Evidence for histidyl and carboxy groups at the active site of the human placental $\text{Na}^+\text{--H}^+$ exchanger

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The $\text{Na}^+\text{--H}^+$ exchanger of the human placental brush-border membrane was inhibited by pretreatment of the membrane vesicles with a histidyl-group-specific reagent, diethyl pyrocarbonate and with a carboxy-group-specific reagent, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline. In both cases the inhibition was irreversible and non-competitive in nature. But, if the membrane vesicles were treated with these reagents in the presence of amiloride, cimetidine or clonidine, there was no inhibition. Since amiloride, cimetidine and clonidine all interact with the active site of the exchanger in a mutually exclusive manner, the findings provide evidence for the presence of essential histidyl and carboxy groups at or near the active site of the human placental $\text{Na}^+\text{--H}^+$ exchanger. This conclusion was further substantiated by the findings that Rose Bengal-catalysed photo-oxidation of histidine residues as well as covalent modification of carboxy residues with *NN'*-dicyclohexylcarbodi-imide irreversibly inhibited the $\text{Na}^+\text{--H}^+$ exchanger and that amiloride protected the exchanger from inhibition caused by *NN'*-dicyclohexylcarbodi-imide.

INTRODUCTION

The $\text{Na}^+\text{--H}^+$ exchanger is a plasma-membrane protein identified in a wide variety of cell types (Aronson, 1985). Under physiological conditions this exchanger couples in an electrically silent manner the influx of Na^+ into the cell with the efflux of H^+ from the cell. In recent years a sudden surge of interest has developed for this protein, because its activity is stimulated by a variety of growth factors (Macara, 1985; Mahnensmith & Aronson, 1985). We have recently discovered the presence of the $\text{Na}^+\text{--H}^+$ exchanger in brush-border membranes isolated from normal term human placenta (Balkovetz *et al.*, 1986). The properties of the human placental $\text{Na}^+\text{--H}^+$ exchanger are similar to those of the exchanger in other cell types. Amiloride and its derivatives are potent inhibitors of the exchanger. During the course of the work with the human placental $\text{Na}^+\text{--H}^+$ exchanger we have demonstrated that cimetidine, an H_2 -receptor antagonist, and clonidine, an α_2 -receptor agonist, also inhibit the exchanger activity (Ganapathy *et al.*, 1986a,b). The inhibition by amiloride, cimetidine and clonidine is freely reversible and is purely competitive in nature. Kinetic analyses have shown that all three inhibitors interact with the external Na^+ -binding site of the exchanger in a mutually exclusive manner (Ganapathy *et al.*, 1986b).

By the use of group-specific reagents many studies have indicated that histidyl and carboxy groups are essential for the activity of the kidney brush-border-membrane $\text{Na}^+\text{--H}^+$ exchanger and that these groups are located at or near the external binding site of the exchanger (Burnham *et al.*, 1982; Grillo & Aronson, 1986; Friedrich *et al.*, 1986; Igarashi & Aronson, 1987).

The present paper provides evidence that the active site of the human placental $\text{Na}^+\text{--H}^+$ exchanger also contains histidyl and carboxy groups that are critical for the exchanger activity.

EXPERIMENTAL

Materials

Carrier-free $^{22}\text{NaCl}$ (200 $\mu\text{Ci/ml}$) was purchased from Amersham/Searle. Diethyl pyrocarbonate (DEP), *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ), *NN'*-dicyclohexylcarbodi-imide (DCCD), Rose Bengal, clonidine and cimetidine were obtained from Sigma Chemical Co. Amiloride was a gift from Dr. K. Green, Department of Physiology, Medical College of Georgia. All other chemicals were of analytical grade.

Methods

Preparation of membrane vesicles. Brush-border-membrane vesicles from normal term human placenta were prepared by an Mg^{2+} precipitation method as previously described (Ganapathy *et al.*, 1985; Balkovetz *et al.*, 1986). The marker enzymes for the brush-border membrane, alkaline phosphatase (EC 3.1.3.1) and 5'-nucleotidase (EC 3.1.3.5), were both enriched 26 ± 9 -fold in these preparations compared with the homogenate of the washed placental tissue.

Treatment with group-specific reagents. Membrane vesicles were treated at pH 6.4 with a histidyl-group-specific reagent, DEP, or with carboxy-group-specific reagents, EEDQ and DCCD, as described by Bindsløv & Wright (1984). Stock solutions of EEDQ and DCCD

Abbreviations used: DEP, diethyl pyrocarbonate; EEDQ, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline; DCCD, *NN'*-dicyclohexylcarbodi-imide.

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were made in ethanol, whereas stock solutions of DEP were made by mixing DEP and ethanol followed by dilution with the buffer. The concentration of ethanol during the pretreatment of the membranes with DEP, EEDQ and DCCD was 0.05%, 1% and 5% (v/v) respectively. Equal concentration of ethanol was added to the corresponding controls. After treatment, the reaction mixture was diluted with the preloading buffer, which was 150 mM-KCl/25 mM-Mes/Tris buffer, pH 5.5. The membrane vesicles were collected by centrifugation and washed one more time with the same buffer. The final membrane pellets were suspended in a small volume of the preloading buffer. The protein concentration was adjusted to 6 mg/ml. When the treatment of the membranes with DEP, EEDQ or DCCD was done in the presence of amiloride, cimetidine or clonidine, these protecting agents were first added to the membranes followed by the group-specific reagents. Since amiloride, cimetidine and clonidine are all freely reversible inhibitors of the exchanger, these agents could be almost completely removed from the membranes by the washing procedure. In these protection experiments, control membranes were treated with the protecting agents alone in the absence of the group-specific reagents.

Photo-oxidation of histidine residues. Membrane vesicles (protein concentration 0.2 mg/ml) were equilibrated at pH 7.0 with 0.1 μ M-Rose Bengal for 2 h in the dark. Photo-oxidation was performed in a Rayonet photochemical reactor (model RPR-400; Southern New England Ultraviolet Co., Hamden, CT, U.S.A.) equipped with 16 RPR-3500 Å lamps and with a fan that maintained the temperature below 35 °C. After irradiation, the membranes were washed twice with 150 mM-KCl/25 mM-Mes/Tris buffer, pH 5.5. All operations, including irradiation, washing and uptake measurements, were done under a red light, because exposure of the control membranes (no irradiation) that were equilibrated with Rose Bengal to the laboratory fluorescent light itself caused complete inactivation of the Na⁺-H⁺ exchanger. If these precautions were taken, Rose Bengal in the absence of irradiation did not affect the exchanger activity.

Uptake studies. ²²Na⁺-uptake measurements were made by using a Millipore filtration technique as described previously (Balkovetz *et al.*, 1986) in the medium 150 mM-KCl/18 mM-Hepes/Tris buffer, pH 7.5. The extravesicular pH at the initiation of the uptake was 7.2 owing to the carry-over of the preloading buffer containing the membranes. For kinetic analyses a 45 s incubation was employed to measure initial uptake rates, since ²²Na⁺ uptake in these vesicles was found to be linear at least up to 1 min over an Na⁺ concentration range of 0.5–50 mM (Balkovetz *et al.*, 1986).

RESULTS AND DISCUSSION

Inhibition of the Na⁺-H⁺ exchanger by group-specific reagents

Fig. 1 describes the time course of Na⁺ uptake (0.5 mM) in control vesicles as well as in vesicles treated with the inhibitors. In all cases Na⁺ uptake was measured in the presence of an outward-directed H⁺ gradient (pH_i 5.5; pH_o 7.2). Control vesicles exhibited the overshoot phenomenon, indicating that the Na⁺

Fig. 1. Effects of DEP and EEDQ on the human placental Na⁺-H⁺ exchanger

Membrane vesicles were incubated with either 2 mM-DEP or 2 mM-EEDQ. After 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 outward-directed H⁺ gradient (pH_i 5.5; pH_o 7.2). ●, Control; ○, DEP; ■, EEDQ.

uptake was energized by the H⁺ gradient. Treatment of the membrane vesicles with either DEP (2 mM) or EEDQ (2 mM) drastically decreased the initial rates of Na⁺ uptake, and the concentrative uptake was abolished in both cases.

We have previously shown that, in the human placental brush-border-membrane vesicles, about 90% of the Na⁺ uptake observed in the presence of an outward-directed H⁺ gradient was due to the activity of the Na⁺-H⁺ exchanger (Balkovetz *et al.*, 1986). The remaining 10% represented the diffusional component. We therefore specifically studied the effect of DEP and EEDQ on the Na⁺ uptake that occurred via the exchanger by determining the amiloride-sensitive Na⁺ uptake. This uptake was calculated by subtracting from the control values the uptake of Na⁺ measured in the presence of 1 mM-amiloride. Fig. 2 shows the progressive inhibition of the amiloride-sensitive Na⁺ uptake by increasing concentrations of DEP or EEDQ. With both inhibitors 50% inhibition was observed at a concentration of 0.5–0.8 mM.

In these experiments the membrane vesicles were incubated with DEP or EEDQ, followed by removal of the residual unused reagent. Since the inhibition of the exchanger was demonstrable in these vesicles, it is apparent that these inhibitors covalently modify histidyl and carboxy groups respectively in the exchanger protein, thereby causing irreversible inhibition. We therefore examined the kinetic nature of the inhibition. We studied the dependence of amiloride-sensitive Na⁺

Fig. 2. Effects of inhibitor concentration on the amiloride-sensitive Na^+ uptake

Initial rates of Na^+ uptake (0.5 mM) were determined in control vesicles and in vesicles that were treated with increasing concentrations of DEP or EEDQ. Uptake measurements were made with 45 s incubations in the presence of an outward-directed H^+ gradient (pH_i 5.5; pH_o 7.2). Uptake of Na^+ in control vesicles under these conditions was 1.9 ± 0.2 nmol/mg of protein. This value was taken as 100% uptake. ●, DEP; ○, EEDQ.

uptake on the concentration of Na^+ in control vesicles and in vesicles that were treated with DEP or EEDQ. The Na^+ concentration was varied from 1 to 20 mM, and the concentration of DEP and EEDQ, when present, was 0.6 mM and 0.7 mM respectively. The results are given as Eadie-Hofstee plots in Fig. 3. The linear plots ($r^2 > 0.94$) indicate that the Na^+ uptake was saturable and that the process obeyed Michaelis-Menten kinetics. Treatment with DEP or EEDQ caused a decrease in the maximal velocity (ordinate-axis intercept of the lines) without changing the affinity of the exchanger for Na^+ (slope of the lines). These data indicate that DEP and EEDQ inhibit the $\text{Na}^+\text{-H}^+$ exchanger in a strictly non-competitive manner. Therefore it appears that the covalent modification of essential histidine residues by DEP as well as the covalent modification of essential carboxy groups by EEDQ inactivate the exchanger irreversibly and hence decrease the concentration of active exchanger molecules in the membrane. The affinity of the unmodified exchanger for one of its substrates, Na^+ , is, however, not affected.

The reaction of DEP with histidine residues is ordinarily reversible with hydroxylamine. Grillo & Aronson (1986) have indeed demonstrated that the inhibition of the renal $\text{Na}^+\text{-H}^+$ exchanger by DEP can be reversed with 0.5 M-hydroxylamine. However, exposure of the membranes to 0.5 M-hydroxylamine alone caused significant inhibition of the exchanger activity. In

Fig. 3. Effects of DEP and EEDQ on the kinetic parameters of the $\text{Na}^+\text{-H}^+$ exchanger

Initial rates of Na^+ uptake (45 s) were measured over an Na^+ concentration range, 1–20 mM, in control vesicles and in vesicles treated with either 0.6 mM-DEP or 0.7 mM-EEDQ. Only the amiloride-sensitive Na^+ uptake was used to construct the Eadie-Hofstee plots (v/s versus v). v , Initial velocity (nmol/45 s per mg of protein); s , concn. of Na^+ (mM). ●, Control; ○, DEP; ■, EEDQ.

the present study we observed that the human placental $\text{Na}^+\text{-H}^+$ exchanger was more sensitive to inhibition by hydroxylamine than was the renal $\text{Na}^+\text{-H}^+$ exchanger. Exposure of the placental brush-border membranes to 0.2 M-hydroxylamine alone caused 55% inhibition of the exchanger. Therefore we could not use this approach to prove that the inhibition of the exchanger by DEP was due to modification of histidine residues. Even though DEP can react with thiol and hydroxy groups in proteins, the reagent is specific for histidine residues in the pH range 5.5–7.5 (Miles, 1977). Since the membranes were treated with DEP at pH 6.4 in the present study, it is reasonable to assume that inactivation of the $\text{Na}^+\text{-H}^+$ exchanger by DEP was the result of modification of the histidine residues.

We employed another approach to substantiate the essential nature of histidine residues in the catalytic activity of the placental $\text{Na}^+\text{-H}^+$ exchanger. Rose Bengal-catalysed photo-oxidation has been used to modify histidine residues in proteins (Westhead, 1972). When the placental brush-border-membrane vesicles were irradiated in the presence of 0.1 μM -Rose Bengal, the $\text{Na}^+\text{-H}^+$ -exchanger activity as measured by the initial rates of amiloride-sensitive Na^+ uptake in the presence of an outward-directed H^+ gradient was drastically inhibited (Fig. 4). However, irradiation in the presence of Rose Bengal also caused disruption of vesicles, resulting in a significant decrease in the equilibrium Na^+ uptake. Nonetheless these results provide additional evidence for the involvement of

Fig. 4. Effect of Rose Bengal-catalysed photo-oxidation on the $\text{Na}^+\text{-H}^+$ exchanger

Membrane vesicles were irradiated in the presence of $0.1\ \mu\text{M}$ -Rose Bengal for different time intervals. Initial uptake (45 s) or equilibrium uptake (2 h) of $^{22}\text{Na}^+$ was measured in these vesicles in the presence of an outward-directed H^+ gradient. ●, Initial uptake; ○, equilibrium uptake.

histidine residues in the activity of the human placental $\text{Na}^+\text{-H}^+$ exchanger.

To substantiate the essential nature of carboxy groups in the activity of the placental $\text{Na}^+\text{-H}^+$ exchanger, another carboxy-group-specific reagent, DCCD, was employed. Like EEDQ, DCCD also covalently modifies carboxy groups in proteins. Fig. 5 shows that treatment of placental brush-border membranes with increasing concentrations of DCCD caused progressive inhibition of the $\text{Na}^+\text{-H}^+$ exchanger. In fact, DCCD was found to be more effective than EEDQ in inhibiting the exchanger activity.

Protection from inhibition caused by group-specific reagents

Amiloride, cimetidine and clonidine are all freely reversible inhibitors of the human placental $\text{Na}^+\text{-H}^+$ exchanger, and they compete with Na^+ and Li^+ for the external binding site of the exchanger (Ganapathy *et al.*, 1986a,b). In order to determine if the essential histidyl and carboxy groups are located at this active site of the exchanger, we investigated the ability of amiloride, cimetidine and clonidine to protect the exchanger from DEP from EEDQ inhibition. In these experiments the membranes were treated with DEP or EEDQ in the presence of the protecting agents, and then the residual unused reagents were removed by washing. The results given in Table 1 show that, in the presence of amiloride, cimetidine or clonidine, the inhibition of amiloride-

Fig. 5. Effect of increasing concentrations of DCCD on the amiloride-sensitive Na^+ uptake

The experimental details are as given in the legend to Fig. 2.

sensitive Na^+ uptake caused by treatment with DEP or EEDQ was either abolished or greatly diminished.

Even though amiloride at a concentration of $0.6\ \text{mM}$ was able to provide almost complete protection from DEP and EEDQ inhibition, when similar attempts were made to protect the $\text{Na}^+\text{-H}^+$ exchanger from DCCD inhibition it was observed that much higher concentrations of the protecting agent were required to offer significant protection. When such high concentrations of amiloride were employed, this reagent could not be completely removed by the washing procedure. For example, membrane vesicles that were treated with $2\ \text{mM}$ -amiloride and then subsequently washed possessed only 50% the $\text{Na}^+\text{-H}^+$ -exchanger activity compared with control vesicles that were treated in a similar way in the absence of amiloride. However, when respective controls were compared, amiloride at a concentration of $2\ \text{mM}$ significantly protected the $\text{Na}^+\text{-H}^+$ exchanger from DCCD inhibition (Fig. 6).

The rationale for these protection experiments was as follows. Amiloride, cimetidine and clonidine all interact with the active site of the placental $\text{Na}^+\text{-H}^+$ exchanger in a freely reversible manner. Therefore, if histidyl and carboxy groups are located at or near the active site, they will be masked in the presence of these inhibitors and rendered unavailable for reaction with the group-specific reagents. Thus these reversible inhibitors can be expected to protect the $\text{Na}^+\text{-H}^+$ exchanger from inhibition by DEP, EEDQ or DCCD. Therefore the data presented in this paper are taken as strong evidence for the location of essential histidyl and carboxy groups at or near the active site of the placental $\text{Na}^+\text{-H}^+$ exchanger. It can be argued that these protecting agents may themselves react

Fig. 6. Protection by amiloride of the Na⁺-H⁺ exchanger from DCCD inhibition

Membrane vesicles were treated with 0.4 mM-DCCD at pH 6.4 in the presence and in the absence of 2 mM-amiloride. After treatment, the vesicles were washed twice with the preloading buffer (150 mM-KCl/25 mM-Mes/Tris buffer, pH 5.5). Uptake of ²²Na⁺ was measured at pH 7.2, in 45 s incubations.

Table 1. Protection of the human placental Na⁺-H⁺ exchanger from DEP and EEDQ inhibition

Placental brush-border-membrane vesicles were treated with either DEP (0.6 mM) or EEDQ (0.7 mM) at pH 6.4. The protecting agents (amiloride, cimetidine and clonidine) were added to the membranes before the addition of DEP or EEDQ. In each case of protecting agent, membrane vesicles treated with the agent in the absence of DEP and EEDQ served as the control. After incubation, membrane vesicles were washed twice with the preloading buffer (150 mM-KCl/25 mM-Mes/Tris buffer, pH 5.5). Uptake of ²²Na⁺ was measured at an extravesicular pH of 7.2, employing 45 s incubations. The results are expressed as means ± S.D. for three determinations.

Protecting agent	Inhibition (%)	
	DEP	EEDQ
None	67 ± 10	50 ± 4
Amiloride (0.6 mM)	-13 ± 7	8 ± 3
Cimetidine (5 mM)	4 ± 3	9 ± 3
Clonidine (12.5 mM)	13 ± 4	9 ± 4

with the group-specific reagents and that the protection offered by these agents is due to a decrease in the effective concentrations of the group-specific reagents. This argument is, however, untenable, because other investigators have demonstrated that amiloride, one of

the protecting agents employed in the present study, does not interact with DEP (Grillo & Aronson, 1986) or with DCCD (Igarashi & Aronson, 1987).

Non-involvement of tyrosyl groups in the Na⁺-H⁺-exchanger activity

The phenolic group of tyrosine has been implicated in the binding of Na⁺ to the Na⁺/glucose and Na⁺/proline co-transporters (Peerce & Wright, 1985). We therefore investigated the possible involvement of tyrosine residues in the binding of Na⁺ to the human placental Na⁺-H⁺ exchanger by studying the effect of a tyrosyl-group-specific reagent, *N*-acetylimidazole, on the exchanger activity. Treatment of the placental brush-border-membrane vesicles with 5 mM reagent failed to inhibit the exchanger (results not shown), showing that tyrosine residues are not essential for the activity of the human placental Na⁺-H⁺ exchanger. This conclusion is, however, only tentative at present, until the effects of other tyrosyl-group-specific reagents on the placental Na⁺-H⁺ exchanger activity are investigated.

In conclusion, the data presented here clearly demonstrate that one or more essential histidyl and carboxy groups are located at or near the active site of the human placental Na⁺-H⁺ exchanger.

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