substrate.

Since Li^+/H^+ antiport activity was significantly lower in the F64Y mutant than in the other mutants (Table 1), we measured the effect of Li^+ (and Na^+) on the growth of *E. coli* KNabc/pTIS216(F64Y) cells. The cells showed significantly slower growth in the presence of 0.4 M LiCl than did cells possessing either wild-type or mutant antiporters (data not shown). This finding supports the idea that the Li^+ extrusion activity of the F64Y mutant antiporter is very low. Thus, it seems that F64 is also very important for Li^+ transport. No significant difference in growth in the presence of 0.4 to 0.7 M NaCl was observed between cells possessing the wild-type NhaA and cells possessing the mutant NhaA (data not shown), although the Na^+/H^+ antiport activity of the F64Y mutant was somewhat lower than that of the others (Table 1).

TABLE 1. Kinetic parameters for Na^+/H^+ and Li^+/H^+ antiports in wild-type and mutant antiporters[a]

Substrate	Antiporter	K_m (mM)	V_{max}[b]	K_i for amiloride (mM)
Na^+	Wild type	2.2	606	0.06
	V62L	3.0	568	0.09
	F63Y	4.1	493	0.13
	F64Y	1.8	402	0.44
	L65F	3.3	633	0.10
Li^+	Wild type	0.14	488	0.09
	V62L	0.15	467	0.09
	F63Y	0.28	368	0.22
	F64Y	0.47	230	0.92
	L65F	0.20	498	0.14

[a] The results are the averages of three experiments. The standard deviations were less than 10%.

[b] V_{max} is expressed as percent fluorescence dequenching per minute per milligram of protein.

After the mutant gene was constructed and expressed, it was necessary to determine whether the mutant protein was present at a level comparable to that in wild-type cells. A Western blot analysis was performed to measure the level of these four mutant NhaAs in cell membranes. A decapeptide corresponding to the C terminus of the MelB protein (PVGAVSDVKA) (26) was connected to the C terminus of the wild-type NhaA protein of *V. parahaemolyticus* by gene fusion. A DNA fragment carrying the 3′ portion of the resulting hybrid gene (0.94 kbp) was cut out and ligated to the corresponding site of each mutant gene. Thus, each mutant NhaA possessing the decapeptide derived from MelB at the C terminus was obtained. The sequences of the constructed hybrid genes were confirmed by sequencing (19). Consequently, a series of NhaA-MelB fusions were obtained. Membrane proteins from KNabc cells harboring each plasmid carrying the fused gene were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12% polyacrylamide gel), and the samples were electrophoretically transferred to polyvinylidene fluoride (GV type; Millipore). Antiserum against the decapeptide of the C terminus of MelB of *E. coli* (2) was used to detect NhaA-MelB fusion proteins. The complex of the NhaA-MelB and the antibody was visualized with the ABC kit (VECTASTAIN; Vector Co.) as suggested by the manufacturer. The antibody bound to the original MelB protein (2). We did not detect any bands with membrane proteins from KNabc (Fig. 2, lane 1), but we did detect a band (about 30 kDa) which corresponds to the NhaA-MelB fusion protein, with membrane proteins from KNabc/pTIS216 (Fig. 2, lane 2). We observed bands of similar density for each mutant antiporter protein that was fused with the MelB decapeptide (Fig. 2, lanes 3 to 6). These results indicate that the change in the mutant antiporters is not due to a change in the quantity of mutant NhaAs in the membranes. We confirmed that each of the fusion proteins showed Na^+/H^+ and Li^+/H^+ antiport activities comparable to those observed with the corresponding mutant NhaA (data not shown).

Our results support the idea that the VFF regions in both the *V. parahaemolyticus* Na^+/H^+ antiporter and the mamma-

lian Na^+/H^+ exchanger are important for amiloride inhibition (perhaps for amiloride binding).

We found a sequence (VFF) which is the same as that of the putative amiloride binding domain of NhaA in the primary structure of the *E. coli* melibiose transporter, a Na^+-coupled symporter (26). We tested the effect of amiloride on the melibiose transporter. As expected, considerable inhibition of melibiose transport by amiloride was observed (12). As reported previously (12), the VFF sequence is present in many Na^+-coupled transport proteins. Thus, it is likely that VFF is one of the motifs involved in Na^+ binding and amiloride binding.

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