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Renal Cortical Basolateral Na^+/HCO_3^- Cotransporter: IV Characterization and Localization with Polyclonal Antibodies

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Abstract. We have previously partially purified the basolateral Na^+/HCO_3^- cotransporter from rabbit renal cortex and this resulted in a 400-fold purification, and an SDS-PAGE analysis showed an enhancement of a protein band with a MW of approximately 56 kDa. We developed polyclonal antibodies against the Na^+/HCO_3^- cotransporter by immunizing Dutch-belted rabbits with a partially purified protein fraction enriched in cotransporter activity. Western blot analysis of renal cortical basolateral membranes and of solubilized basolateral membrane proteins showed that the antibodies recognized a protein with a MW of approximately 56 kDa. The specificity of the purified antibodies against the Na^+/HCO_3^- cotransporter was tested by immunoprecipitation. Solubilized basolateral membrane proteins enriched in Na^+/HCO_3^- cotransporter activity were incubated with the purified antibody or with the preimmune IgG and then reconstituted in proteoliposomes. The purified antibody fraction caused a concentration-dependent inhibition of the Na^+/HCO_3^- cotransporter activity, while the preimmune IgG failed to elicit any change. The inhibitory effect of the antibody was of the same magnitude whether it was added prior to (inside) or after (outside) reconstitution in proteoliposomes. In the presence of the substrates ($NaHCO_3$ or Na_2CO_3) for the cotransporter, the inhibitory effect of the antibody on cotransporter activity was significantly blunted as compared with the inhibition observed in the absence of substrates. Western blot analysis of rabbit kidneys showed that the antibodies recognized strongly a 56 kDa protein band in microsomes of the inner stripe of outer medulla and inner medulla, but not in the outer stripe of outer medulla. A 56 kDa protein band was recognized in microsomes of the stomach, liver, esophagus, and small intestine but was not detected in red blood cell membranes. Localization of the Na^+/HCO_3^- cotransporter protein by immunogold technique revealed specific labeling of the cotransporter on the basolateral membranes of the proximal tubules, but not in the brush border membranes. These results demonstrate that the polyclonal antibodies against the 56 kDa basolateral protein inhibit the activity of the Na^+/HCO_3^- cotransporter suggesting that the 56 kDa protein represents the cotransporter or a component thereof. These antibodies interact at or near the substrate binding sites. The Na^+/HCO_3^- cotransporter protein is expressed in different regions of the kidneys and in other tissues.

Key words: Na^+/HCO_3^- cotransporter — Polyclonal antibody — Immunolocalization — Cellular transport —Acid-base balance

Introduction

The Na^+/HCO_3^- cotransporter has been recognized as the main system responsible for HCO_3 transport out of the proximal tubule cells into the blood and plays an important role in HCO_3 transport under physiologic and pathophysiologic conditions [1, 3, 10, 15]. The ionic mechanism of the Na^+/HCO_3^- cotransporter has been characterized and it has been well established that the system functions via a 1:1:1 cotransport of $CO_3^=$, HCO_3^- and Na^+ on different sites [19]. The system functions in an integrated fashion with the brush border Na-H antiporter and the activities of these two systems vary in parallel [2]. We have partially purified the Na^+/HCO_3^- cotransporter protein by hydroxylapatite chromatography followed by glycerol gradient centrifugation [5]. The purified protein was reconstituted in asolectin and the Na^+/HCO_3^- cotransporter activity was enriched 400-fold compared to the basolateral membranes. The purified fraction exhibited kinetic properties and inhibitor sensitivity similar to

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the original membranes. SDS-PAGE analysis of the purified protein fraction collected from the different purification steps showed an enhancement of a protein doublet of molecular weight of approximately 56 kDa which we believe represents an active component of the Na^+/HCO_3^- cotransporter [5]. We have also shown that fluorescein isothiocyanate specifically labeled the 56 kDa fraction and inhibited the activity of the cotransporter and the inhibition was prevented in the presence of substrates [21].

Despite the importance of the Na^+/HCO_3^- cotransporter in physiologic and pathophysiologic conditions, no antibodies against this protein are available. The availability of antibodies against the Na^+/HCO_3^- cotransporter would allow immunopurification, localization and characterization of the regulation of this important protein. In the present study, we have developed polyclonal antibodies against the renal Na^+/HCO_3^- cotransporter and characterized the inhibitory effect of these antibodies, their interaction with substrate binding sites and also used them for localization of cotransporter in different regions of the kidney and in other tissues by Western blot analysis.

Materials and Methods

The methods used in the present study which have been published are only briefly summarized while the new techniques are described in detail.

Solubilization and Reconstitution

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 [15]. This procedure resulted in highly purified basolateral membranes enriched on the average of 12- to 14-fold in Na-K-ATPase activity compared with homogenates, and with less than 5% cross contamination with brush border membranes. Solubilization of basolateral membrane proteins and reconstitution were done as previously described [5]. One part basolateral membrane protein (5 mg/ml) was incubated with 1.25 parts 2% n-octyl β-D-glucopyranoside (octylglucoside) in 50 mM HEPES and 250 mM mannitol (buffer A), pH 7.4, on ice for 15 min. The protein-detergent mixture was spun for 30 min at 110,000 ×*g*. The solubilized protein (2.5 mg/ml) was reconstituted into liposomes by mixing 1.6 parts of protein with 1 part of L-α-phosphatidylcholine (35 mg/ml) which was sonicated for 10 min. The combination of proteins and lipids was dialyzed for 18 hr in buffer A using a membrane restricting the passage of 6–8 kDa molecules. The protein concentration of the proteoliposomes was measured. ^{22}Na uptake was performed in the presence of $NaHCO_3$ or Na gluconate in the proteoliposomes by the rapid filtration technique as will be described below.

Measurement of Na^+/HCO_3^- Cotransporter Activity

Native basolateral membrane vesicles or proteoliposomes were preincubated for 1–2 hr at room temperature in a solution containing (in mM): 200 sucrose, 50 HEPES, pH 7.50 with Tris and 1 Mg gluconate as previously described [5]. The suspension was spun at 30,000 revolutions/min with a Beckman Ti 50.2 rotor for 30 min at 4°C, and the resulting pellet was resuspended in the same solution. The assay was started by addition of 100–150 μg protein 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 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. Na^+/HCO_3^- cotransporter activity was taken as the difference in ^{22}Na uptake in the presence or absence of an inwardly directed HCO_3^- gradient (HCO_3^- was replaced by gluconate).

Measurement of Na^+/HCO_3^- Cotransporter Activity in Primary Cultures of Rabbit Proximal Tubule Cells

Primary cultures of proximal tubule cells were prepared as previously described [18]. The cells were preincubated in an incubation buffer containing (in mM): 100 K gluconate, 80 sucrose, 10 HEPES, pH 7.40, 1 Mg gluconate, 2 $CaSO_4$, 5.5 glucose and 5.0 alanine in presence of 10^{-4}M ouabain and 50 mM ethylisopropyl amiloride for 1 hr at 37°C. Reaction was started by addition of 20 μl of cell suspension of 90 μl of uptake buffer containing (in mM): 100 K gluconate, 10 HEPES, pH 7.40, 1 Mg, gluconate, 2 $CaSO_4$, 5.5 glucose, 5 alanine, 1 μCi ^{22}Na, and 40 Na-HCO_3 or 40 Na-gluconate. The reaction was stopped after 5 min with 3 ml ice-cold incubation solution and then poured on a 0.45-μm pore size prewetted Millipore filter. The filters were washed three times more and radioactivity measured by scintillation spectroscopy. HCO_3-dependent ^{22}Na uptake was taken as the difference in ^{22}Na uptake in the presence of HCO_3^- and gluconate. Cell viability in the presence of the polyclonal antibody or preimmune IgG was examined by tryphan blue method. The results show that the polyclonal antibody as well as the preimmune IgG were not cytotoxic to the primary culture of proximal tubule cells used in the experiments.

Production of Polyclonal Antibodies Against Na^+/HCO_3^- Cotransporter

Purified protein fraction highly enriched in Na^+/HCO_3^- cotransporter activity (100 μg protein) emulsified 1:1 with complete Freund's adjuvant was injected intradermally into different sites in the back of the Dutch-belted rabbits using a gauge 25 needle. Preimmune sera were collected and purified as will be described below from each rabbit and frozen at −70°C in small aliquots for future use as controls. Booster injections were done monthly with 50 μg of purified glycerol gradient protein fraction emulsified in incomplete Freund's adjuvant. Immune sera were collected and purified as follows: Saturated ammonium sulfate $(NH_4)_2SO_4$ was added slowly to the sera until precipitation of protein occurs. The sera were dialyzed extensively to remove excess ammonium sulfate against 0.02M phosphate buffer. Further purification was done by DEAE-Sephacel ion-exchange chromatography. Initial elution was done by applying 0.05 M NaCl in phosphate buffer. The eluates were analyzed by the UV monitor and IgG peaks noted. Repeat application of 0.1M, 0.2M, and 0.5M and finally 1M NaCl in phosphate buffer was done to get all the protein off the column. SDS-PAGE analysis of each fraction was done to ascertain the presence of 25 kDa and 55 kDa proteins that represent the light and heavy chains of IgG.

The specificity of the polyclonal antibody was determined by the Western blot technique and immunoprecipitation.

Western Blot Analysis

When indicated protein aliquots were subjected to 12% SDS-PAGE gel according to the method of Laemmli [12]. After electrophoresis, the gel and the polyvinylidine difluoride (PVDF) transfer membrane which were pre-equilibrated in Towbin transfer buffer consisting of 25 mM Tris, 192 mM glycine, pH 8.3 were sandwiched between two filter papers and placed in the electroblotting apparatus with the membrane on the anode side. Transfer was done for 1–4 hr at 50 V. Blot was washed with TTBS (Tween 20 and Tris buffered saline) and then incubated with 2% nonfat dry milk in TTBS overnight. Primary antibodies (10 μg/ml) or preimmune IgG (10 μg/ml) were added to the blot for 1½-hr incubation. After thorough washings with TTBS, goat anti-rabbit IgG antibodies (conjugated with alkaline phosphatase) were added at dilution of 1:3000. Development was performed utilizing 5-bromo-4-chloro-3 indolyl phosphate/p-nitro blue tetrazolium chloride as substrate.

Western blot was performed in plasma membranes prepared from the kidney, and in microsomes from esophagus, stomach small intestine, liver and in red blood cell membranes. The renal membranes were prepared from the outer stripe of outer medulla, inner stripe of outer medulla, and inner medulla (papilla) [17]. The different tissue homogenates were prepared using the techniques described previously by our laboratory and others [7, 8, 9, 14, 20, 22].

Immunoprecipitation of the Na^+/HCO_3^- Cotransporter by the Polyclonal Antibody

Immunoprecipitation was carried out to demonstrate that binding of the antibody to the protein fraction enriched in Na^+/HCO_3^- cotransporter activity altered the function of this cotransporter. Immunoprecipitation of Na^+/HCO_3^- cotransporter protein was performed according to the method described for Na-Ca exchanger by Carafoli and Longoni [13]. Briefly, octylglucoside-solubilized proteins enriched in Na^+/HCO_3^- cotransporter activity were incubated with the same concentrations of either the purified antibody or preimmune IgG at various dilutions for 30 min at 4°C. The incubation was stopped by addition of HEPES/mannitol buffer. L-α-phosphatidylcholine was added to reconstitute the protein into proteoliposomes. The proteoliposomes were resuspended in buffer A and assayed for Na^+/HCO_3^- cotransporter activity as described above [5]. Antibody alone, preimmune IgG or the purified cotransporter fraction alone was reconstituted into liposomes as appropriate controls.

Additional immunoprecipitation studies on the native basolateral membrane vesicles were carried out as described previously [23]. Briefly, different dilutions of the purified polyclonal antibody against the Na^+/HCO_3^- cotransporter protein or preimmune IgG were incubated with protein A Sepharose beads in phosphate buffered saline (PBS), pH 7.4 with 0.3% ovalbumin for 18 min at 4°C. The antibody-protein A Sepharose complex was washed with PBS two times. Basolateral membrane proteins (2.5 mg/ml) were incubated with the above complex for 4 hr at 20°C with gentle agitation. The beads were isolated by centrifugation and the supernatants were obtained for ^{22}Na uptake measurements as described. When indicated, basolateral membrane vesicles were incubated with protein A alone and processed in the same way as experimental groups.

To examine whether the antibodies inhibit the cotransporter when added to the inside or the outside of proteoliposomes, the following experimental protocol was used. When indicated, aliquots of solubilized basolateral membrane proteins (2.5 mg/ml) were incubated with equal amounts of polyclonal antibody or preimmune IgG at various dilutions as indicated for 30 min at 4°C. The reaction was terminated by the addition of HEPES/mannitol buffer and reconstituted into proteoliposomes (inside) as described above. In other experiments, aliquots of solubilized basolateral membranes proteins (2.5 mg/ml) were reconstituted into proteoliposomes as described. The formed proteoliposomes were incubated with the same amounts of polyclonal antibody (outside) or preimmune IgG at various dilutions as needed for 30 min at 4°C. The reaction was terminated by addition of buffer A and centrifuged at 30,000 × *g* for 30 min and the pellet containing the proteoliposomes was resuspended in a buffer containing 200 mM sucrose, 50 mM HEPES, pH 7.5 with Tris. Thereafter, HCO_3-dependent ^{22}Na uptake was measured as described below.

To study the interaction of substrate/antibody with the Na^+/HCO_3^- cotransporter protein, the following experiments were done. The proteoliposomes were incubated with the same concentration of antibody or preimmune IgG at different dilutions in the presence of 40 mM $NaHCO_3$ or Na_2CO_3 or 40 mM Tris for 30 min at 4°C. The reaction was terminated by addition of buffer A, centrifuged at 30,000 × *g* for 30 min and the pellet containing the proteoliposomes was resuspended in a buffer containing 200 mM sucrose, 50 mM HEPES, pH 7.5 with Tris and assayed for HCO_3-dependent $^{22}Na^+$ uptake as described.

Immunoelectron Microscopy

Rabbits were anesthetized with nembutal and the kidneys were initially flushed arterially with PBS (phosphate buffered saline, pH 7.4) followed by perfusion with PLP fixative (2% paroformaldehyde, 75 mM lysine, 10 mM sodium periodate in PBS, pH 7.4) for 5 min. The kidneys were excised and the cortex cut into 1 mm blocks and fixed overnight in PLP at 4°C. These blocks were rinsed in PBS and then dehydrated in graded series of ethanol and embedded in LR white resin. Sections cut from these blocks were collected onto nickel grids, and further processed by floating the section side down on 20 μl drops of solution B (1% BSA and 0.5% gelatin in PBS) for 20 min × 2. The polyclonal antibody diluted to 5 μg/ml with solution B was applied for 18 hr at 4°C, then 1 hr at 25°C. Alternatively, equal amounts of preimmune IgG or nonimmune rabbit IgG were used at the same solutions as controls. Sections were rinsed in solution B for 10 min × 2. The blocked sections were incubated with protein A gold (5 nm) diluted 1:50 in solution B for 3 hr at 4°C, then 1 hr at 25°C. Successive washes were done with solution B for 20 min × 2, PBS for 10 min × 2, then 2.5% glutaraldehyde in PBS for 5 min and finally with double distilled H_2O, 10 min × 3. Sections were stained with 2% aqueous uranyl acetate and viewed at 80kV on a JEOL 100S transmission electron microscope.

SDS-PAGE Analysis

SDS-PAGE was carried out on discontinuous systems as described by Laemmli [12]. Low molecular weight standards were used (from 14 *kDa* to 92 *kDa*). Electrophoresis was done on slab gels of 12% acrylamide. Coomassie Brilliant Blue or silver stains were used as indicated.

Materials

^{22}Na was purchased from Amersham. DEAE-Sephacel was obtained from Pharmacia LKB Biotechnology. L-α-phosphatidylcholine, Freund's adjuvant, protein A gold, octyglucoside, molecular weight standards, Coomasie R-250, SDS, acrylamide and bisacrylamide, were

bought from Sigma. Tween 20, alkaline phosphatase immunoblot assay kit were purchased from Biorad. PVDF (Immobilon$_{TM}$), were obtained from Millipore. General laboratory chemicals were of research and analytical grade and were obtained from Sigma Chemicals.

Data Analysis

Results are presented as mean ±SEM and represent the average of 4–6 experiments. The *t*-test for paired or unpaired data was used to analyze the data wherever appropriate.

Results

Polyclonal Antibodies that Recognize the Renal Basolateral Na^+/HCO_3^- Cotransporter in Basolateral Membranes and in Primary Cultures of the Proximal Tubule

Polyclonal antibodies against the Na^+/HCO_3^- cotransporter were generated by immunizing Dutch-belted rabbits with a purified protein fraction from the glycerol gradient centrifugation which was highly enriched in Na^+/HCO_3^- cotransporter activity. Antibodies were purified by ammonium sulfate precipitation and ion-exchange chromatography. SDS-PAGE analysis of purified antibodies revealed two distinct bands of heavy and light chains of IgG (*not shown*). The specificity of the purified antibodies against the Na^+/HCO_3^- cotransporter was tested by Western blot analysis and by immunoprecipitation. Octylglucoside-solubilized basolateral membrane proteins, enriched in Na^+/HCO_3^- cotransporter activity, were incubated with the purified antibodies or with the preimmune IgG and then reconstituted into proteoliposomes. Several pools of polyclonal antibodies were screened for inhibition of Na^+/HCO_3^- cotransporter activity in octylglucoside-solubilized basolateral protein fraction. We found that different antibodies inhibited Na^+/HCO_3^- cotransporter activity with inhibition ranging from 40% to 80% at 1:10 dilution. We examined the effect of the polyclonal antibody and preimmune IgG on the Na^+/HCO_3^- cotransporter activity. The polyclonal antibody (at 1:100 dilution) significantly inhibited the bicarbonate dependent ^{22}Na uptake measured nmoles/mg protein/3 seconds (antibody 23.80 ± 1.90 *vs.* preimmune IgG 35.40 ± 2.74, $P < .001$) without altering the ^{22}Na uptake in the presence of gluconate (antibody 17.18 ± 1.94 *vs.* preimmune IgG, 15.68 ± 2.44). In addition, as compared to control, the preimmune IgG did not alter either the bicarbonate dependent ^{22}Na uptake (control 38.6 ± 2.83 *vs.* preimmune 35.40 ± 2.74) or gluconate (control 15.98 ± 2.28, preimmune 15.68 ± 2.44). Although different batches of antibodies were used in the different experiments, the experiments were always performed in a paired fashion and utilized the same concentration of the antibody thus accounting for a variable degree of inhibition by different pools of antibodies.

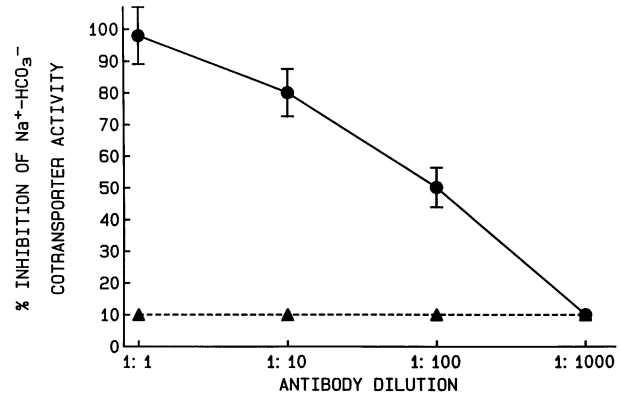

Fig. 1. Effect of the polyclonal antibody on HCO_3-dependent ^{22}Na uptake in proteoliposomes. Immunoprecipitation of the Na^+/HCO_3^- cotransporter activity was examined by the polyclonal antibody against the Na^+/HCO_3^- cotransporter. Octylglucoside-solubilized basolateral membrane proteins were incubated with same concentrations of polyclonal antibody or preimmune IgG at various dilutions. The incubation was stopped by the addition of HEPES/mannitol buffer and subsequently reconstituted with L-α-phosphatidylcholine to form proteoliposomes. Octylglucoside-solubilized proteins were reconstituted into proteoliposomes as control and HCO_3-dependent ^{22}Na uptake was measured at 3 sec. Each point in the graph represents triplicate experiments on different proteoliposome preparations immunoprecipitated by the same antibody. Closed circle ●, polyclonal antibody; shaded triangle ▲, pre-immune IgG.

The additional immunoprecipitation studies were done to demonstrate the inhibitory effect of the antibody on the Na^+/HCO_3^- cotransporter in the native basolateral membrane vesicles. Incubation of the native basolateral membrane vesicles with the antibody (1:100 dilution) against the cotransporter resulted in significant inhibition of Na^+/HCO_3^- cotransporter activity as compared to the control (control, 2.47 ± 0.40 *vs.* Ab 1.11 ± 0.26 $P < .03$) $n = 5$. Furthermore, the preimmune IgG did not inhibit the Na^+/HCO_3^- cotransporter (IgG 2.42 ± 0.41 *vs.* control 2.47 ± 0.40). Experiments involving the incubation of native basolateral membrane vesicles with protein A alone did not result in inhibition of the Na^+/HCO_3^- cotransporter activity (control, 2.45 ± 0.40 *vs.* protein A 2.35 ± 0.38).

Figure 1 shows that the polyclonal antibody inhibited Na^+/HCO_3^- cotransporter activity in a concentration-dependent fashion with 100% inhibition at 1:1 dilution and 50% inhibition at 1:100 dilution. The preimmune IgG in the same concentration as the antibody did not cause any inhibition of the Na^+/HCO_3^- activity. Figure 2 shows that the polyclonal antibody that inhibited the Na^+/HCO_3^- activity also recognized a distinct protein band of 56 *kDa* MW in basolateral membranes (lane 1) and in octylglucoside-solubilized protein fraction (lane 3), while the preimmune IgG failed to detect any protein

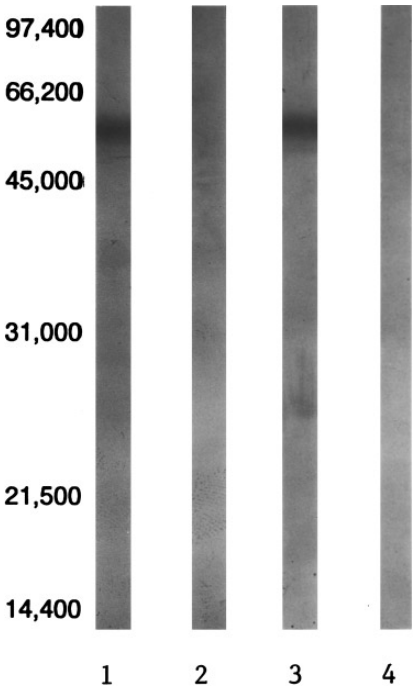

Fig. 2. Western blot analysis of the basolateral and octylglucoside solubilized proteins by the polyclonal antibody and preimmune IgG. Fifteen μg of basolateral membrane vesicle proteins and 30 μg octylglucoside-solubilized proteins were separated in 12% polyacrylamide gel, transferred to polyvinylidine difluoride (PVDF) membranes and screened by the polyclonal antibody against the Na^+/HCO_3^- cotransporter and preimmune IgG. Molecular weights are indicated on the left side of the figure. Lane 1, basolateral membrane vesicle proteins (BLMV) screened by polyclonal antibody; Lane 2, BLMV screened by preimmune IgG; Lane 3, octylglucoside-solubilized proteins screened by polyclonal antibody; Lane 4, octylglucoside-solubilized proteins screened by the preimmune IgG.

band in basolateral and in solubilized proteins (lanes 2 and 4 respectively). This antibody also recognized the same 56 *kDa* protein band in basolateral membrane vesicles and in fractions from hydroxylapatite chromatography and glycerol gradient centrifugation which have been shown previously to result in increased purification of the Na^+/HCO_3^- cotransporter (*not shown*).

We have recently demonstrated the presence of Na^+/HCO_3^- cotransporter activity in primary cultures of proximal tubule cells of the rabbit [18]. In these cells, there is HCO_3-dependent ^{22}Na uptake which is inhibited by the disulfonic stilbene, DIDS. Studies were undertaken to determine if the antibody or the preimmune IgG had cytotoxic effects on these cells. Neither the antibodies against the Na^+/HCO_3^- cotransporter or the IgG were cytotoxic as assessed by tryphan blue method (preimmune IgG, 95% of cells were viable *vs.* antibody, 98% of cells were viable). We measured HCO_3-dependent ^{22}Na uptake in the presence of IgG and in the presence of the polyclonal antibody at 1:10 dilution. The polyclonal antibody significantly inhibited the Na^+/HCO_3^- cotransporter activity (IgG 1.37 ± .59 *vs.* Ab 0.35 ± 0.17 nm/mg protein/5 min, $P < 0.05$, $n = 4$).

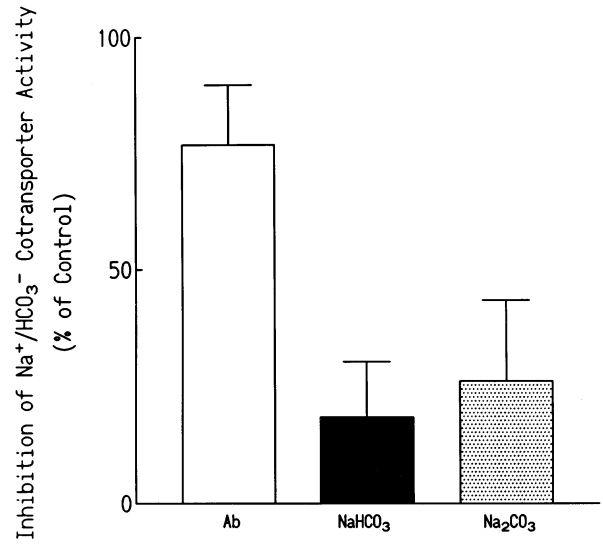

Fig. 3. The effect of polyclonal antibody on the Na^+/HCO_3^- cotransporter activity in the presence of substrates $NaHCO_3$ or Na_2CO_3. Solubilized proteins reconstituted into proteoliposomes were incubated with the polyclonal antibodies in the presence of a solution containing 40 mM Tris or a solution containing 40 mM $NaHCO_3$ or Na_2CO_3. The data presented shows the inhibition of $NaHCO_3$ cotransporter activity as % of control. Experiments are done in triplicates in different proteoliposomes preparation (Ab *vs.* $NaHCO_3$, $P < 0.001$; Ab *vs.* Na_2CO_3, $P < 0.01$).

Interaction of the Antibody with the Substrates Binding Sites in the Cotransporter

To examine the sidedness of the inhibitory effect of polyclonal antibodies on the cotransporter, the antibody (1:10 dilution) was added either prior to the reconstitution (inside) or after the reconstitution (outside) into proteoliposomes. In six paired experiments utilizing the same polyclonal antibody the inhibitory effect of the antibody on the Na^+/HCO_3^- cotransporter activity was the same magnitude whether it was added to the outside or to the inside (inside 59 ± 10%, outside 51 ± 15% of control). These results suggest that the antibody recognized similar antigenic determinants from outside as well as from inside when the protein is reconstituted and oriented in the transport mode in the proteoliposomes. This finding allowed the study of the interaction of the antibody with the bindings sites for $NaHCO_3$ or Na_2CO_3 on the cotransporter.

To examine the interaction of substrates with the binding sites for the antibody the solubilized proteins were incubated in a standard solution containing Tris only or in a solution containing either 40 mM $NaHCO_3$ or Na_2CO_3 with the antibody. Figure 3 shows that in the

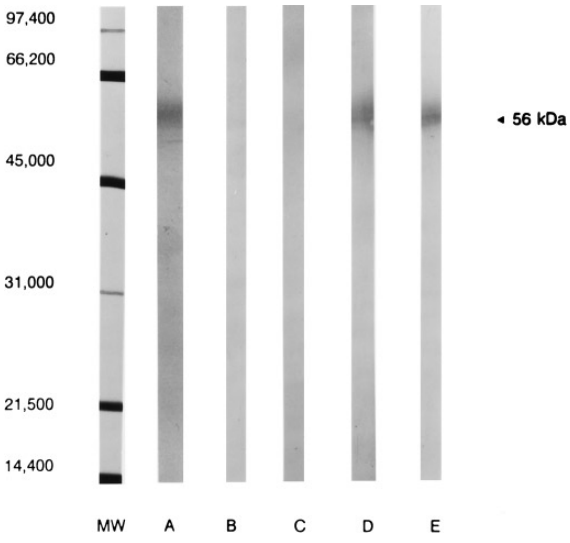

Fig. 4. Western blot analysis of different regions of the kidney by polyclonal antibody against the 56 *kDa* Na^+/HCO_3^- cotransporter. Membrane proteins preparations (50 μg/well) from different regions of the kidney were electrophoresed onto 12% Laemmli gels. The proteins were transferred onto PVDF membrane and screened with polyclonal antibody (1:1000) against the Na^+/HCO_3^- cotransporter proteins. Lane MW, molecular weight marker; Lane A, cortical basolateral membrane; Lane B, cortical brush border membrane; Lane C, outer stripe of the outer medulla; Lane D, inner medulla, Lane E, inner stripe of outer medulla. The antibody did not recognize the 56 kDa protein in cortical brush border membranes (Lane B) and in microsomes of the outer stripe of the outer medulla. (Lane C).

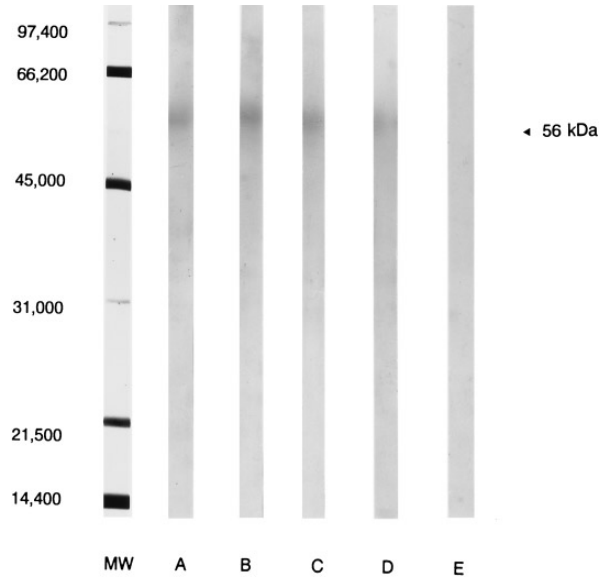

Fig. 5. Western blot analysis of different tissues by the polyclonal antibody against the 56 kDa Na^+/HCO_3^- cotransporter. Microsomal preparations of different tissues (50 μg/well) were electrophoresed onto 12% Laemmli gels. The proteins were transferred onto PVDF membrane and screened with polyclonal antibody (1:1000) against the Na^+/HCO_3^- cotransporter proteins. Lane MW, molecular weight markers; Lane A, esophagus; Lane B, stomach; Lane C, small intestine; Lane D, liver; Lane E, red blood cell membranes. The antibody did not recognize the 56 kDa protein in the red blood cell membranes (Lane E).

absence of substrates during incubation, the polyclonal antibody (1:10) inhibited the Na^+/HCO_3^- cotransporter activity by 76.8 ± 12.9% of control levels. In the presence of $NaHCO_3$ or Na_2CO_3 during incubation with the antibody, the inhibitory effect of the antibody was significantly blunted ($NaHCO_3$ 18.3 ± 11.9%, $P < .001$; Na_2CO_3 26.0 ± 17.3%, $P < 0.01$ of control levels) respectively.

Western Blot Analysis of 56 kDa Protein in Different Regions of the Kidney and Different Organs

To gain further insight on the expression of the Na^+/HCO_3^- cotransporter protein we subjected different regions of the kidneys and different tissues to Western blot analysis with polyclonal antibody against the 56 *kDa* protein. Figure 4 shows that polyclonal antibody recognizes a 56 *kDa* protein in the inner stripe of outer medulla and inner medulla, but not in the outer stripe of outer medulla or in cortical brush border membrane. Figure 5 shows that the polyclonal antibodies also recognize a 56 *kDa* protein in microsomes of the esophagus, stomach, small intestine and liver. In contrast, there was no staining in red blood cell membranes suggesting that our polyclonal antibody does not recognize the Cl-HCO_3 exchanger protein. Figure 6 shows the localization of 56 *kDa* protein by immunogold electron microscopy technique. The presence of distinct specific immunogold staining in the basolateral membranes can be seen with the antibody but not with IgG. There was no staining of the brush border membranes using the antibodies (*not shown*). The preimmune IgG did not show staining in both the basolateral membranes and in the brush border membranes (*not shown*).

Discussion

The Na^+/HCO_3^- cotransporter is the main protein responsible for bicarbonate removal out of the proximal tubule into the blood. This protein has been extensively studied in basolateral membrane vesicles and its regulation by physiological states, kinases, and hormones have also been the subject of intense study [4, 16]. Despite the extensive studies in the regulation of this protein little is known about this protein. We have recently partially purified an active component of the renal Na^+/HCO_3^- cotransporter and showed that this protein fraction was enriched in cotransporter activity (400-fold) and had an apparent molecular weight of 56 *kDa* [5].

In the present study, we have further characterized

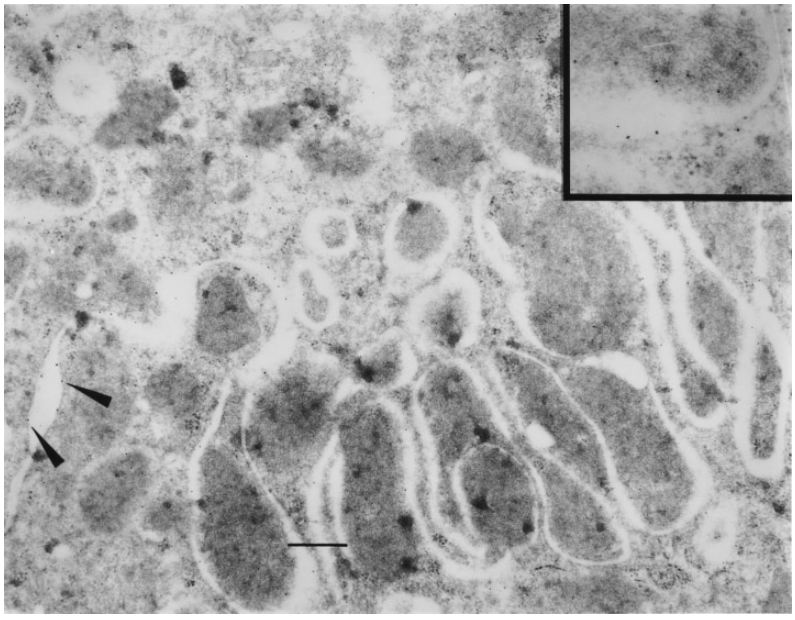

Fig. 6. Localization of 56 kDa protein by the immunogold electron microscopy technique. The presence of distinct specific immunogold staining (arrows) in the basolateral membranes can be seen with the antibody against the 56 kDa protein at 1:10 dilution but not with the IgG (*not shown*). There was no labeling in the brush border membranes (*not shown*). Inset shows immunogold particles at higher magnification. Bar = .5 μm.

the 56 *kDa* protein component of the Na^+/HCO_3^- cotransporter by developing polyclonal antibodies against the partially purified 56 *kDa* protein. This antibody recognized a 56 *kDa* band in renal cortical basolateral membranes and in purified protein fractions obtained from the purification steps using octylglucoside solubilized basolateral membrane vesicles, hydroxylapatite chromatography and glycerol gradient centrifugation. Of interest is the fact that the antibody did not recognize this protein in brush border membranes from the renal cortex which does not contain cotransporter activity. In addition, the protein was expressed in microsomal preparations of the inner stripe of outer medulla and inner medulla, but not in the outer stripe of outer medulla. The polyclonal antibodies against the 56 *kDa* protein was also used to probe other tissues where evidence of the functional presence cotransporter has been described [6, 14]. The antibody recognized a 56 *kDa* protein in the microsomal preparations of the stomach, liver, esophagus and small intestines. Of great interest is the fact that the antibody did not recognize the protein in red cell membranes, indicating that it does not cross react with the Cl^-/HCO_3 exchanger protein.

The availability of the polyclonal antibody against the 56 *kDa* protein allowed us to characterize the interaction of substrate with the transport protein. Several pools of specific antibodies were characterized and we chose to study in detail the effect of the inhibiting antibodies. Although the different pools of antibody displayed variable degree of inhibition, the experiments were always performed in paired fashion thus allowing comparison of the inhibitory effect of the different pools. It is of interest that the antibodies inhibited the Na^+/HCO_3^- cotransporter activity equally when added to the outside or inside of the proteoliposomes. This suggest that the antibody recognized similar antigenic determinants from the inside as well as from the outside, when the protein is reconstituted in the transport mode in the proteoliposomes. In addition, these antibodies also inhibited the Na^+/HCO_3^- cotransporter activity when added to the outside of the cells of primary cultures of proximal tubule. Likewise, the antibodies also inhibited the activity of the Na^+/HCO_3^- cotransporter in the native basolateral membranes.

The finding that the antibody inhibited the Na^+/HCO_3^- cotransporter activity when added to the outside of the proteoliposomes allowed us to study the interaction of the antibody with substrate binding sites for $NaHCO_3$ or Na_2CO_3. The cotransporter has distinct sites for interaction of Na^+, HCO_3^-, and CO_3^- [19]. The present studies show that in the presence of $NaHCO_3$ or Na_2CO_3 during incubation with the antibody, the inhibitory effect of the antibody was significantly decreased. In these experiments, the pH of the media was carefully controlled to avoid a nonspecific effect of pH on the binding of the antibody. These findings strongly suggest that the antibody interacts with the binding sites for the $NaHCO_3$ or Na_2CO_3.

In summary, the present studies have characterized in detail the development of inhibitory polyclonal antibodies against the 56 *kDa* protein component of the Na^+/HCO_3^- cotransporter. The results show that the antibodies interact at or near the substrate binding sites and that the cotransporter protein is expressed in different regions of the kidney and in other tissues.

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