Biochimica et Biophysica Acta, 1024 (1990) 385–389
Elsevier

BBAMEM 70655

BBA Report

An essential role for vicinal dithiol groups in the catalytic activity of the human placental Na^+-H^+ exchanger

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(Received 15 December 1989)

Key words: Sodium-proton exchanger; Vicinal dithiol group; Phenylarsine oxide; Amiloride; Brush border; (Human placenta)

We examined the effects of phenylarsine oxide, a reagent specific for vicinal dithiol groups, on the catalytic activities, Na^+ influx and H^+ efflux, of the human placental Na^+-H^+ exchanger. Treatment of the placental brush-border membrane vesicles with the reagent markedly inhibited both the activities. The inhibition was partially reversible by dithiols. The effect of phenylarsine oxide was to reduce the maximal velocity of the exchanger without influencing its affinity for Na^+. The exchanger was partially protected from this inhibition by amiloride but not by cimetidine even though both these compounds interacted with the Na^+-binding site. The data demonstrate that vicinal dithiol groups are essential for the catalytic function of the placental Na^+-H^+ exchanger and that the critical dithiol groups are located at a site distinct from the Na^+-binding site.

Na^+-H^+ exchanger is an ubiquitous plasma membrane transport system which catalyzes the influx of Na^+ coupled to the efflux of H^+ in a cell under physiological conditions [1]. The exchanger participates in a variety of cellular functions such as regulation of intracellular pH and cell volume, transport of acid and base equivalents, modulation of intracellular Ca^{2+} and Na^+ activities, and cellular proliferation [1–6]. The role of amino acid residues in the catalytic activity of the Na^+-H^+ exchanger has been probed in many laboratories using group-specific reagents. These studies have established the essential nature of carboxyl [7–12], histidyl [9,13], sulfhydryl [11,14], and amino [15] groups in the funciton of the exchanger. The purpose of the current investigation was to study the nature of the essential sulfhydryl groups. Vicinal dithiol groups have been implicated in recent years in the catalytic activity of many H^+-coupled transport systems [16–18]. The present study was designed to determine whether vicinal dithiol groups are essential for the activity of the Na^+-H^+ exchanger. These experiments were done with the human placental Na^+-H^+ exchanger [19] using brush-border membrane vesicles purified from normal term human placentas. The chemical probe employed in these experiments was phenylarsine oxide (PAO), a reagent specific for vicinal dithiols [20,21].

Normal term human placentas, obtained within 3 h of delivery were used to prepare brush-border membrane vesicles. The detailed procedure for the preparation has been described earlier [19,22]. Alkaline phosphatase activity in the final membrane preparation was enriched 20–25-fold compared to the activity in the homogenate.

Membrane vesicles were treated with PAO in 20 mM K_2HPO_4/KH_2PO_4 buffer (pH 8) containing 280 mM mannitol for 30 min at 37°C. Stock solutions of PAO were made in ethanol and control vesicles received an equal amount of ethanol alone. In experiments where the reversal of inhibition by thiols was studied, control and treated membranes were again incubated with or without thiols at room temperature (21–22°C) for 30 min. In protection experiments, membranes were first incubated with the protecting agent at room temperature for 15 min before the addition of PAO. In all cases, the reagents were removed following incubation by dilution and centrifugation. The membrane vesicles were finally preloaded with 25 mM 4-morpholineethanesulfonic acid (Mes)-Tris buffer (pH 5.5) containing 300 mM mannitol. Protein concentration of the membrane suspension was adjusted to 5 mg/ml before used in assays for the Na^+-H^+ exchanger activity.

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 4-morpholineethanesulfonic acid; PAO, phenylarsine oxide.

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Two different procedures were used to determine the exchanger activity, one quantifying Na^+ influx using $^{22}Na^+$ and the other monitoring H^+ efflux using Acridine orange. The $^{22}Na^+$ influx measurements were made at room temperature (21–22° C) by a rapid filtration technique [23]. Millipore filters (DAWP, 0.65 μm pore size) were employed in these experiments. Uptake of Na^+ was initiated by mixing 40 μl membrane suspension with 160 μl of uptake buffer containing unlabeled Na^+ (as NaCl) and traces of $^{22}Na^+$. Final concentration of Na^+ during uptake was 0.5 mM. The uptake buffer was 18 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes)-Tris, 300 mM mannitol (pH 7.5). Under these conditions, the extravesicular pH was 7.2. After incubation for a desired time at room temperature, uptake was terminated by adding 3 ml of ice-cold stop buffer (5 mM Hepes-Tris, 160 mM KCl (pH 7.5)) and the mixture was filtered. The filter was washed with 3 × 5 ml of stop buffer and the radioactivity associated with filter was counted. Na^+ uptake which occurred via the Na^+-H^+ exchanger was calculated by subtracting dimethylamiloride-insensitive uptake from the total uptake.

The H^+ efflux was monitored at room temperature (21–22° C) by the changes in the absorbance of the ΔpH indicator, acridine orange [24]. Briefly, 1 ml of 18 mM Hepes-Tris (pH 7.5) containing 300 mM mannitol, 6 μM Acridine orange and either 40 mM NaCl or 40 mM KCl was taken in a cuvette and the absorbance of the dye as measured by dual wavelength spectrophotometry (492–540 nm) was monitored. When the absorbance had stabilized, 20 μl of membrane suspension (protein concentration, 1.5 mg/ml) was added to the cuvette. Since the membrane vesicles were preloaded with pH 5.5 buffer, the absorbance of the dye decreased indicating the ΔpH across the membrane. Following this initial decrease, the absorbance began to increase with time due to dissipation of the pH gradient. This time-dependent increase in the absorbance was recorded and the initial rates of the increase were calculated from the slopes of the curves. The Na^+-H^+ exchanger activity was quantified by subtracting the absorbance change in the presence of K^+ from the absorbance change in the presence of Na^+.

Each experiment was done in duplicate or triplicate using two or three different membrane preparations. The data are presented as mean ± S.E. The statistical significance was calculated by Student's *t*-test. A *P* value less than 0.05 was considered significant.

Carrier-free $^{22}NaCl$ (radioactivity, 200 μCi/ml) and D-[1-^{14}C]mannitol (spec. act., 16.8 mCi/mmol) were purchased from the Radiochemical Center, Amersham. Acridine orange and nigericin were obtained from Sigma. Phenylarsine oxide was from Aldrich Chemical Co. All other chemicals were of analytical grade.

Fig. 1 describes the time courses of Na^+ uptake in

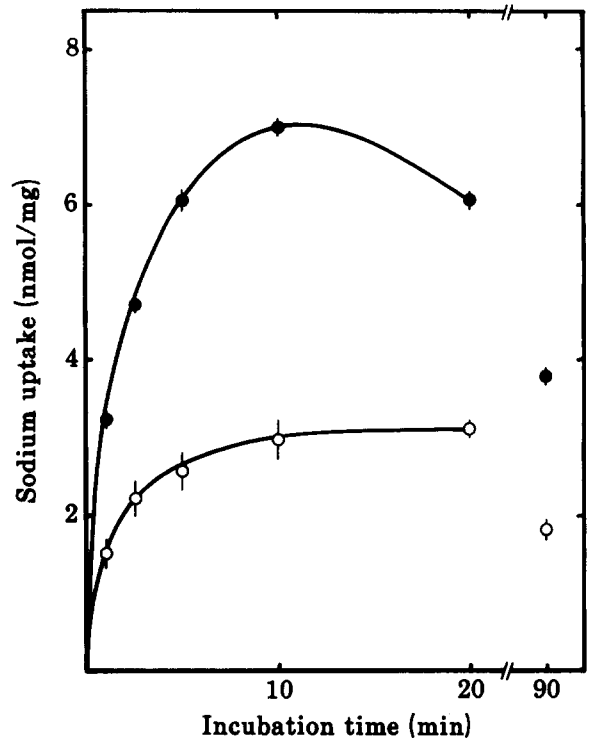

Fig. 1. Effect of PAO on the placental Na^+-H^+ exchanger. Membrane vesicles were incubated with or without 0.25 mM PAO at 37° C for 30 min. Following the incubation, the vesicles were washed twice with a pH 5.5 buffer to remove any residual unused reagent and finally suspended in the buffer. Uptake of Na^+ (0.5 mM) was measured in the presence of an outwardly directed H^+ gradient ($pH_i = 5.5$; $pH_o = 7.2$). ●———●, control; ○———○, PAO.

control and PAO-treated membrane vesicles. Uptake was measured in the presence of an outwardly directed H^+ gradient ($pH_i = 5.5$; $pH_o = 7.2$). Na^+ uptake was markedly reduced in treated membrane vesicles compared to control vesicles. At 1 min incubation, the uptake rate in treated vesicles was only 47% of the control uptake rate (1.48 ± 0.21 versus 3.17 ± 0.03 nmol/mg of protein). The uptake values measured at equilibriuim (90 min incubation) were also not identical in control and treated vesicles. This raised the possibility that the inhibitory effect of PAO on the initial rates Na^+ uptake might be due to decreased intravesicular volume rather than a direct effect on the Na^+-H^+ exchanger system. However, when the intravesicular volume was calculated using radiolabeled D-mannitol, the values were comparable in control and PAO-treated membrane vesicles (control, 0.97 ± 0.03 μl/mg; PAO, 0.90 ± 0.05 μl/mg). These results demonstrate that PAO treatment did not affect the integrity of the membrane vesicles. In one of our earlier papers [19], we have shown that binding of Na^+ to the membranes accounted for approx. 40% of the equilibrium value measured under the same experimental conditions as employed in the present study. Since there was no evidence for decreased intravesicular volume following PAO treat-

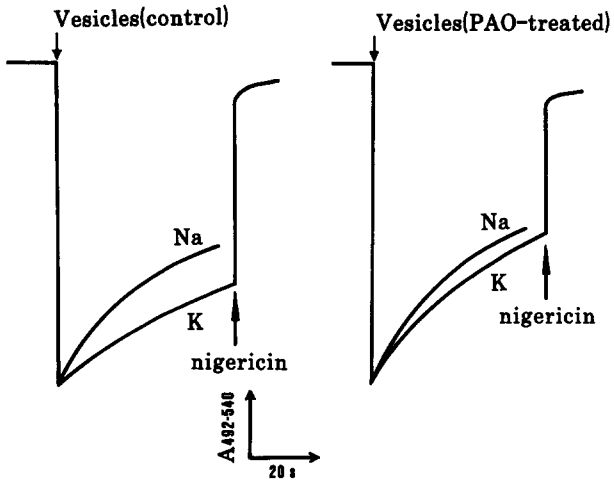

Fig. 2. Effect of PAO on Na^+-induced and K^+-induced H^+ efflux. Control and PAO (0.2 mM)-treated membrane vesicles were preloaded with a pH 5.5 buffer. The pH of the cuvette buffer was 7.5. The concentration of Na^+ or K^+ was 40 mM.

ment, it appears that the difference between the equilibrium values in control and PAO-treated vesicles was the result of changes in the binding component brought about by PAO treatment. This binding component was, however, negligible when uptake was measured with short incubations because, under these conditions, more than 90% of Na^+ uptake was inhibitable by the inhibitors of Na^+-H^+ exchanger such as dimethylamiloride. Since PAO treatment caused inhibition of Na^+ uptake measured with short incubations, it is concluded that the inhibition was due to a direct effect of PAO on the Na^+-H^+ exchanger.

We also determined the effect of PAO treatment on the Na^+-H^+ exchanger by measuring H^+ efflux in control and PAO-treated membrane vesicles. In this experiment, the difference between the Na^+-induced H^+ efflux and the K^+-induced H^+ efflux indicated the exchanger activity. As can be seen in Fig. 2, the exchanger was inhibited by PAO treatment. Also shown in Fig. 2 is the effect of nigericin, an ionophore for Na^+, K^+ and H^+. Addition of the ionophore induced a rapid efflux of H^+, indicating that there were no changes in the ionic gradients across the membranes in control and treated vesicles. Even though this experiment provided a clear evidence for the inhibition of the Na^+-H^+ exchanger by PAO, there was also indication that the H^+ permeability was significantly greater in PAO-treated vesicles compared to control vesicles. In order to eliminate any influence of the alterations in H^+ permeability on the exchanger, the activity of the exchanger was quantified from the slopes of the curves recorded over a short period of 2 s. The pooled data from two experiments showed that the Na^+-H^+ exchanger activity, measured as the difference between the Na^+-induced and the K^+-induced changes in the acridine orange absorbance was (in absorbance/mg per s) 0.0473 in control vesicles and 0.0277 in PAO-treated vesicles. These results provide direct evidence for the inhibition of the placental Na^+-H^+ exchanger by PAO.

The effect of increasing concentrations of PAO during treatment on the rates of Na uptake was studied using 30 s incubations. A 50% inhibition was observed when the concentration of PAO was 150–200 μM (data not shown).

PAO-induced inhibition of the Na^+-H^+ exchanger should be reversible with thiols which can regenerate the exchanger in the unmodified original form. Since dithiols are known to be more effective than monothiols in reversing the PAO reaction [20], we examined the ability of dimercaptopropanol (2 mM) and dithiothreitol (20 mM) to reverse the PAO-induced inhibition of the exchanger activity. We measured Na^+ uptake in membrane vesicles which were treated in four different ways to provide proper controls for the assessment of the reversal of the inhibition: (a) control vesicles, (b) vesicles treated with PAO, (c) vesicles treated with dithiol, and (d) vesicles treated with PAO followed by treatment with dithiol. This strategy allowed us to take into account the direct effect, if any, of the dithiols on the exchanger activity. The data given in Table I show that both dimercaptopropanol and dithiothreitol were able to reverse the PAO-induced inhibition of the Na^+-H^+ exchanger to a significant extent.

The kinetics of the Na^+-H^+ exchanger in control and PAO-treated membrane vesicles were investigated to determine whether the PAO-induced inhibition was due to a reduction in the affinity of the exchanger for Na^+ or to a decrease in the maximal velocity of the exchanger. In these experiments, membrane vesicles were treated in the presence or absence of 250 μM PAO. After removal of the unreacted reagent, rates of Na^+ uptake were measured in these vesicles in the presence of an outwardly directed H^+ gradient (pH_i 5.5, pH_o 7.2) using 30 s incubations. The concentration of Na^+ was varied between 1 and 40 mM. The results in Fig. 3, given as Eadie-Hofstee plots (uptake rate/Na^+ concentration versus uptake rate), demonstrate that the

TABLE I

Reversal of PAO-induced inhibition of the Na^+-H^+ exchanger by dithiols

Experimental condition	Na^+ uptake			
	dimercaptopropanol (2 mM)		dithiothreitol (20 mM)	
	nmol/mg per 30 s	%	nmol/mg per 30 s	%
Control	1.76 ± 0.04	100	2.61 ± 0.05	100
PAO (0.25 mM)	0.59 ± 0.02	34	0.87 ± 0.03	33
Dithiol	1.92 ± 0.04	100	1.74 ± 0.08	100
PAO + dithiol	1.13 ± 0.07	59	0.98 ± 0.08	56

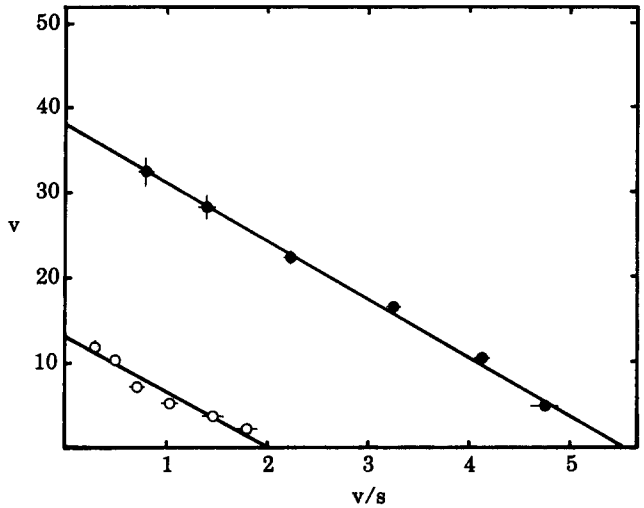

Fig. 3. Effect of PAO on the kinetic parameters of the placental Na^+-H^+ exchanger. Rates of Na^+ uptake (30 s) were measured over a Na^+ concentration range of 1–40 mM in control and PAO (0.25 mM)-treated vesicles. v, uptake rate (nmol/mg per 30 s); s, Na^+ concentration (mM). ●———●, control; ○———○, PAO.

exchanger obeyed Michaelis-Menten kinetics describing a single uptake system (i.e., the plots were linear in both control and treated vesicles; $r^2 > 0.95$). The apparent dissociation constant (K_t) for Na^+ was 6.8 ± 0.2 mM in control vesicles which was not different from the corresponding value in treated vesicles (6.5 ± 0.8 mM). On the contrary, the value for the maximal velocity (V_{max}) was markedly reduced in treated vesicles compared to control vesicles (12.9 ± 0.9 versus 37.8 ± 0.6 nmol/mg of protein/30 s). It has to be mentioned, however, that since the uptake rates measured with a 30 s incubation did not strictly represent the initial rates (see Figs. 1 and 2), the kinetic constants reported here are only approximate values.

Reversible inhibitors of the Na^+-H^+ exchanger such as amiloride and its derivatives, cimetidine and clonidine have been shown to interact with the Na^+-binding site of the exchanger molecule [2,22,23]. These inhibitors have been successfully employed to determine if an amino acid group, which has been shown to be essential for the exchanger activity using group-specific reagents, is located at the Na^+-binding site [7–13]. We employed a similar strategy to define the location of the essential vicinal dithiol groups. For this purpose, the ability of amiloride and cimetidine to protect the placental Na^+-H^+ exchanger from PAO-induced inhibition was investigated. Because these inhibitors are freely reversible, they could be completely removed by the washing procedure prior to uptake measurements. The results of the experiments, given in Table II, show that amiloride offered a significant protection, but cimetidine did not. However, we have used cimetidine in an earlier study to demonstrate the presence of essential histidyl and carboxyl groups at the Na^+-binding site of the placental Na^+-H^+ exchanger [9]. Therefore, the essential vicinal dithiol groups do not appear to be located at this site. The protection by amiloride is intriguing and interesting. Igarashi and Aronson [11] have shown that the renal Na^+-H^+ exchanger was inhibited by *N*-ethylmaleimide (NEM), a thiol group-specific reagent, but the exchanger was not protectable from this inhibition by amiloride. PAO, in contrast to NEM, is very specific for vicinal dithiols and hence it is possible that the nature and location of the NEM-reactive and the PAO-reactive thiol groups are different. This might explain the observed differences in the ability of amiloride to protect the exchanger from the inhibition induced by these reagents. Alternately, the molecular structures of the renal and placental Na^+-H^+ exchangers may not be identical. This view is supported by the pharmacological distinction between the two exchangers recently observed in our laboratory [25]. The protection of the placental Na^+-H^+ exchanger by amiloride but not by cimetidine indicates that the reactive vicinal dithiol groups are located at a site which interacts exclusively with amiloride. Even though amiloride and cimetidine commonly interact with the Na^+-binding site, there may be different subsites invovled in the binding of these two inhibitors. There is evidence that amiloride interacts with a modifier site which is distinct from the Na^+-binding site [26,27]. This modifier site is a potential location for the essential vicinal dithiol groups.

TABLE II

Ability of amiloride and cimetidine to protect the placental Na^+-H^+ exchanger from PAO-induced inhibition

Experimental condition	Na^+ uptake	
	nmol/mg per 30 s	%
Control	1.66 ± 0.17	100
PAO (0.25 mm)	0.63 ± 0.07	38
Amiloride (0.6 mM)	1.66 ± 0.18	100
Amiloride + PAO	0.91 ± 0.10	55 ($P < 0.002$)
Cimetidine (5 mM)	1.84 ± 0.23	100
Cimetidine + PAO	0.66 ± 0.06	36 ($P > 0.5$)

In conclusion, the data presented here clearly demonstrate that vicinal dithiol groups are essential for the catalytic activity of the Na^+-H^+ exchanger present in the human placental brush-border membrane. In contrast to the essential histidyl and carboxyl groups which are located at the Na^+-binding site of the exchanger, the vicinal dithiol groups are not present at the Na^+-binding site but at a site which specifically interacts with amiloride.

Acknowledgements

This work was supported in part by NIH Grant HD 22103. The authors thank Mrs. Kira M. Smith and Mrs.

Maria Rendondo for expert secretarial assistance. This is contribution No. 1217 from the Department of Cell and Molecular Biology.

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