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 \( K_{\text{d}} \) of 6 and 15 \( \mu \text{M} \), 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 (\( K_{\text{d}} = 200 \mu \text{M} \)) while cimetidine had no effect on the antiporter up to concentrations 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]. NhaA is essential for growth at high salt concentrations, a necessity which is increased with the pH [4]. NhaB 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 \( K_{\text{m}} \) 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' antiporter 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' antiporter activity of *E. coli*. It was reported that the Na'+H' exchange activity is competitively inhibited by amiloride (\( K_{\text{d}} = 40 \mu \text{M} \)) when measured with inverted membrane vesicles derived from wild type (nhaA nhaB) 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 \( \Delta \text{pH} \) driven Na' transport [13,14].

Purified NhaA reconstituted into liposomes, was found resistant to amiloride; there was no significant inhibition of down-hill 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 \( \mu \text{M} \). 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 nhaB/nhaA. It was isolated from *D. salina* (nhaA nhaB) strain [14]. E. coli K12 [7] is a derivative of pGP1-2 [15] and contains the T7 polymerase gene and *tetR* instead of *kanR*. Cells were grown in enriched LB media containing 2% bacto tryptone, 1% yeast extract 0.5% KCl, 0.2% glycerol and 50 mM KPi, pH 7.2. Medium used for labeling of NhaB with [35S]methionine was minimal Medium A without sodium citrate supplemented with thia-
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 OD600 = 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 [35S]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, (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 300 μl of 150 mM choline-Cl, 15 mM Tris, pH 8.5, and 0.5–1 μCl/ml [35S]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, [35S]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 μl), 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. [35S]NaCl was from Dupont-New England Nuclear and [35S]methionine was from Amer sham 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 [35S]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 [35S]Na and diluted to a buffer of the same composition but free of sodium, a rapid [35S]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 [35S]Na+:Na+ exchange are inhibited by amiloride. (A) NhaB proteoliposomes (120 μg protein/ml) loaded with 100 mM potassium acetate, 10 mM KP, 0.1 mM NaP, and [35S]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 (d) or without (c) 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 proteoliposomes (120 μg protein/ml) loaded with 0.15 M ammonium chloride and 15 mM Tris, pH 8.5, and 0.1 mM [35S]NaCl. Under these conditions a ΔpH dependent Na+ uptake occurred (d). 120 seconds after the reaction was started, NaCl was added to 10 mM to begin a [35S]Na exchange reaction with (d) or without (c) the addition of 1 mM amiloride prior to the salt addition.](image-url)
Table 1

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>NhaB</th>
<th>NHE-1</th>
<th>NHE-3</th>
<th>D. salina</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amiloride</td>
<td>6.0 × 10^{-4}</td>
<td>1.6 × 10^{-6}</td>
<td>1.0 × 10^{-4}</td>
<td>1.5 × 10^{-5}</td>
</tr>
<tr>
<td>EIPA</td>
<td>5.3 × 10^{-3}</td>
<td>1.5 × 10^{-5}</td>
<td>2.4 × 10^{-6}</td>
<td>8.0 × 10^{-5}</td>
</tr>
<tr>
<td>HMA</td>
<td>4.3 × 10^{-3}</td>
<td>5.0 × 10^{-5}</td>
<td>8.0 × 10^{-3}</td>
<td>2.3 × 10^{-5}</td>
</tr>
<tr>
<td>MIBA</td>
<td>6.7 × 10^{-3}</td>
<td>2.3 × 10^{-8}</td>
<td>1.4 × 10^{-5}</td>
<td></td>
</tr>
<tr>
<td>Benzamil</td>
<td>1.0 × 10^{-4}</td>
<td>1.2 × 10^{-4}</td>
<td>1.0 × 10^{-4}</td>
<td></td>
</tr>
<tr>
<td>Harmaline</td>
<td>1.5 × 10^{-4}</td>
<td>1.4 × 10^{-6}</td>
<td>1.0 × 10^{-4}</td>
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</tr>
<tr>
<td>Clonidine</td>
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<td>6.2 × 10^{-3}</td>
<td></td>
</tr>
<tr>
<td>Cimetidine</td>
<td>1.0 × 10^{-4}</td>
<td>2.6 × 10^{-3}</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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 proteoliposomes was tested. Addition of 1 mM harmaline into the dilution buffer slowed 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_i 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_i = 75 μM). These results confirm that harmaline is a potent inhibitor of NhaB (K_i = 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 22Na+ 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_i of 200 μM (data not shown). On the other hand cimetidine, a strong inhibitor of the Na+/H+ exchanger in M. Results for rat NHE-1 and NHE-3 are taken from Orlovsky [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 22NaCl and Na+/H+ antiporter of D. salina was assayed in the presence of 30 μM 22NaCl.
ApH driven Na\(^+\) uptake experiments were conducted as described in
4. Discussion and neutral pH \([5,21]\). Another relevant conclusion from our
inverted membrane vesicles derived from a wild type
inhibition is competitive with an apparent \(K_i\) of 20/\(\mu\)M. This
tested at varying concentrations and it was found that the
22Na flux and 22Na : Na exchange and ApH-driven reactions, namely
the use of a reconstituted system allows monitoring of activity
particularly important since in the wild type cell, two specific
E. coli.
Na+/H + exchanger \([20]\).
Both LiCl and MnCl\(_2\) inhibit NhaB dependent
antiporters, NhaA and NhaB, have been described. In addition,
to study the pharmacology of NhaB, a Na+/H + antiporter from
the use of a purified protein was
useful data for the epithelial Na\(^+\) channel [18]
was found in this report to inhibit
NhaB specifically with a \(K_{0.5}\) of 15/\(\mu\)M. When the inhibition
of Na+/H + antiporter activity by harmaline. Initial rates of
phenomenon results from the inhibition of Na+/H + activity
E. coli
mucous clearance and to retard the decline in lung function
possible implications for this drug other than its classical one.
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
nhaA and nhaB 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\(^+\) antiporter 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

Fig. 3. Concentration-response profiles for inhibition of NhaB or
monensin mediated Na\(^+\) uptake activity by harmaline. Initial rates of
4pH driven Na\(^+\) uptake were tested. The \(K_{0.5}\) values at a
sodium concentration of 100 \(\mu\)M 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: \(\Delta\phi\)-independent reactions namely \(22Na^+\) ef-
flux and \(22Na^+\) : Na exchange and \(\Delta\phi\)-driven reactions, namely
\(22Na^+\) uptake. NhaB activity is inhibited by amiloride in all the
modes. Inhibition of \(\Delta\phi\) 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 \(\mu\)M.
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 (nhaA\(~nhaB\)) [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\(^+\) an-
tiporter 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, \(\Delta\phi\) driven Na\(^+\) uptake.

Table 1 summarizes the half-maximal inhibition concentra-
tions 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/\(\mu\)M, 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 \((1.6 \text { and } 1.4/\mu\)M, respectively) but much lower than the one found for
NHE-3 \((100/\mu\)M) \([18,22]\). 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 \((K_{0.5} = 43-
67/\mu\)M). 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 \((K_{0.5} = 10/\mu\)M) And similar to the D. salina antiporter
(3 \(\mu\)M).

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 \(\mu\)M. When the inhibition
percentage was measured in a \(\Delta\phi\) driven Na\(^+\) uptake assay,
a \(K_{0.5}\) of 15 \(\mu\)M 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 \(\mu\)M. Clonidine was found to inhibit NhaB activity
with a \(K_{0.5}\) of 200 \(\mu\)M and cimetidine did not have any effect
on NhaB activity.

Interestingly Mn\(^2+\) has quite a high affinity to NhaB, compara-
tible 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 \([20]\).

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possible implications for this drug other than its classical one.
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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
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: (LVGGXX) which is located in the middle of the fourth putative trans-membrane segment. We found in the sequence of NhaB the pentamer (LV) 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, 53% similarity) [29] and the product of the mouse pink-eyed dilution gene (21% identity, 53% similarity) [30] and the product of the mouse pink-eyed dilution gene (21% identity, 53% similarity) [30]. A conserved motif is 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: (LVGGXXxDD) [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: (LVGGXXxDD) [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: (LVGGXXxDD) [29] and the product of the mouse pink-eyed dilution gene (21% identity, 53% similarity) [30].

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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