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Amiloride and harmaline are potent inhibitors of NhaB, a Na^+/H^+ antiporter from *Escherichia coli*

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Abstract The diuretic drug amiloride is a specific inhibitor of sodium transporting proteins in several cell types. Attempts to inhibit this activity in membrane vesicles derived from various bacteria, did not yield clear results. Therefore, we tested the effect of amiloride and its derivatives on the purified Na^+/H^+ antiporters of *E. coli* reconstituted in functional form in proteoliposomes. Whereas NhaA is not inhibited by amiloride, both amiloride and harmaline are potent inhibitors of NhaB with $K_{0.5}$ of 6 and 15 μM, respectively. The pattern of inhibition by amiloride derivatives is different from that reported for mammalian antiporters but similar to that reported for the Na^+/H^+ antiporter of *D. salina* [Katz, A., Kleyman, T.R. and Pick, U. (1994) Biochemistry 33, 2389–2393]. Clonidine is a poor inhibitor ($K_{0.5}$ = 200 μM) while cimetidine had no effect on the antiporter up to concentration of 1 mM. These new potent inhibitors provide us with important tools for the study of the mechanism of action of NhaB.

Key words: Transport; Salinity; Membrane protein; Proteoliposome; Reconstitution

1. Introduction

Sodium extrusion and maintenance of a sodium concentration gradient, directed inward, is a feature common to all living cells. This gradient is generated by primary sodium pumps or by secondary transporters, namely Na^+/H^+ antiporters. In *E. coli* there are two specific Na^+/H^+ antiporters namely NhaA and NhaB [1]. The structural genes coding for the two systems were cloned and sequenced [2,3]. *Nha*A is essential for growth at high salt concentrations, a necessity which is increased with the pH [4]. *Nha*B is vital for growth on sodium/cotransported substrates such as glutamate and proline on low salt media at low pH [5]. A mutant in which both genes were deleted is extremely sensitive to sodium and lithium but is able to grow throughout the pH range of the wild type provided that the salt content is kept low [5].

Both proteins were studied in a purified and reconstituted system in proteoliposomes [6,7]. NhaA activity is extremely pH dependent, changing its rate by three orders of magnitude when the pH is changed from 6.5 to 8.5. The pH affects also the apparent K_m of NhaB to Na^+ which increases by 10-fold when the pH is shifted from 8.5 to 7.2. Both NhaA and NhaB are electrogenic with stoichiometries of $2H^+/1Na^+$ and $3H^+/2Na^+$, respectively [6,7].

To study the contribution of each antiporter at different growth conditions and to understand their mechanism of action, it is important to characterize other ligands and inhibitors. Because of the presence of two antiporters, a reconstituted system is most suitable to test properties which could not easily or unequivocally be tested in intact cells or isolated membrane vesicles. Sensitivity of the antiporter to amiloride and other drugs is an example.

The diuretic drug amiloride is a specific inhibitor of sodium transporting proteins in several cell types. It is a competitive inhibitor of plasma membrane Na^+/H^+ antiporter in animal cells as well as epithelial Na^+ channels [8]. In algae and plants it has been shown to inhibit tonoplast [9] and plasma membrane [10] Na^+/H^+ antiport activity. The Na^+/H^+ antiporter of methanogenic bacteria was also found to be inhibited by both amiloride and harmaline [11].

There is some controversy whether amiloride inhibits also the Na^+/H^+ antiport activity of *E. coli*. It was reported that the Na^+/H^+ exchange activity is competitively inhibited by amiloride (K_i = 40 μM) when measured with inverted membrane vesicles by monitoring quenching of acridine orange [12]. However, attempts to inhibit this activity with amiloride in membrane vesicles derived from wild type (*nha*A^+*nha*B^+) strains did not yield clear results [1,13]. It was suggested that amiloride and some of its derivatives may inhibit due to their uncoupling activity and therefore it was hard to differentiate between specific and non-specific effects of amiloride on ΔpH driven Na^+ transport [13,14].

Purified NhaA reconstituted into liposomes, was found resistant to amiloride; there was no significant inhibition of downhill sodium efflux catalyzed by purified NhaA [6]. In this study, using the purified and reconstituted NhaB, we found that NhaB is specifically and competitively inhibited by amiloride and harmaline at concentrations of 10–20 μM. Analysis of the potency of amiloride derivatives reveals a new pattern distinct from that displayed by mammalian antiporters.

2. Materials and methods

2.1. Bacterial strains, plasmids and culture media

The bacterial strain used is NM81 [4], an *E. coli* K12 derivative which is *mel*BLid Δ*lac*ZY Δ*nha*A1 *kan*^{R} *thr*1. pGP1-3 [7] is a derivative of pGP1-2 [15] and contains the T7 polymerase gene and *tet*^{r} instead of *kan*^{r}. Cells were grown in enriched LB media containing 2% bacto tryptone, 1% yeast extract 0.5% KCl, 0.2% glycerol and 50 mM KP_i, pH 7.2. Medium used for labeling of NhaB with [^{35}S]methionine was minimal Medium A without sodium citrate supplemented with thia-

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Abbreviations: HTP, hydroxylapatite; DTT, dithiothreitol; PL, *E. coli* phospholipids; OG, *n*-octyl glucoside; DM, *n*-dodecyl β-D-maltoside; MOPS, 3-(*N*-morpholino)propanesulphonic acid; EIPA, 5-(*N*-ethyl-*N*-isopropyl)amiloride; MIBA, 5-(*N*-methyl-*N*-isobutyl)amiloride; HMA, 5-(*N,N*-hexamethylene)amiloride; DMA, 5-(*N,N*-dimethyl)amiloride.

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mine (2.5 μg/ml), threonine (50 μg/ml) and glycerol (0.5%). Ampicillin and tetracycline were added to 100 and 10 μg/ml, respectively.

2.2. Overproduction, membrane preparation and protein purification

All overproduction and preparation steps were as described in Pinner et al. [7]. NM81/pGP1-3/pEL76 (NhaB overexpressing strain) cells were grown in rich LB media and induced by a heat shock at OD_{600} = 1.5. Membrane vesicles were prepared by sonication and washed with 5 M urea. The membrane vesicles were mixed with membranes from the same strain (grown in minimal medium) in which NhaB was specifically labeled with [^{35}S]methionine [7]. Membrane vesicles (1 ml) were solubilized by addition of 2 ml of a mixture containing 1.5% dodecyl maltoside (DM), 7.5 mg/ml *E. coli* phospholipids, 150 mM MOPS, pH 7, 30% glycerol and 3 mM DTT. The extract was diluted and applied on hydroxylapatite (HTP) column. Elution was carried out by washing the column with a linear gradient of KP_i (pH 7) from 0.4 to 1 M. Fractions with the highest amount of radioactivity were pooled and frozen after the glycerol concentration was raised to 20%. At this point NhaB is highly enriched although not homogeneous [7].

2.3. Reconstitution

As described by Taglicht et al. [6] and Pinner et al. [7]. Protein after HTP (400 μl) was added to a sonicated mixture (300 μl) containing 8 mg of PL in 1.2% OG, 100 mM MOPS, pH 7. The mixture was diluted 36-fold into the appropriate buffer, depending on the assay used (this buffer determines the composition of the internal volume). After 20 min at room temperature, proteoliposomes were pelleted by centrifugation at 257,000 × *g* for 1 h, resuspended to 100 μl with the dilution buffer, frozen in liquid air and stored at −70°C. When assayed, an aliquot was thawed at room temperature and sonicated briefly until clear.

2.4. ΔpH driven sodium uptake

4 μl proteoliposomes, loaded with 150 mM ammonium chloride, 1 mM DTT and 15 mM Tris, pH 7, were diluted into 500 μl of 150 mM choline-Cl, 15 mM Tris, pH 8.5, and 0.5–1 μCi/ml ^{22}NaCl with the appropriate drug. The reaction was done at room temperature and stopped by addition of 2 ml of ice-cold dilution buffer, filtered on 0.2 μm Schleicher and Schuell filters and washed with additional 2 ml buffer. Radioactivity on the filter was measured in a γ-counter. Zero times were made with the addition of 1 mM amiloride and subtracted from all experimental points.

2.5. Sodium efflux

Proteoliposomes were made by dilution into a buffer containing 100 mM potassium acetate, 1 mM DTT, buffered with 10 mM Tris or phosphate, pH 7 or 7.5, and sodium at the concentration mentioned in the figure legend. After thawing, ^{22}NaCl was added to the proteoliposomes to 15 μCi/ml and the mixture was sonicated to clarity. Dilution of 4 μl proteoliposomes was made to 500 μl of the above buffer but without sodium. The assay was terminated with ice-cold dilution buffer (2 ml), filtered and washed. Infinite time equilibrium values obtained after incubation of 1.5–2 h were subtracted from all experimental points.

2.6. Protein determination

Protein was determined by the methods of Bradford [16] and Peterson [17].

2.7. Materials

Hydroxylapatite and octyl glucoside were from Calbiochem; dodecyl maltoside, amiloride, HMA and harmaline were from Sigma; EIPA was a gift from Dr. Steven Karlish (Weizmann Institute of Science, Rehovot); MIBA a gift from Dr. Marc Solioz (University of Bern); and DMA and benzamyl amiloride from Merck and Co. ^{22}NaCl was from Dupont-New England Nuclear and [^{35}S]methionine was from Amersham Co.

3. Results

Since it has been reported that amiloride has uncoupling activity [13,14], the effect of this drug was first tested on ΔpH independent reactions, namely Na^+ efflux and $^{22}Na^+$:Na^+ exchange. The preparation we used for all the experiments described in this communication is purified NhaB reconstituted into proteoliposomes [7]. When NhaB proteoliposomes were loaded with K-acetate and ^{22}Na and diluted to a buffer of the same composition but free of sodium, a rapid $^{22}Na^+$ efflux was measured (Fig. 1A: $t½$ = 25 s). When the experiment was done in the presence of 1 mM of amiloride, the efflux rate decreased significantly (Fig. 1A: $t½$ > 120 s). When NhaB proteolipo-

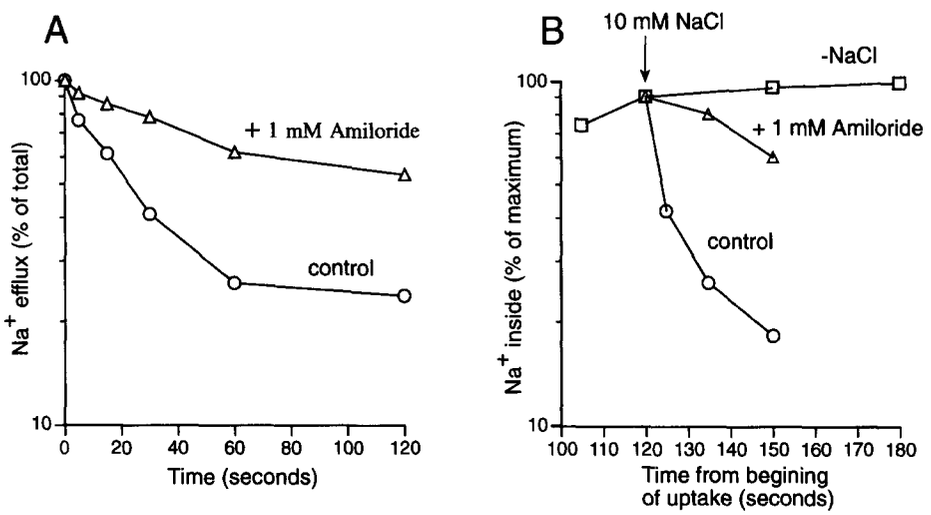

Fig. 1. NhaB mediated Na^+ efflux and $^{22}Na^+$:Na^+ exchange are inhibited by amiloride. (A) NhaB proteoliposomes (120 μg protein/ml) loaded with 100 mM potassium acetate, 10 mM KP_i, 0.1 mM NaP_i and ^{22}NaCl at pH 7 were prepared as described in section 2. The experiment was initiated by dilution of 4 μl proteoliposomes into 500 μl of buffer with the same composition but devoid of Na^+ with (Δ) or without (○) 1 mM amiloride. The reaction was stopped by addition of 2 ml of cold buffer and filtration through 0.2 μM filters which were counted by γ-counter. The experiment was done in duplicates. (B) NhaB proteliposomes (120 μg protein/ml) loaded with 0.15 M ammonium chloride and 15 mM Tris, pH 7, were diluted 125 fold into 0.15 M choline chloride, 15 mM Tris, pH 8.5, and 0.1 mM ^{22}NaCl. Under these conditions a ΔpH dependent Na^+ uptake occured (■). 120 seconds after the reaction was started, NaCl was added to 10 mM to begin a ^{22}Na:Na exchange reaction with (Δ) or without (○) the addition of 1 mM amiloride prior to the salt addition.

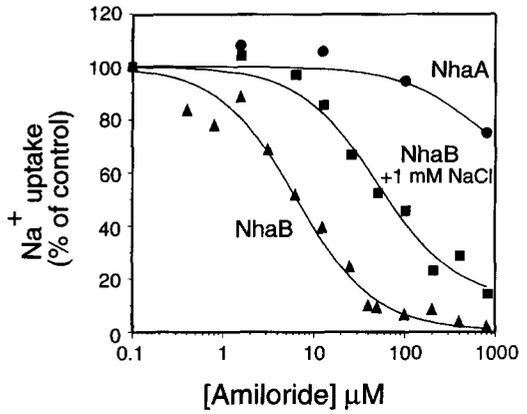

Fig. 2: Concentration–response profiles for inhibition of NhaB and NhaA mediated Na^+ uptake activity by amiloride. ΔpH driven Na^+ uptake experiments were done by dilution of proteoliposomes (4 μl of 120 μg protein/ml) loaded with 0.15 M ammonium chloride and 15 mM Tris, pH 7, 125-fold into 0.15 M choline chloride, 15 mM Tris, pH 8.5, carrier-free $^{22}NaCl$ and different concentrations of amiloride. Initial rate of uptake by NhaB proteoliposomes, was measured for 20 s without addition of unlabelled NaCl (▲) or with the addition of 1 mM NaCl to the reaction buffer (■). The initial rate of Na^+ uptake by NhaA proteliposomes was measured for 30 s without addition of unlabelled NaCl (●).

somes were loaded with ammonium chloride and diluted to choline chloride with ^{22}Na, Na^+ was taken up in a time- dependent manner and reached a steady state level after 2–3 min [7]. If unlabeled NaCl was then added to 10 mM, a rapid $^{22}Na^+$:Na^+ exchange reaction was detected (Fig. 1B: $t½ < 5$ s). The rate of this reaction was markedly decreased by the addition of 1 mM amiloride prior to the NaCl addition (Fig. 1B). These experiments strongly indicate that the NhaB protein is specifically inhibited by amiloride since the reactions described above are not driven by $\Delta\mu_{H+}$ and therefore the inhibition cannot be attributed to uncoupling activity.

The inhibition of ΔpH driven $^{22}Na^+$ uptake by amiloride was then tested (Fig. 2). Under these conditions NhaB was inhibited by amiloride with a $K_{0.5}$ of about 6 μM. When the reaction was performed in the presence of 1 mM NaCl the inhibition profile shifted to the right with a $K_{0.5}$ of 70 μM. A Dixon plot of the data shown in Fig. 2 and additional data (not shown) with other NaCl concentrations, indicated that amiloride is a competitive inhibitor of NhaB with an apparent K_i of 20 μM. Since amiloride does not inhibit NhaA [6] we could use NhaA proteoliposomes as a control to confirm that the inhibition of NhaB under these conditions is specific and not due to an effect on the ΔpH. Indeed we were able to confirm our previous finding that amiloride does not influence NhaA activity significantly when measured under the same conditions as NhaB with concentrations up to 1 mM (Fig. 2).

Amiloride analogs, that bear branched carbon chains on the 5-amino group, like ethyl isopropyl amiloride (EIPA) and methyl isobutyl amiloride (MIBA), are 100–1000-fold more potent inhibitors of the mammalian Na^+/H^+ exchanger than amiloride itself [8,18]. We therefore checked the inhibition pattern of some derivatives on NhaB activity and the values for apparent half-maximal inhibition of ΔpH driven ^{22}Na uptake are summarized in Table 1. In contrast to NHE-type antiporters, we found that 5-amino substitutions do not increase the inhibition potency of amiloride, and even slightly decrease it, regardless of the type of carbon group added at this position. All the derivatives of this type that we checked, namely, EIPA, MIBA, HMA (hexamethylene) and DMA (dimethyl) had $K_{0.5}$ in the range of 43–70 μM (Table 1). Further difference in the pharmacology of NHE antiporters and NhaB is seen with another derivative in which a benzene group is added to the guanidino moiety (benzamil): in the case of NhaB it has a similar inhibition potency to that of amiloride although it has much less potency in inhibition of the mammalian exchanger than amiloride (Table 1).

The hallucinogenic drug, harmaline, was reported to inhibit mammalian Na^+/H^+ exchanger [18,19]. The effect of harmaline on the Na^+ efflux reaction from NhaB proteliposomes was tested. Addition of 1 mM harmaline into the dilution buffer slows the efflux rate 5-fold, practically to the same extent, as the addition of 1 mM amiloride (not shown). From an analysis of the concentration dependence of the inhibition of ΔpH driven Na^+ uptake by different concentrations of harmaline a $K_{0.5}$ of 15 μM was found (Fig. 3). However, since harmaline is a hydrophobic weak base and therefore a potential protonophore that could exert its inhibition by abolishing the ΔpH, we performed the same experiment with liposomes to which monensin was added. Monensin is a Na^+/H^+ exchanging ionophore and it catalyzes Na^+ uptake at the expense of the pH gradient generated by ammonium. Monensin driven Na^+ uptake reaction is also inhibited by harmaline (Fig. 3) though at higher concentrations than the NhaB catalyzed reaction ($K_{0.5}$ = 75 μM). These results confirm that harmaline is a potent inhibitor of NhaB ($K_{0.5}$ = 15 μM), although it also shows quite a significant protonophoric activity. NhaA driven Na^+ uptake was inhibited at intermediate concentrations between the NhaB and monensin driven uptake (data not shown). Therefore from these results we cannot learn whether harmaline inhibits NhaA directly.

Two other organic compounds that are known to inhibit mammalian Na^+/H^+ antiporters are clonidine and cimetidine [18,19]. When ΔpH driven $^{22}Na^+$ uptake was tested in the presence of clonidine, a concentration-dependent inhibition was observed although at concentrations higher than those observed for amiloride and harmaline with a $K_{0.5}$ of 200 μM (data not shown). On the other hand cimetidine, a strong inhibitor

Table 1
Inhibition constants of Na^+ uptake activity mediated by different Na^+/H^+ antiporters for amiloride derivatives and other compounds[a]

Inhibitor	NhaB	NHE-1	NHE-3	*D. salina*
Amiloride	6.0×10^{-6}	1.6×10^{-6}	1.0×10^{-4}	1.5×10^{-5}
EIPA	5.3×10^{-5}	1.5×10^{-8}	2.4×10^{-6}	8.0×10^{-5}
HMA	4.3×10^{-5}			
MIBA	5.0×10^{-5}			8.2×10^{-5}
DMA	6.7×10^{-5}	2.3×10^{-8}	1.4×10^{-5}	
Benzamil	1.0×10^{-5}	1.2×10^{-4}	1.0×10^{-4}	3.0×10^{-6}
Harmaline	1.5×10^{-5}	1.4×10^{-4}	1.0×10^{-3}	
Clonidine	2.0×10^{-4}	2.1×10^{-4}	6.2×10^{-4}	
Cimetidine	$> 1 \times 10^{-3}$	2.6×10^{-5}	6.2×10^{-3}	

[a] The values shown are the half-maximal inhibition concentration ($K_{0.5}$) in M. Results for rat NHE-1 and NHE-3 are taken from Orlowsky [18] and for the Na^+/H^+ antiporter of *D. salina* from Katz et al. [10]. NhaB, NHE-1 and NHE-3 were assayed with carrier-free $^{22}NaCl$ and Na^+/H^+ antiporter of *D. salina* was assayed in the presence of 30 μM $^{22}NaCl$.

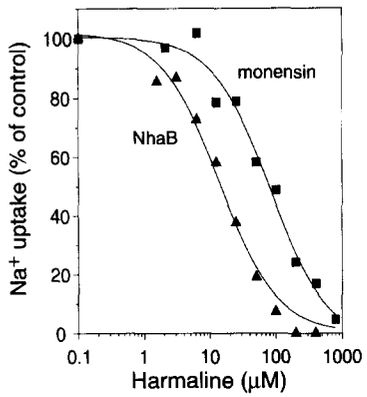

Fig. 3. Concentration–response profiles for inhibition of NhaB or monensin mediated Na^+ uptake activity by harmaline. Initial rates of ΔpH driven Na^+ uptake experiments were conducted as described in Fig. 2 with different concentrations of harmaline in the reaction solution. The experiment was done in duplicates with NhaB proteliposomes (▲) or empty liposomes with addition of 2 μM monensin to the reaction mixture (■). Reaction was terminated after 20 s (NhaB mediated) or 15 s (monensin mediated).

of rat NHE-1 isoform [18], did not have any effect on NhaB activity at a concentration of 1 mM.

Li^+ is a known substrate of both Na^+/H^+ antiporters of *E. coli* [4] and Mn^{2+} is a substrate of mitochondrial Na^+/H^+ exchanger [20]. Both LiCl and $MnCl_2$ inhibit NhaB dependent Na^+ uptake ([7] and data not shown). In order to assess the relative potency the effect of different cations concentrations on ΔpH driven Na^+ uptake was tested. The $K_{0.5}$ values at a sodium concentration of 100 μM were found to be 1.2 and 5 mM for Mn^{2+} and Li^+, respectively. These data indicate that, surprisingly, NhaB has a higher affinity for Mn^{2+} than to Li^+, a situation similar to that observed for the mitochondrial Na^+/H^+ exchanger [20].

4. Discussion

In this report we used a purified and reconstituted system [7] to study the pharmacology of NhaB, a Na^+/H^+ antiporter from *E. coli*. In the case of *E. coli* the use of a purified protein was particularly important since in the wild type cell, two specific antiporters, NhaA and NhaB, have been described. In addition, the use of a reconstituted system allows monitoring of activity at various modes: ΔpH-independent reactions namely $^{22}Na^+$ efflux and $^{22}Na:Na$ exchange and ΔpH-driven reactions, namely ^{22}Na uptake. NhaB activity is inhibited by amiloride in all the modes. Inhibition of ΔpH driven Na^+ uptake by amiloride was tested at varying concentrations and it was found that the inhibition is competitive with an apparent K_i of 20 μM. This value is similar to the K_i found for Na^+/H^+ antiporter activity in inverted membrane vesicles derived from a wild type *E. coli* strain (*nha*A^+*nha*B^+) [12]. Since NhaA is resistant to amiloride [6] the latter findings fit the notion that NhaB is the 'house keeping' antiporter and constitutes most of the Na^+/H^+ antiporter activity of *E. coli* cells grown in low sodium medium and neutral pH [5,21]. another relevant conclusion from our studies is that amiloride has no significant uncoupling activity in the concentration range tested (up to 1 mM) since it has no effect on NhaA mediated, ΔpH driven Na^+ uptake.

Table 1 summarizes the half-maximal inhibition concentrations for the different compounds tested for NhaB and for other Na^+/H^+ antiporters. It can be seen that there is some resemblance in the inhibition pattern between NhaB and the Na^+/H^+ antiporter of *D. salina* [10]. The $K_{0.5}$ for inhibition by amiloride is 6 and 15 μM, respectively, for NhaB and the *D. salina* antiporter. These inhibition constants are relatively close to those found for the Rat NHE-1 and NHE-2 (1.6 and 1.4 μM, respectively) but much lower than the one found for NHE-3 (100 μM) [18,22]. The amiloride binding characteristics of NhaB are different from that of the mammalian exchanger isoforms since amiloride derivatives with a branched alkyl residue at the 5-amino nitrogen are more potent than amiloride at least by two orders of magnitude in inhibiting mammalian NHE isoforms [8]. In this communication we found that such derivatives (EIPA, MIBA, HMA and DMA) are less potent than amiloride in inhibiting NhaB by about 10 fold ($K_{0.5}$ = 43–67 μM). The potency of EIPA and MIBA toward the *D. salina* antiporter is very similar. The amiloride sensitive sodium channel from epithelial cells has also a reduced affinity to these derivatives and the decline in inhibition potency due to these substitutions in the case of the channel is much more extreme than for NhaB [8]. The benzamyl derivative (addition of methyl benzene group to the guanidino moiety) is one of the most potent inhibitors of the epithelial Na^+ channel but has reduced affinity to mammalian Na^+/H^+ exchanger. We found that this derivative inhibits NhaB at concentrations slightly higher than amiloride ($K_{0.5}$ = 10 μM) And similar to the *D. salina* antiporter (3 μM).

Harmaline, a hallucinogenic drug that was also found to inhibit NHE isoforms [18,22], was found in this report to inhibit NhaB specifically with a $K_{0.5}$ of 15 μM. When the inhibition percentage was measured in a ΔpH driven Na^+ uptake assay, a $K_{0.5}$ of 15 μM was obtained. However, we also found significant inhibition of monensin-mediated Na^+ uptake showing that this compound has considerable protonophore activity with a $K_{0.5}$ of 75 μM. Clonidine was found to inhibit NhaB activity with a $K_{0.5}$ of 200 μM and cimetidine did not have any effect on NhaB activity.

Interestingly Mn^{2+} has quite a high affinity to NhaB, comparable to that of Na^+ and significantly higher than that of Li^+. The only Na^+/H^+ antiporter reported to interact with Mn^{2+} is the mitochondrial one [20].

It was reported before that amiloride inhibits growth of *E. coli* cells at alkaline pH [23,24]. It was suggested that this phenomenon results from the inhibition of Na^+/H^+ activity which is responsible for pH regulation. This is probably not the case because of two reasons: (a) *E. coli* strain in which both *nha*A and *nha*B are deleted can grow at alkaline pH [2]; (b) growth of this strain is also inhibited by amiloride (not shown), indicating that this growth inhibition is not due to inhibition of specific Na^+/H^+ antiport activity.

The growing interest in the diuretic amiloride is due to some possible implications for this drug other than its classical one. Amiloride inhalation as a treatment for cystic fibrosis (CF) patients, has been shown in independent studies to increase mucous clearance and to retard the decline in lung function [25]. Amiloride was also suggested as an anti cancer drug since it was found to inhibit growth of colon cancer cells [26] and

angiogenesis [27], in vitro. Therefore the structure of amiloride binding sites is an important issue and was the subject of a few reports that used different approaches. In a study aimed to characterize the amiloride binding site of NHE-1, Pouyssegur and co-workers developed a procedure to isolate mutations which confer resistance to the amiloride analog MPA [28]. Two NHE-1 mutants with decreased sensitivity to MPA were isolated and both had a replacement of Leu-167 to Phe. This Leu is found in a sequence which is conserved among NHE isoforms: ^{164}VFF**L**FLLPPI^{173} which is located in the middle of the fourth putative trans-membrane segment. We found in the sequence of NhaB the pentamer ^{445}FLFLL^{450} which is identical to 5 amino acid of the putative amiloride binding site. However, it should be noted that in contrast to NHE-1, this sequence resides in the middle of the eleventh putative trans-membrane segment of NhaB and the homology between NhaB and NHE-1 is not extended beyond that in this region.

Of the two *E. coli* antiporters NhaB seems to be the most archaic one and is part of a novel family of proteins. It displays a weak but distinct homology to a membrane protein with unknown function in *Mycobacterium leprae* (Accession no. L10660; 24% identity, 55% similarity). In addition there is a limited homology to two transporters found in animal cells: the rat sodium-dependent sulfate transporter (24% identity, 50% similarity) [29] and the product of the mouse pink-eyed dilution gene (21% identity, 53% similarity) [30]. A conserved motif is observed in the four proteins: (S,T)X(L,I)GGXXTXX-GX(P,S)XNX(I,V). The significance of this motif is not known yet.

The availability of these new potent inhibitors provides us with important tools to study ligand–protein interactions and the relative levels of NhaB activity under various physiological conditions.

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