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The Renal Cortical Na^+/HCO_3^- Cotransporter VI: The Effect of Chemical Modification in Cotransporter Activity

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Abstract. The Na^+/HCO_3^- cotransporter is the main system that mediates bicarbonate removal out of the proximal tubule cell into the blood. We have previously partially purified this protein and showed that chemical modification of the α-amino groups by fluorescein isothiocyanate (FITC) inhibited the activity of the Na^+/HCO_3^- cotransporter. The inhibition was prevented by the presence of Na and bicarbonate suggesting that this compound binds at or near the substrate transport sites of the cotransporter. We examined the effect of agents that modify the sulfhydryl group (dithiothreitol), carboxyl groups (*n*-n′dicyclohexyl carbodiimide) and tyrosine residues (*p*-nitrobenzene sulfonyl fluoride, *n*-acetyl imidazole and tetranitromethane) on the activity of the cotransporter to gain insight into the chemical residues which may be important for transport function. The sulfhydryl residues modifier, carboxyl group modifier, and tyrosine modifier significantly inhibited bicarbonate dependent ^{22}Na uptake in basolateral membranes by 50–70% without altering the ^{22}Na uptake in the presence of gluconate indicating that these agents directly affected the cotransporter without affecting diffusive sodium uptake. The effect of the tyrosine modifier *n*-acetylimidazole was not prevented by the presence of Na and bicarbonate suggesting that the tyrosine residues are not at the substrate binding sites. To determine the presence and role of glycosylation on the Na^+/HCO_3^- cotransporter protein, we examined the effects of different glycosidases (endoglycosidase F and H, *N*-glycosidase F, O-glycanase) on the cotransporter activity. All glycosidases caused a significant 50–80% inhibition of cotransporter activity. These data demonstrate that N-glycosylation as well as O-glycosylation are important for the function of the Na^+/HCO_3^- cotransporter protein. Taken together, these results suggest that chemical modifiers of tyrosine, carboxyl and sulfhydryl groups as well as glycosylation are important for expression of full functional activity of the cotransporter.

Key words: Protein modification — Carboxyl groups — Sulfhydryl groups — Tyrosine modification — Glycosylation

Introduction

The Na^+/HCO_3^- cotransporter has been recognized as the main mechanism responsible for the transport of bicarbonate from the proximal tubule cells into the blood [1, 3]. The cotransporter plays an important role in the regulation of intracellular pH as evidenced by alterations in its activity under physiologic and pathophysiologic conditions [2, 22]. The cotransporter activity is enhanced by acidosis and decreased in alkalosis. In addition, cyclic AMP and calmodulin inhibit while protein kinase C stimulates the Na^+/HCO_3^- cotransporter [5, 23]. We have partially purified the renal cortical basolateral Na^+/HCO_3^- cotransporter with approximately 300-fold purification of the cotransporter protein [4]. In addition, we have shown that chemical modification of the alpha amino groups by fluorescein isothiocyanate (FITC) inhibited the activity of the Na^+/HCO_3^- cotransporter [24]. The inhibition was prevented by the presence of Na^+ and bicarbonate suggesting that this compound binds at or near the substrate transport sites of the cotransporter.

Despite the important role of the Na^+/HCO_3^- cotransporter in acid base physiology very limited data are available about the physicochemical properties and structure of the cotransporter. Different group-specific chemical modifying agents have been used successfully and extensively to identify the important structures and amino acid residues of membrane transport proteins for

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full functional expression of their activity (6–10, 12–21, 25–27). We studied the effects of chemical agents like dithiothreitol which act on the sulfhydryl groups, and modifiers like *p*-nitrobenzene sulfonyl fluoride, *n*-acetyl imidazole, and tetranitromethane which act on tyrosine residues. We also investigated the role of carboxyl group modifier, *n*-*n*′ dicyclohexyl carbodiimide, on the Na^+/HCO_3^- cotransporter activity. Furthermore, it is known that carbohydrate residues have been found to be important for secretion and targeting of proteins as well as in the expression of full functional activity of membrane proteins. Thus, we employed several glycosidases (endoglycosidase F and H, N-glycosidase F, O-glycanase) to study the roles of glycosylation on the Na^+/HCO_3^- cotransporter protein. The aim of the present studies was to elucidate the role of the sulfhydryl and tyrosine residues, carboxyl groups and carbohydrate moieties on the activity of the Na^+/HCO_3^- cotransporter.

Materials and Methods

Materials

Endoglycosidases F and H and *N*-glycosidase were purchased from Boehringer Mannheim (Indianapolis, IN). O-glycanase was bought from Genzyme (Cambridge, MA). Dithiothreitol, *p*-nitrobenzene sulfonyl fluoride, *n*-acetyl imidazole, tetranitromethane, and *n*-*n*′-dicyclohexyl carbodiimide were obtained from Sigma (St. Louis, MO). ^{22}Na was purchased from Amersham (Arlington Heights, IL). Sephadex LH-20 was bought from Pharmacia (Piscataway, NJ). General laboratory chemicals were of analytical grade and were acquired from Sigma or Fisher.

Basolateral Membrane Preparations

Renal cortical basolateral membrane vesicles were prepared from New Zealand white rabbits by means of differential and gradient centrifugation with ionic precipitation as described previously [22]. The procedure results in highly purified basolateral membrane enriched on the average of 12- to 14-fold in Na-K-ATPase activity compared with homogenates and with less than 5% contamination with brush border membranes.

Modification of Sulfhydryl Groups with Dithiothreitol

Freshly prepared basolateral membrane vesicles (2.5 mg/ml) were incubated with dithiothreitol (DTT) at 5 mM final concentration of DTT for 30 min. The incubation was carried out at 37°C with gentle constant shaking. The control experiments were performed under the same conditions in the presence of vehicle alone. After incubation, the vesicles were diluted with buffer A containing 250 mM mannitol, 50 mM HEPES, pH 7.5 and centrifuged in Beckman Ti 50 for 20 min at 30,000 × *g*. The pellet was resuspended in appropriate buffers for measurement of Na^+/HCO_3^- cotransporter activity as described below.

Modification of Carboxyl Groups with DCCD

Freshly prepared basolateral membrane vesicles (2.5 mg/ml) were incubated with *n*-*n*′-dicyclohexyl carbodiimide (DCCD) at final concentration of 0.5 mM DCCD for 30 min at 20°C. The treatment of BLMV with DCCD was carried out as described previously with minor modifications (14). Briefly 2.5 ml unit volume of Sephadex LH-20 was pre-equilibrated with buffer A. It was centrifuged thereafter at 1,000 × *g* for 3 min to remove excess buffer. The reaction mixture above was applied to the column then centrifuged at 50 × *g* for 10 min and thereafter at 2,000 × *g*. The above modified protocol was adequate to stop the reaction based on our preliminary experiments since 0.5 mM addition of DCCD resulted in vesicles with 95% intact transport activity in contrast to control vesicles without DCCD pretreatment. There was no significant protein loss during the process. Protein recovery amounted consistently to about 90%. The eluted vesicles were resuspended in buffer containing 250 mM mannitol and 50 mM HEPES pH 7.5 and washed twice by centrifugation. HCO_3^- dependent ^{22}Na uptake was measured as described below.

Modification of Tyrosine Residues

Freshly prepared purified basolateral membranes vesicles (2.5 mg/ml) were incubated with either tetranitromethane, TNM (to final concentration of 5 mM TNM) or *p*-nitrobenzene sulfonyl fluoride, NBSF (at final concentration of 250 μM NBSF) for 30 min at 22°C. Likewise, fresh basolateral membrane vesicles (2.5 mg/ml) were treated with *n*-acetyl imidazole, NAI (to final concentration of 75 mM NAI), in the presence (substrate protection) or in the absence of 100 mM $NaHCO_3$. The incubation was done for 60 min at 22°C. Following incubation with each respective reagent, the reaction was stopped by addition of ice-cold buffer containing 250 mM mannitol, 50 mM HEPES pH 7.5. The mixtures were centrifuged at 3,000 × *g* for 30 min. The pellets were resuspended in appropriate buffer for measurement of the HCO_3^- dependent ^{22}Na uptake.

Treatment of Basolateral Membranes Vesicles with Glycosidases

Freshly prepared purified basolateral membrane vesicles (2.5 mg/ml) were incubated with the following glycosidases in the presence of protease inhibitors as described earlier [13]. Individual samples were treated with either one of the following: endoglycosidase H, endo-H (2 units/ml final concentration), endoglycosidase F, endo-F (at final concentration of 2 units/ml); *N*-glycosidase F, PNF gase F (at final concentration of 2 units/ml); and O-glycanase, O-gly (at final concentration of 4 units/ml). All incubations were done at 4°C for 18 hours. The reaction was stopped by addition of ice-cold buffer containing 250 mM mannitol and 50 mM HEPES pH 7.5. The reaction mixture was centrifuged at 10,000 × *g* for 10 min and the pellet was obtained for measurement of the HCO_3^- dependent ^{22}Na uptake.

Measurement of Na^+/HCO_3^- Cotransporter Activity

HCO_3 dependent ^{22}Na uptake (a measurement of Na^+/HCO_3^- cotransporter activity) was measured at 3 sec by the rapid filtration method as

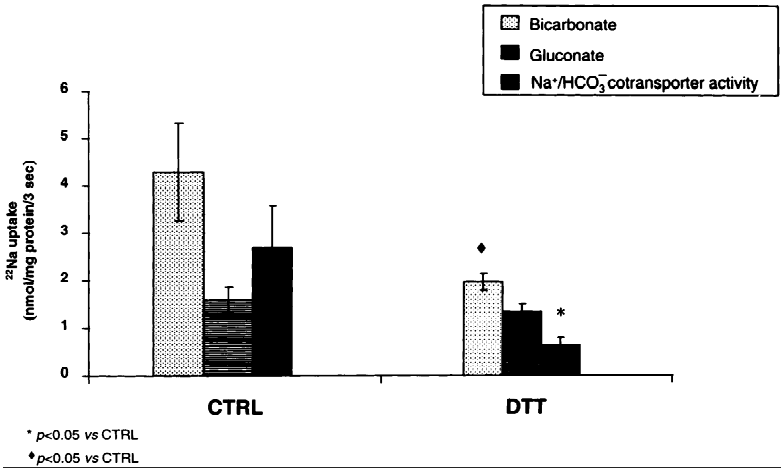

Fig. 1. Purified renal cortical basolateral membranes (2.5 mg/ml) were incubated with dithiothreitol (DTT), right panel, at 5 mM final concentration of DTT for 30 min at 37°C. The control (CTRL) experiments, left panel, were performed under the same conditions in the presence of vehicle alone. ^{22}Na uptake was done to measure the activity of the Na^+/HCO_3^- cotransporter. The data represent the mean ± SE for six separate experiments on different membrane preparations.

previously described [4, 22]. Briefly, basolateral membrane vesicles were preincubated for 1 hr at room temperature in a solution containing 200 mM sucrose, 50 mM HEPES, pH 7.5 with Tris and 1 mM mg gluconate. The suspension was centrifuged at 30,000 revolutions per minute with a Beckman Ti 50 rotor for 30 minutes at 4°C. The resulting pellet was resuspended in the same solution. The assay was started by addition of processed proteins to uptake medium containing (in mM) 40 Na gluconate, 60 K gluconate, 1 mg gluconate, and 50 HEPES, pH 7.50 with KOH as well as 1 μCi $^{22}NaCl$ in the presence of 25 mM HCO_3 or gluconate. After 3-sec incubation at room temperature the reaction was stopped by adding 4 ml ice-cold stop solution containing 200 mM sucrose and 50 mM HEPES, pH 7.50 with Tris, and subsequently poured on a 0.45-μM pore size prewetted Millipore filter. Filters were washed three times more, and radioactivity was measured by scintillation spectroscopy.

Data Analysis

Results are presented as mean ± SEM. The *t* test for paired data was used to analyze the results. In the figures the results are represented as ^{22}Na uptake in the presence of HCO_3^- and gluconate. Na^+/HCO_3^- cotransporter activity (HCO_3^- dependent ^{22}Na uptake) was taken as the difference in ^{22}Na uptake in the presence or in the absence of an inwardly directed HCO_3^- gradient (HCO_3 was replaced by gluconate). ^{22}Na uptake in the presence of gluconate was taken as diffusive Na uptake and was used to assess nonspecific effect of these agents on membrane vesicle integrity.

Results

Modification of the Sulfhydryl Groups with Dithiothreitol

To study the effect of dithiothreitol (DTT), a sulfhydryl group modifier, freshly prepared highly purified basolateral membrane vesicles were incubated with DTT for 30 min. After termination of the reaction and adequate washing, the activity of the Na^+/HCO_3^- cotransporter was measured as the difference in ^{22}Na uptake in the presence of bicarbonate and gluconate. Figure 1 shows that basolateral membranes vesicles pretreated with DTT significantly inhibited the ^{22}Na uptake in the presence of HCO_3 as compared to controls, but did not affect the diffusive ^{22}Na uptake in the presence of gluconate. The Na^+/HCO_3^- cotransporter activity (nanomoles/mg protein/3 sec) decreased from 2.70 ± .87 control, to 0.63 ± 0.15, DTT ($P < 0.02$).

Modification of the Carboxyl Groups with DCCD

To examine the role of the carboxyl groups on the activity of the Na^+/HCO_3^- cotransporter, highly purified basolateral membrane vesicles were treated with .5 mM *n-n'*dicyclohexyl carbodiimide (DCCD). Previous investigators have described that termination of the reaction with Sephadex LH-20 need to be monitored for its adequacy and the effects on protein recovery [14]. Preliminary experiments showed that the reaction was adequately terminated by filtration with Sephadex and the protein recovery was excellent. Figure 2 shows that DCCD pretreatment significantly inhibited the activity of the Na^+/HCO_3^- cotransporter activity as compared to untreated controls (control, 3.02 ± 0.62 *vs.* DCCD, 1.86 ± 0.50, $P < .03$). Incubation of the basolateral membranes with DCCD inhibited the Na^+/HCO_3^- cotransporter activity in the presence of bicarbonate but not in the presence of gluconate indicating that the effect of the carboxyl modifier DCCD is specific.

Effect of Tyrosine Residues Modifiers on the Na^+/HCO_3^- Cotransporter

Different chemical agents known to modify tyrosine residues in a protein have been utilized in different experiments. TNM (tetranitromethane) at final concentration of 5 mM was used to treat freshly prepared highly purified basolateral membrane vesicles. TNM had no effect

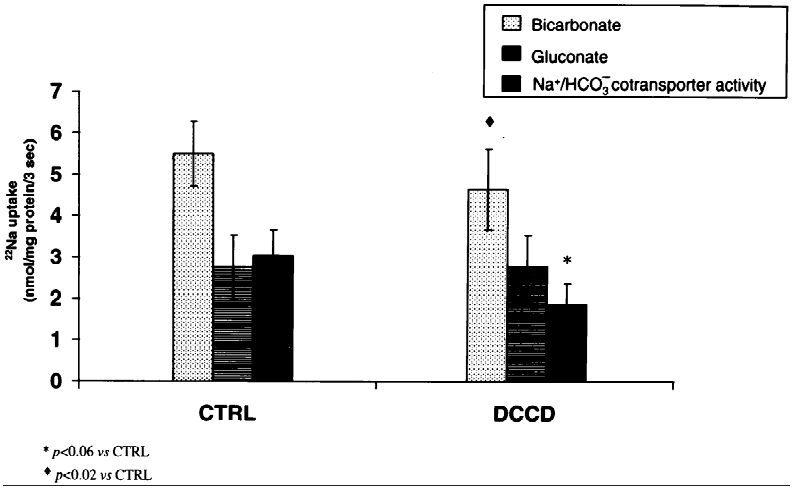

Fig. 2. Purified renal cortical basolateral membranes (2.5 mg/ml) were treated with *n-n'*-dicyclohexyl carbodiimide (DCCD), right panel, at final concentration of 0.5 mM DCCD, for 30 min at 20°C. The reaction was stopped by centrifugation and elution of the vesicles from Sephadex LH-20 column. Matched control (CTRL) experiments, left panel, were done under the same conditions. ^{22}Na uptake was done to measure the activity of the Na^+/HCO_3^- cotransporter. The data represent the mean ± SE for six separate experiments on different membrane preparations.

on the ^{22}Na uptake in the presence of gluconate in the experimental as well as the control group indicating that the agent has no effect on diffusive ^{22}Na uptake. As shown in Fig. 3, the addition of TNM caused a significant decrease in the activity of the Na^+/HCO_3^- cotransporter as compared to controls (control 1.31 ± 0.23 *vs.* TNM 0.28 ± 0.10, $P < .001$). NBSF (*p*-nitrobenzene sulfonyl fluoride) a different tyrosine residues modifier agent was also used. Incubation of the basolateral membrane vesicles with NBSF (Fig. 4) resulted in significant inhibition of the cotransporter activity, control, 1.33 ± 0.38 *vs.* NBSF treatment, 0.43 ± 0.12 ($P < .01$).

To understand the mechanism whereby tyrosine group modifiers may affect the activity of the cotransporter, NAI (*n*-acetyl imidazole) treatment of basolateral membrane vesicles were undertaken in the presence (substrate protection) or absence of $NaHCO_3$. Figure 5 shows that NAI treatment in the absence of $NaHCO_3$ inhibited the activity of the Na^+/HCO_3^- cotransporter, control 1.25 ± 0.30 *vs.* NAI treatment, 0.66 ± 0.20 ($P < .01$). In the presence of substrate protection during treatment with NAI (Fig. 6), the tyrosine modifying agent also inhibited the Na^+/HCO_3^- cotransporter (1.33 ± 0.31 *vs.* 0.67 ± 0.27, $P < .01$).

Effect of Glycosylation on the Na^+/HCO_3^- Cotransporter

To determine the role of different carbohydrate residues on the activity of the cotransporter protein, several glycosidases were utilized. Purified basolateral membrane vesicles were incubated with either the agents that act on the *N*-oligosaccharide groups (endo-F, endo-H, PNF gase F) or on the O-linked oligosaccharide groups (O-glycanase). As shown on Fig. 7, treatment of the basolateral membrane vesicles with endo-F (endoglycosidase F) led to significant inhibition of the Na^+/HCO_3^- cotransporter activity as compared to the untreated controls (control 2.11 ± 0.25 *vs.* Endo-F 0.80 ± 0.27, ($P < .001$). Likewise, incubation of the basolateral membranes with endo-H (endoglycosidase H) Fig. 8, resulted in the inhibition of the Na^+/HCO_3^- cotransporter activity control 1.97 ± 0.23 *vs.* 0.65 ± 0.07 endo-H ($P < 0.01$). In addition, PNF gase F (recombinant *N*-glycosidase F) also caused a significant reduction in the activity of the Na^+/HCO_3^- cotransporter activity in treated vesicles as compared to controls (Fig. 9). Another agent which acts on the O-linked saccharide groups, O-glycanase was also utilized to study the importance of O-linked glycosylation on the activity of the cotransporter. The results of the experiments indicated that O-glycanase caused a significant reduction in the activity of the Na^+/HCO_3^- cotransporter (Fig. 10).

Discussion

The important role of the Na^+/HCO_3^- cotransporter protein in the regulation of intracellular pH is well established [1, 3]. Likewise, studies have been published lately on the mechanisms of regulation of the cotransporter protein by the signal transduction pathways [5, 23]. However, despite the important role of the cotransporter protein, very limited data are available concerning its protein structure. The use of different chemical modifiers to probe the roles of several components of a protein has helped investigators to understand the chemical structures of a protein that are important in their physiologic functions. In this paper we investigated the roles of the sulfhydryl, and carboxyl groups, tyrosine residues, and carbohydrate moieties in the activity of Na^+/HCO_3^- cotransporter protein.

Previous studies have suggested that sulfhydryl groups are essential for the activity of membrane trans-

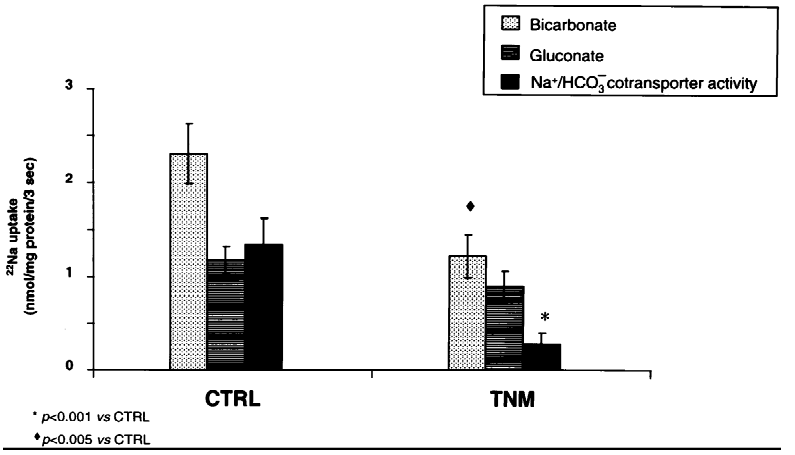

Fig. 3. Purified renal cortical basolateral membrane vehicles (2.5 mg/ml) were treated with tetranitromethane (TNM), right panel, at final concentration of 5 mM of TNM for 30 min at 22°C. The control (CTRL) experiments, left panel, were done under the same conditions in the presence of vehicle. ^{22}Na uptake was done to measure the activity of the Na^+/HCO_3^- cotransporter. The data represent the mean ± SE for ten separate experiments on different membrane preparations.

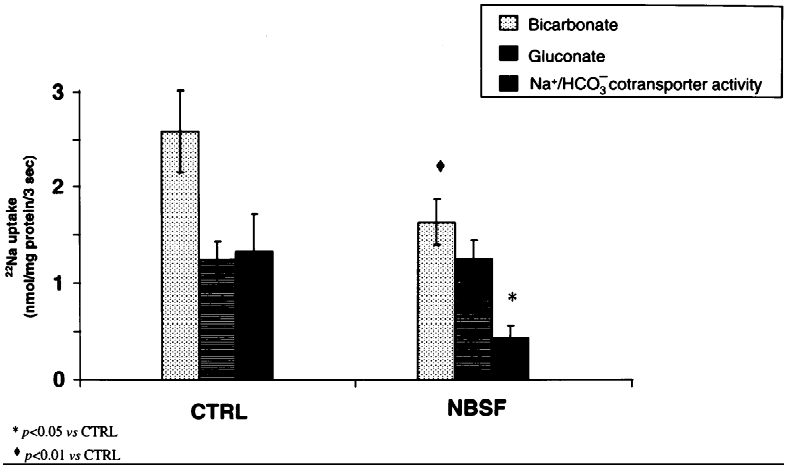

Fig. 4. Purified renal cortical basolateral membrane (2.5 mg/ml) incubated with *p*-nitrobenzene sulfonyl fluoride (NBSF), right panel, at final concentration of NBSF to 250 μM. The incubation was done for 30 min at 22°C. The control (CTRL) experiments, left panel, were performed under the same conditions. ^{22}Na uptake was done to measure the activity of the Na^+/HCO_3^- cotransporter. The data represent the mean ± SE for six separate experiments on different membrane preparations.

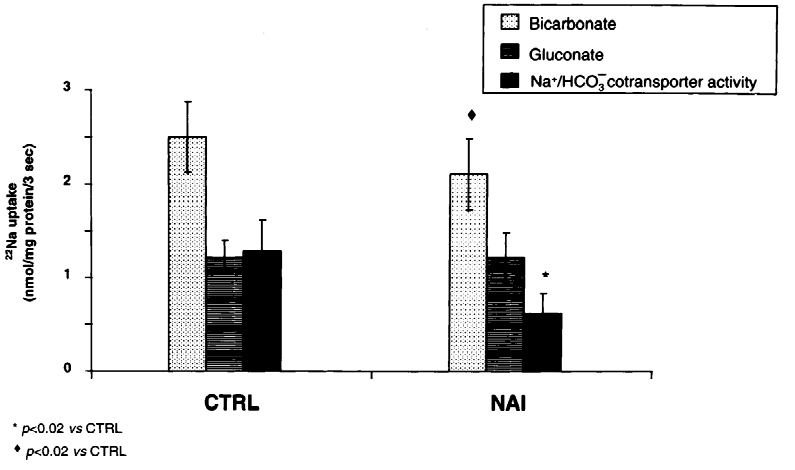

Fig. 5. Purified renal cortical basolateral membrane vesicles (2.5 mg/ml) were treated with *n*-acetylimidazole (NAI), right panel, at final concentration of 75 mM NAI in the absence of 100 mM $NaHCO_3$. The incubation was done for 60 min at 22°C. The control (CTRL) experiments, left panel, were done under the same conditions in the presence of vehicle alone. ^{22}Na uptake was done to measure the activity of the Na^+/HCO_3^- cotransporter. The data represent the mean ± SE for six separate experiments on different membrane preparations.

port proteins such as the Na/H exchanger [9, 16]. To study the role of sulfhydryl groups, we utilized DTT on freshly prepared highly purified basolateral membrane vesicles. The sulfhydryl modifying agent caused a significant decrease in the activity of the cotransporter protein. This suggests that the sulfhydryl groups play an essential role in maintaining the full functional activity of the Na^+/HCO_3^- cotransporter. It has been proposed that sulfhydryl groups form bridges resulting in formation of intramolecular mixed disulfides, thus affecting

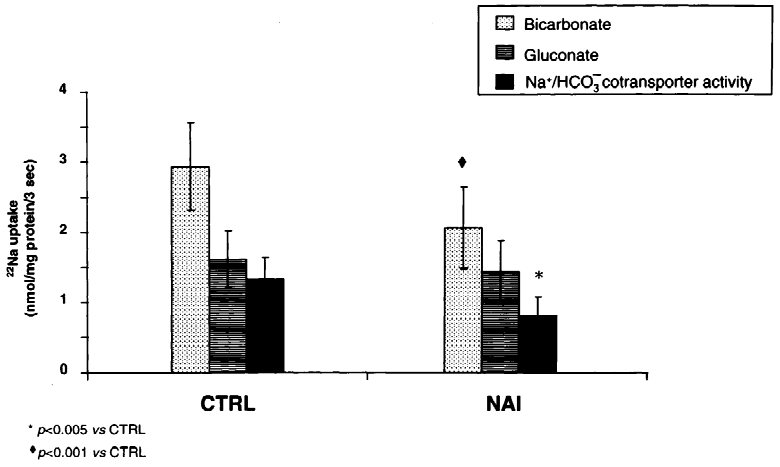

Fig. 6. Purified renal cortical basolateral membrane vesicles (2.5 mg/ml) were incubated with *n*-acetylimidazole (NAI), right panel, at final concentration of 75 mM NAI in presence of 100 mM $NaHCO_3$ (substrate protection). The incubation was done for 60 min at 22°C. The control (CTRL) experiments, left panel, were performed under the same conditions in the presence of vehicle alone. ^{22}Na uptake was done to measure the activity of the Na^+/HCO_3^- cotransporter. The data represent the mean ± SE for six separate experiments on different membrane preparations.

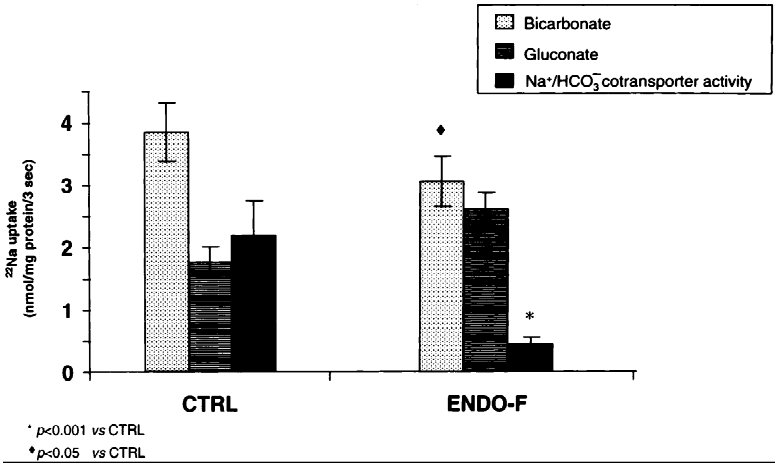

Fig. 7. Purified basolateral membrane vesicles (2.5 mg/ml) were incubated with endoglycosidase F (Endo-F), right panel, at final concentration of 2 units/ml in the presence of protease inhibitors. Incubation was done at 4°C for 18 hr. The controls (CTRL), left panel, were subjected to the same conditions in the presence of vehicles alone. The Na^+/HCO_3^- cotransporter activity was measured by rapid filtration technique. The data represent the mean ± SE for eight separate experiments on different membrane preparations.

the tertiary structures and functions of proteins. The activity of cardiac K channels is known to be affected by agents that modify sulfhydryl groups [7]. Of interest is the finding that SH group modifiers have also resulted in inhibition of the activity of the Na/H antiporter in placental brush border membranes [9].

To study the role of carboxyl group on the function of the Na^+/HCO_3^- cotransporter activity, we used the carboxyl group specific reagent, DCCD, which has been used previously by other investigators [6, 10, 14] and these studies showed that DCCD inhibited the anion exchanger protein and Na/H antiporter. The effect of another carboxyl reagent *N*-ethoxycarbomyl-2-ethoxyl-1-2-dihydroagrinoline (EEDQ) on the Na- glucose transporter was also investigated [25]. EEDQ decreased the sodium-dependent phlorizin binding of the Na-glucose transporter. This inhibition was prevented by glucose. In addition, inactivation of the red cell anion exchanger by another carbodiimide has been observed [6]. While the above effects on the activities of the different transport proteins were demonstrated to be due to modification of the carboxyl group, another group of investigators showed that the inactivation of the Na-K-ATPase by EPC (1-, lethyl-3 (3-dimethyl amino propyl 1 carbodiimide) was a consequence of internal cross-linking and not carboxyl group modification [20]. Our studies show that this compound inhibits the activity of the Na^+/HCO_3^- cotransporter, but did not elucidate the mechanism of the effect.

To further understand the importance of the different structural components of the Na^+/HCO_3^- cotransporter protein, experiments were carried out utilizing tyrosine residue-specific reagents. These active modifiers, *N*-acetyl imidazole (NAI), tetranitromethane (TNM) and *p*-nitrobenzene sulfonyl fluoride (NBSF) were used. All these tyrosine group modifiers resulted in the inhibition of the Na^+/HCO_3^- cotransporter activity. The tyrosine group modifier tetranitromethane caused the greatest inhibition while the *p*-nitrobenzene sulfonyl fluoride had the least inhibition. The inhibitory effect of NAI was not abolished by the presence of substrate, $NaHCO_3$. This suggests that the tyrosine modifier NAI acts on the residues that may be remote from the binding sites for Na and/or HCO_3.

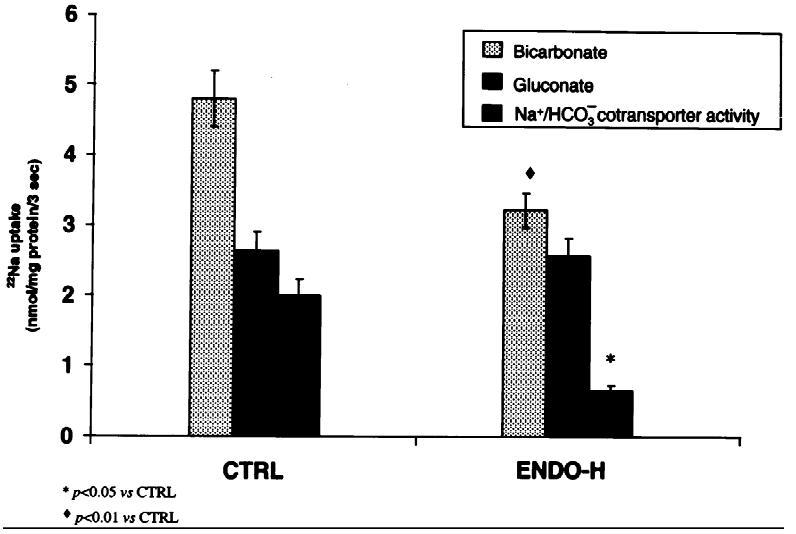

Fig. 8. Highly purified basolateral membrane vesicles (2.5 mg/ml) were incubated with endoglycosidase H (ENDO-H), right panel, at 2 units/ml final concentration of Endo H in the presence of protease inhibitors. The incubation was done for 18 hr at 4°C. The control (CTRL) experiments, left panel, were done in the presence of vehicles alone under the same conditions. ^{22}Na uptake was done to measure the activity of the Na^+/HCO_3^- cotransporter. The data represent the mean ± SE for six separate experiments on different membrane preparations.

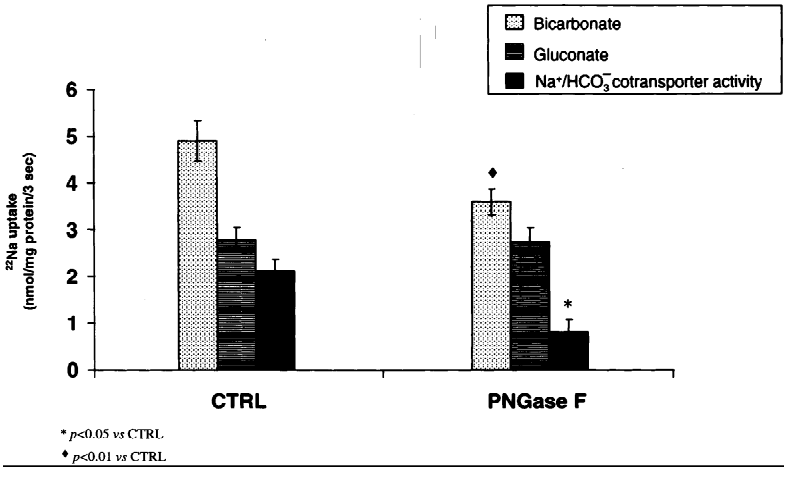

Fig. 9. Purified basolateral membrane vesicles (2.5 mg/ml) were treated with *N*-glycosidase F (PNFGase F), right panel, at final concentration of 2 units/ml of PNFGase F in presence of protease inhibitors. The incubation was done for 18 hr at 4°C. The control (CTRL) experiments, left panel, were performed under the same conditions in the presence of vehicles alone. ^{22}Na uptake was done to measure the activity of the Na^+/HCO_3^- cotransporter. The data represent the mean ± SE for six separate experiments on different membrane preparations.

We studied the role of glycosylation on the functional activities of the Na^+/HCO_3^- cotransporter protein. Previous studies have shown an essential role for glycosylation in membrane transport systems such as the Na/H antiporter and the Na^+ pump [13, 19, 21, 26, 27]. Agents that act on the *N*-glycosidic bonds (endoglycosidase F and H and *N*-glycosidase F) inhibited the activity of the Na^+/HCO_3 cotransporter. In addition, O-glycanase which acts on O-linked oligosaccharides also resulted in significant decrease in the activity of the cotransporter protein.

From these studies, it can not be determined with certainty the membrane surface location of the amino acid residues in the Na^+/HCO_3^- cotransporter modified by the reagents used. We speculate that the amino acids are located on the extracellular domain of the basolateral membrane because most of the vesicles are oriented right side out. In addition, one could raise the question as to whether other transporters localized to the basolateral membranes (e.g., sodium-dependent decarboxylate transporter, sodium phosphate cotransporter) would also be altered by the reagents used. We believe that the effects of the reagents may not be ''specific'' for the Na^+/HCO_3^- cotransporter because many other transporters that contain the amino acids or carbohydrate residues being altered by the reagents used have been shown to be affected by the chemical modifiers [9, 10, 12, 14, 16, 21].

Taken together, we have shown that sulfhydryl group modifiers (dithiothreitol) as well as carboxyl group modifier (*n-n'*-dicyclohexyl carbodiimide) significantly inhibited the Na^+/HCO_3^- cotransporter activity. Chemical modifiers of the tyrosine residues (*p*-nitrobenzene sulfonyl fluoride, *n*-acetyl imidazole, tetranitromethane) also significantly decreased the Na^+/HCO_3^- cotransporter activity by 50–70%. The effect of tyrosine modifier (NAI) was not prevented by the presence of Na and bicarbonate suggesting that tyrosine residues are not at the binding sites. Finally, treatment of basolateral membrane proteins with the modifiers of the

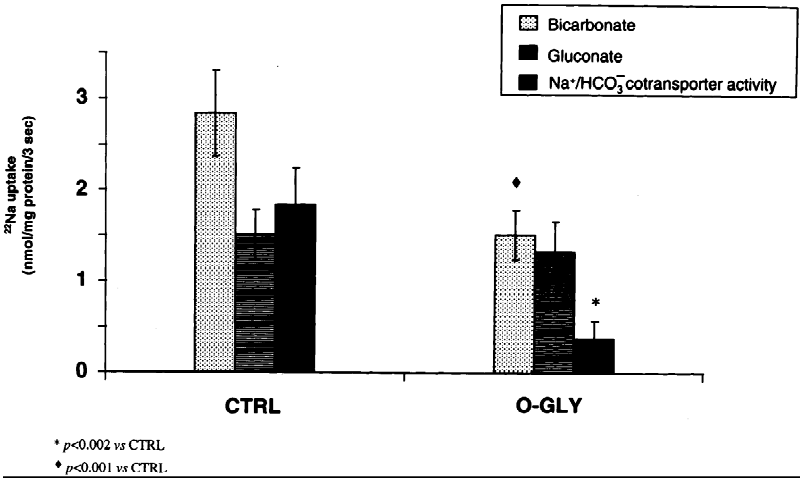

Fig. 10. Purified basolateral membrane vesicles (2.5 mg/ml) were treated with O-glycanase (O-GLY), right panel, at 4 units/ml final concentration of O-GLY in the presence of protease inhibitor. Incubation condition was maintained at 4°C for 18 hr. The control (CTRL) experiments, left panel, were performed under the same conditions in the presence of vehicles. ^{22}Na uptake was done to measure the activity of the Na^+/HCO_3^- cotransporter. The data represent the mean ± SE for seven separate experiments on different membrane preparations.

N and O-glycosidic linked residues (endoglycosidase F and H, N-glycosidase F, O-glycanase) resulted in 50–80% inhibition of cotransporter activity.

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