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Renal Cortical Basolateral Na^+/HCO_3^- Cotransporter III. Evidence for a Regulatory Protein in the Inhibitory Effect of Protein Kinase A

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Abstract. The activity of the Na-H antiporter is inhibited by cyclic AMP-dependent protein kinase A (cAMP-PKA). The inhibitory effect of PKA on the Na-H antiporter is mediated through a regulatory protein that can be dissociated from the antiporter by limited protein digestion. PKA also inhibits the activity of the Na^+/HCO_3^- cotransporter. We investigated whether the activity of Na^+/HCO_3^- cotransporter and the effect of PKA on this transporter may also be regulated by limited protein digestion. In rabbit renal cortical basolateral membranes (BLM) and in solubilized BLM reconstituted in liposomes (proteoliposomes), trypsin (100 μg) increased ^{22}Na uptake in the presence of HCO_3 but not in the presence of gluconate, indicating that trypsin does not alter diffusive ^{22}Na uptake but directly stimulates the Na^+/HCO_3^- cotransporter activity. In proteoliposomes phosphorylated with ATP, the catalytic subunit (CSU) of cAMP-PKA decreased the activity of the Na^+/HCO_3^- cotransporter (expressed as nanomoles/mg protein/3s) from 23 ± 10 to 14 ± 6 ($P < 0.01$). In the presence of trypsin, the inhibitory effect of CSU of cAMP-PKA on the activity of Na^+/HCO_3^- cotransporter was blunted. To identify a fraction that was responsible for the inhibitory effect of the CSU on the Na^+/HCO_3^- cotransporter activity, solubilized proteins were separated by size exclusion chromatography. The effect of CSU of cAMP-PKA on the Na^+/HCO_3^- cotransporter activity was assayed in proteoliposomes digested with trypsin with the addition of a fraction containing the 42 kDa protein (fraction S+) or without the 42 kDa protein (fraction S–). With the addition of fraction S–, the CSU of cAMP-PKA failed to inhibit the Na^+/HCO_3^- cotransporter activity (control 27 ± 6, CSU 27 ± 3) while the addition of fraction S+ restored the inhibitory effect of CSU (27 ± 6 to 3 ± 0.3 $P < 0.01$). The CSU of cAMP-PKA phosphorylated several proteins in solubilized protein including a 42 kDa protein. Fluorescein isothiocyanate (FITC) labels components of the Na^+/HCO_3^- cotransporter including the 56 kDa and 42 kDa proteins. In trypsin-treated solubilized protein the 42 kDa protein was not identified with FITC labeling. The results demonstrate that the activity of the Na^+/HCO_3^- cotransporter is regulated by protein(s) which mediates the inhibitory effect of PKA. Limited protein digestion can dissociate this protein from the cotransporter.

Key words: Protein kinase A — Na^+/HCO_3^- cotransporter — Trypsin digestion — Regulatory protein — Protein phosphorylation

Introduction

Bicarbonate transport from proximal tubule cells into the blood is mediated by a basolateral electrogenic Na^+/HCO_3^- cotransporter [1]. This system functions in an integrated fashion with the brush border Na-H antiporter and the activities of these two systems vary in parallel [2]. The activities of the renal Na^+/HCO_3^- cotransporter and of the Na-H antiporter are stimulated by acidosis and are inhibited by alkalosis [10]. In addition, cyclic AMP and calmodulin dependent protein kinases inhibit, while protein kinase C stimulates both transporters [11].

Weinman et al. have studied extensively the regulation of the Na-H antiporter by cAMP-PKA system [8, 14, 15, 16, 17]. They demonstrated that limited protein digestion of renal brush border membranes with trypsin enhances the activity of the Na-H antiporter. They also showed that trypsin digestion abolishes the inhibitory effect of PKA on the Na-H antiporter. They have isolated a peptide fraction that restored the inhibitory effect of PKA and showed that this peptide is also phosphor-

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ylated by PKA. More recently, these investigators have purified the putative regulatory protein of the Na-H antiporter, determined its partial amino acid sequence and developed a polyclonal antibody against this peptide [17]. In brush border membranes immunodepleted of the regulatory protein with this polyclonal antibody, PKA failed to inhibit the Na-H antiporter. On the other hand, the addition of the 55 kDa polypeptide to brush border membranes depleted of the regulatory protein restored the inhibitory effect of PKA on the Na-H antiporter.

We have recently partially purified and then reconstituted the basolateral renal cortical Na^+/HCO_3^- cotransporter into liposomes and showed the enhancement of 56 kDa protein band by SDS-PAGE analysis [4]. Fluorescein isothiocyanate labeling of the purified cotransporter revealed two major bands of 56 kDa, and 42 kDa molecular weights [13]. We have provided initial evidence that catalytic subunit of cAMP-PKA inhibits the purified Na^+/HCO_3^- cotransporter [4]. The present studies were aimed at studying whether trypsin digestion alters the baseline activity of Na^+/HCO_3^- cotransporter and the inhibitory effect of PKA on this cotransporter.

Materials and Methods

Materials

^{22}Na was purchased from Amersham. L-α-phosphatidylcholine, octylglucoside, molecular weight standards, Coomassie R-250, SDS, acrylamide, bisacrylamide, silver staining kit, trypsin, trypsin inhibitor, catalytic subunit of protein kinase A, phenylisothiocyanate (PITC) and fluorescein isothiocyanate (FITC) were bought from Sigma. Centricon 3 and 10 were bought from Amicon. Sephacryl S-100 was purchased from Pharmacia. General laboratory chemicals were of analytical grade and were obtained from Sigma or Fisher Chemicals.

Basolateral Membranes Preparation

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 [4, 10]. 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 Renal Cortical Basolateral Membrane and Purification by Size-exclusion Chromatography

Solubilization of basolateral membrane vesicles was done as previously described [4]. One part purified basolateral membrane vesicles (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 7.2, on ice for 15 min. The concentration of the detergent was 1.1% and the protein concentration was 2.2 mg/ml. The protein-detergent mixture was centrifuged for 30 min at 110,000 ×*g*. The supernatant which was recovered was dialyzed extensively for 18 hr in buffer A using a membrane with molecular weight cut off of 6-8 kDa. Thereafter, the octylglucoside solubilized proteins were fractionated according to size. Size exclusion chromatography was done by loading 40 mg of solubilized proteins onto a Sephacryl S-100 column, after appropriate preparation and equilibration. Elution was done with buffer containing 50 mM Tris, pH 7.4, 0.1 mM EDTA, 0.1 mM DTT and 0.2% octylglucoside at the rate of .5 ml/min. Individual fractions were collected and concentrated using Centricon 3. Thereafter protein concentrations were measured, and used in subsequent experiments as indicated.

Reconstitution of Solubilized and Purified Protein Fraction into Liposomes

The reconstitution of solubilized and purified protein fractions into liposomes was performed as described before [4]. Briefly, the solubilized or purified protein fractions (2.5 mg/ml) were reconstituted into liposomes by mixing 1.6 part of protein (v/v) with one 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 proteoliposomes thus formed were used in subsequent uptake studies. In all uptake assays, the amount of reconstituted solubilized protein was kept constant at 5 μg in each tube. ^{22}Na uptake was performed in the presence of $NaHCO_3$ or Na gluconate in the proteoliposomes, as will be described below.

Trypsin Digestion

When indicated, solubilized proteins or basolateral membranes (2.5 mg/ml) were treated with varying amounts of trypsin at varying time and temperature to find the optimum condition for limited trypsin digestion. Soybean trypsin inhibitor was added at 10-fold molar excess of the trypsin concentration to stop the proteolytic process. As described by Weinman et al. [14], studies on the effect of PKA and trypsin were done by incubating the solubilized protein in immobilized trypsin. To stop the reaction, trypsin was removed by filtration using 50 μm pore size polyethylene disc.

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 technique as previously described [4, 10]. In brief, basolateral membrane vesicles or proteoliposomes were preincubated for 1–2 hr at room temperature in a solution containing (mM) 200 sucrose, 50 HEPES, pH 7.50 with Tris and 1 Mg gluconate. The suspension was spun at 30,000 revolutions/min with 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 reconstituted 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 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 (HCO_3-dependent ^{22}Na uptake) was taken as the difference in ^{22}Na

uptake in the presence or absence of inwardly directed HCO_3^- gradient (HCO_3^- was replaced by gluconate).

Regulation of the Reconstituted Na^+/HCO_3^- Cotransporter by cAMP-PKA

To determine the effect of trypsin digestion on cAMP-dependent PKA regulation of Na^+/HCO_3^- cotransporter, solubilized proteins or trypsin treated solubilized proteins (2.5 mg/ml) were incubated with 50 μM ATP, 100 μM MgCl (pH 7.4) in the presence or absence of 40 mU/ml of catalytic subunit of cAMP-PKA for 15 min at 30° and then reconstituted into proteoliposomes as described before. When indicated, fractions from size exclusion chromatography with 42 kDa proteins (fraction S+) and those without the 42 kDa protein (fraction S–) were added to solubilized protein or trypsin-treated solubilized proteins and phosphorylated as above in the presence or absence of catalytic subunit of cAMP-PKA and also reconstituted into proteoliposomes. HCO_3^- dependent ^{22}Na uptake was then measured in each experimental condition as previously noted.

Protein phosphatase activity was measured in solubilized proteins and trypsin treated solubilized proteins according to the method described by Shriner and Brautigan [12]. Studies on the inhibition of phosphatase activity by zinc and vanadate were also undertaken.

SDS-PAGE Analysis and Autoradiography

To determine the specific proteins phosphorylated by cAMP-PKA, labeling is done by substitution of [γ-^{32}P] ATP for unlabeled ATP. Phosphorylation reaction was terminated by addition of sample buffer containing 60 mM Tris-glycine (pH 8.3), 3% SDS (wt/vol) and 30% glycerol (wt/vol). SDS-PAGE analysis was carried out on discontinuous systems as described by Laemmli [9]. Electrophoresis was done on slab gels of 10–12% acrylamide. The gels were developed when indicated with Coomassie blue or silver stain. When autoradiography is needed, the stained gels are dried and processed. These gels were exposed to x-ray film with tungsten phosphate intensifier screen at –70°C. Phosphorylation of each peptide band was quantified by the use of laser densitometry (Ambis, USA).

FITC Labeling and Limited Trypsin Digestion

FITC labeling and limited trypsin digestion were performed as described previously [13]. Briefly, solubilized protein (2.5 mg/ml) was exposed to 2 mM PITC in the presence of 50 mM $NaHCO_3$, 200 mM PITC at room temperature for 60 min with gentle agitation. The reaction was stopped by addition of a 10-fold volume of solution M (250 mM mannitol, 50 mM HEPES/Tris base, pH 7.5) and dialyzed against solution M at 4°C overnight to remove excess PITC. The PITC treated proteins (1000 μl) was then exposed to 30 μM FITC in presence of 50 mM $NaHCO_3$, for 40 min at room temperature. All steps involving FITC were done in the dark. The reaction was stopped and excess FITC removed by dialysis as above. The FITC treated solubilized protein was then concentrated using Centricon 10 to a final concentration of 2.5 mg/ml. The FITC labeled protein was then treated with trypsin (100 μg) for 15 min at 4°C. The reaction was stopped by addition of trypsin inhibitor at tenfold the molar concentration of trypsin. Protein aliquots were subjected to SDS-PAGE analysis, Coomassie blue staining, and visualization of fluorescent bands by ultraviolet light when indicated.

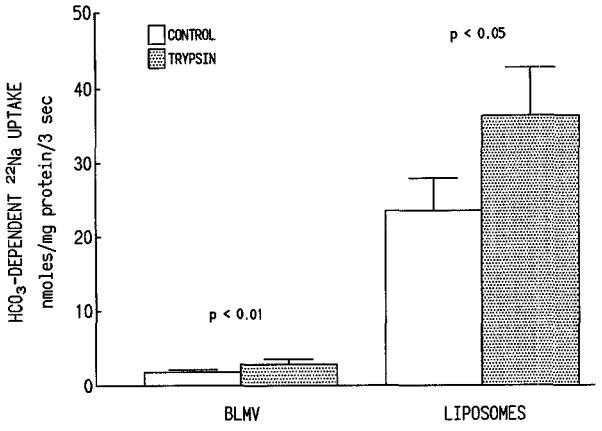

Fig. 1. Basolateral membranes (BLMV) or solubilized protein were incubated with 100 μg of trypsin. The solubilized proteins were then reconstituted into proteoliposomes. HCO_3^- dependent ^{22}Na uptake was measured in BLMV and liposomes as described in Materials and Methods. The figure depicts an average of five different experiments measured in triplicates.

Analysis of Data and Results

Results are presented as mean ± SEM. The *t*-test for paired or unpaired data was used to analyze the data wherever possible.

Abbreviations

cAMP-PKA, 3′5′ Cyclic adenosine monophosphate-Protein Kinase A; SDS-PAGE, Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis; HEPES, (*N*-[2- Hydroxyethyl]Piperazine-*N*′-[2-Ethane Sulfonic Acid]); PITC, Phenyl Isothiocyanate; EDTA, Ethylenediaminetetraacetic Acid; DTT, Dithiothreitol; CSU, Catalytic Subunit, FITC, Fluorescein Isothiocyanate.

Results

Effect of Trypsin on Na^+/HCO_3^- Cotransporter Activity in Basolateral Membranes and in Proteoliposomes

In basolateral membranes incubated with 100 μg of trypsin for 30 min, the activity of the Na^+/HCO_3^- cotransporter (expressed as HCO_3^- dependent ^{22}Na uptake) was significantly increased as compared to controls (trypsin 2.8 ± 0.7, control 1.8 ± 0.3 nanomoles/mg protein/3s $P < 0.01$) (Fig. 1 left panel). These results show that limited trypsin digestion has qualitatively similar effects on the Na^+/HCO_3^- cotransporter and on the Na-H antiporter activity [14].

In proteoliposomes, the activity of the Na^+/HCO_3^- cotransporter was, as previously reported [4], increased on the average 18-fold as compared to the native baso-

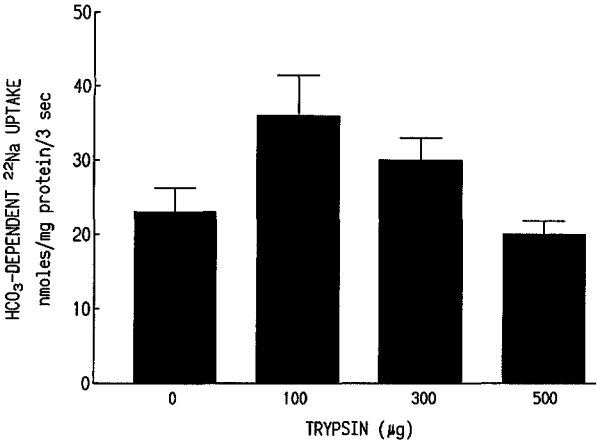

Fig. 2. Solubilized proteins were incubated at 40°C for 15 min with varying amounts of trypsin, and subsequently reconstituted into proteoliposome. HCO_3^- dependent ^{22}Na uptake was measured by rapid filtration technique as described above (Materials and Methods). The results shown represent the average of six different experiments done in triplicate in various proteoliposome preparations.

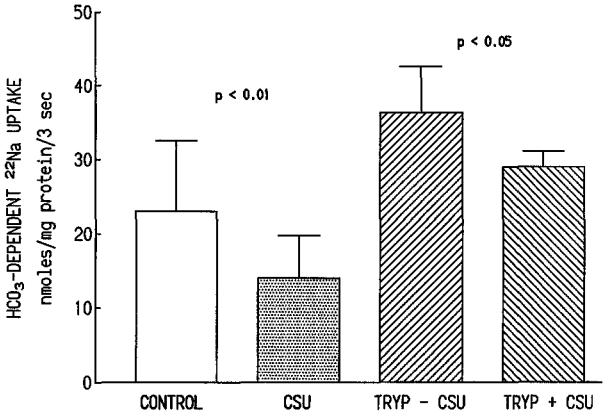

Fig. 3. HCO_3^- dependent ^{22}Na uptake was determined in solubilized proteins reconstituted into proteoliposomes in the absence (CONTROL) and presence of catalytic subunit of cAMP-PKA (CSU). Na/HCO_3 cotransporter activity was also measured in trypsin-treated solubilized proteins in the presence (TRYP + CSU) and absence (TRYP – CSU) of catalytic subunit of cAMP-PKA. The values shown represent the average of five experiments measured in triplicates in different proteoliposome preparations.

lateral membranes (range 8–44-fold). Figure 2 shows the effect of various concentrations of trypsin on the activity of Na^+/HCO_3^- in proteoliposomes.

Preliminary experiments were undertaken to determine the optimum conditions of trypsin digestion by varying the incubation time, temperature and the concentration of trypsin (varying from 20 µg–500 µg). Our initial data indicated that 15 min incubation at 4°C with 100 µg of trypsin yielded the optimum condition for limited trypsin digestion. Lower concentrations of trypsin did not cause the same degree of increase of Na^+/HCO_3 activity compared to 100 µg. It is clear that 100 µg or trypsin enhances the activity of the Na^+/HCO_3^- and higher concentrations of trypsin did not elicit further increases in the activity. For this reason, all subsequent experiments were performed with 100 µg of trypsin. Figure 1, right panel summarizes the effect of 100 µg trypsin on the activity of the Na^+/HCO_3^- cotransporter in six different preparations of proteoliposomes. Trypsin significantly increased HCO_3^- dependent ^{22}Na uptake (a measure of Na^+/HCO_3^- cotransporter activity) as compared to controls (36.3 ± 6.3 *vs.* 23.5 ± 4.4 nanomoles/mg protein/3s, $P < 0.005$). Trypsin did not affect ^{22}Na uptake in presence of gluconate (*not shown*), indicating that trypsin does not affect diffusive ^{22}Na uptake. Trypsin enhances the baseline activity of Na/HCO_3 cotransporter and blunted the inhibitory effect of CSU (*see below*). This suggests that the Na/HCO_3 cotransporter may be phosphorylated under baseline conditions and the effect of trypsin could be mediated by an effect on phosphatase activity that would act to phosphorylate/desphosphorylate the Na/HCO_3 cotransporter. We tested for the presence of phosphatase activity in control proteoliposomes and found an average specific protein phosphatase activity of 1.46 ± 1 units/mg protein which was inhibited 53% by vanadate and 46% by ZnCl. The effect of trypsin on phosphatase activity could not be determined with certainty because it appeared to interfere with the assay for the enzyme.

Effect of the Catalytic Subunit (CSU) of Protein Kinase A on Na^+/HCO_3^- Cotransporter Activity in the Presence of Trypsin

We have previously reported that in basolateral membranes and in proteoliposomes phosphorylated with 50 µM ATP, the catalytic subunit of cAMP-PKA significantly inhibits the activity of the Na^+/HCO_3^- cotransporter [4]. Figure 3 shows that the CSU inhibits the Na^+/HCO_3^- cotransporter activity in control proteoliposomes from 23.0 ± 9.5 to 14.0 ± 5.7 nanomoles/mg protein/3 sec, $P < .001$ in agreement with previous results. In trypsin treated proteoliposomes, the baseline activity of the Na^+/HCO_3^- cotransporter increased and yet the inhibitory effect of the CSU was blunted (36.3 ± 6.3 *vs.* 29.0 ± 2.1, $P < .05$). The percentage of inhibition in trypsin-treated proteoliposomes in the presence of CSU was blunted in comparison with proteoliposomes not subjected to limited protein digestion (43.6 ± 5.8% *vs.* 19.8 ± 6.3%, $P < 0.01$).

To identify the protein fraction that may mediate the inhibitory effect of CSU on the Na^+/HCO_3^- cotransporter solubilized proteins were separated by size exclusion chromatography and the ability of different fractions to restore the inhibitory effect of CSU was tested. The effect of the CSU on the Na^+/HCO_3^- was assayed in pro-

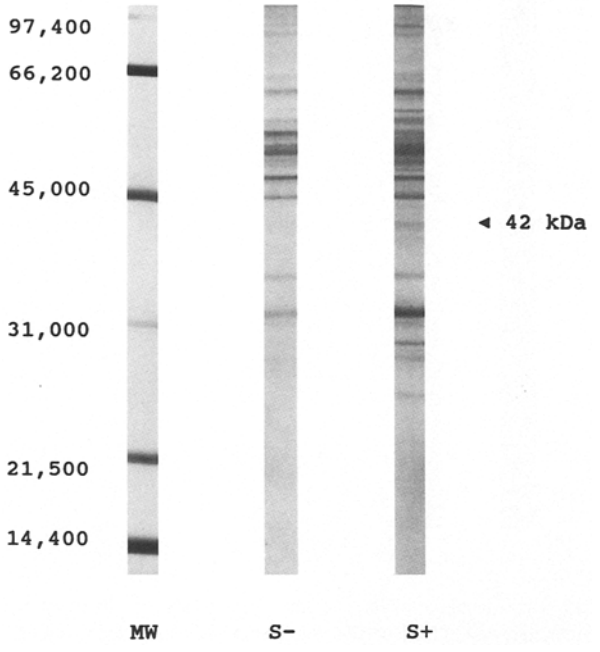

Fig. 4. Representative SDS-PAGE analysis of protein fractions from size exclusion chromatography of the solubilized proteins. Solubilized proteins were loaded onto Sephacryl S-100 column and eluted as described in Materials and Methods. Lane MW is the reference molecular weight, Lane 2 represents Fraction S–, protein fractions which do not contain 42 kDa band. Lane 3, Fraction S+ represents the fractions with the 42 kDa protein. The 42 protein is indicated by the arrow. Ten (10) μg of proteins were loaded on each well.

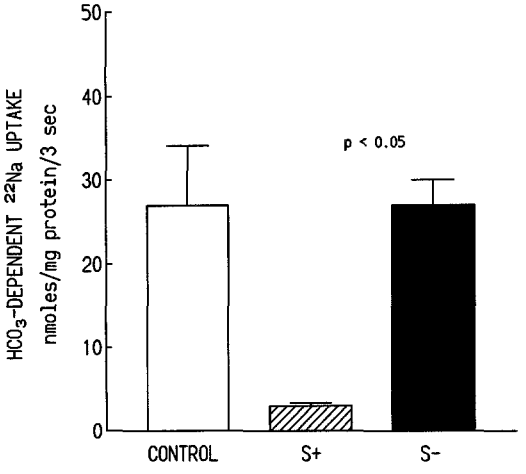

Fig. 5. HCO_3-dependent ^{22}Na uptake was measured in proteoliposomes after incubation of trypsin-treated solubilized protein with CSU of cAMP-PKA with addition of fraction S+ (S+) or fraction S– (S–) from size exclusion chromatography. The results shown represent the average of three separate experiments measured in triplicates in different proteoliposome preparations.

teoliposomes incubated with 100 μg trypsin with and without the addition of fractions collected from the size exclusion chromatography. Fraction S+ and S– were collected and characterized as described in the methods. Figure 4 is the SDS-PAGE analysis of the size exclusion chromatography of the solubilized protein. It can be seen that fraction S– does not contain proteins with molecular weight of 42 kDa, while fraction S+ has this specific protein. Figure 5 shows that with the addition of fraction S–, the CSU failed to inhibit the Na^+/HCO_3^- cotransporter (control 27 ± 6.0 *vs.* S–, 27 ± 3.0) while the addition of fraction S+ containing the 42 kDa proteins restored the inhibitory effect of CSU (3.0 ± 0.3 nanomoles/mg protein/3s).

In octylglucoside solubilized protein phosphorylated with [γ^{32}P]ATP, the CSU enhanced the phosphorylation of several bands including 92, 66, 56, 45 and 42 kDa bands (Fig. 6). Of note was the fact that by densitometric analysis, the CSU increased the phosphorylation of the 42–45 kDa and 56 kDa bands by threefold. Although limited trypsin digestion enhanced the activity of the Na^+/HCO_3^- cotransporter and abolished the inhibitory effect of CSU on this protein, no protein band was consistently absent by SDS-PAGE analysis. We have previously shown that FITC labels protein bands which may be putative components of the Na^+/HCO_3^- cotransporter. We therefore labeled trypsin treated solubilized protein with FITC and subjected this preparation to SDS-PAGE analysis and visualized the fluorescent bands with ultraviolet light. Figure 7 shows that in the control-solubilized proteins, FITC labeled the 56 and 42 kDa bands prominently whereas with trypsin treatment the 42 kDa is notably absent.

Discussion

cAMP-dependent protein kinase A (cAMP-PKA) inhibits the Na-H antiporter in brush border membranes. Weinman et al. have solubilized and reconstituted the rabbit brush border sodium proton antiporter and showed that cAMP dependent protein kinase A inhibits this antiporter in proteoliposomes [14, 15]. These investigators further demonstrated that limited protein digestion with trypsin of the solubilized brush border membranes enhanced the activity of the Na-H antiporter when assayed in proteoliposomes.

Of great interest was the finding that in trypsin-treated membranes, the CSU of cAMP- dependent protein kinase A failed to inhibit the activity of the Na-H antiporter. The inhibitory effect of cAMP was restored by addition of a protein fraction of approximate molecular weight of 42 kDa collected from the size exclusion chromatography and ion-exchange chromatography. These results were interpreted by the investigators as indicating that the activity of Na-H antiporter is regulated by a cofactor which can be dissociated from the antiporter and which represents the site of action of

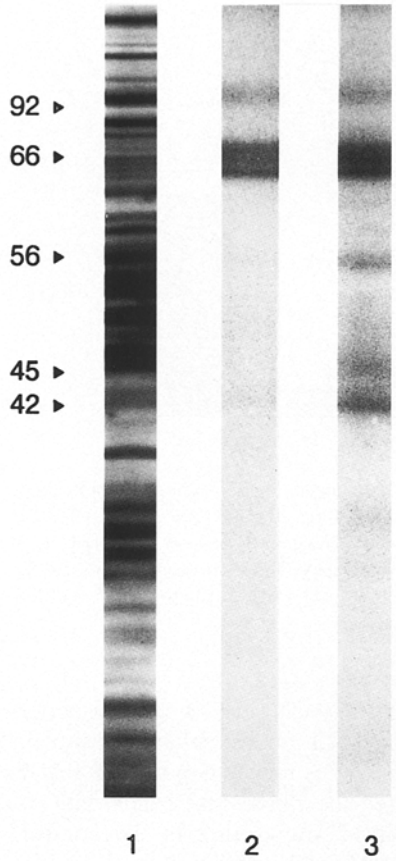

Fig. 6. Representative SDS-PAGE autoradiography of solubilized protein incubated with phosphorylating solution containing Mg and (γ-^{32}P]ATP in the presence (Lane 3) or absence (Lane 2) of the catalytic subunit of cAMP-PKA. Silver stain of SDS-PAGE analysis of the solubilized proteins (Lane 1) is also shown. Phosphorylation of the 42, 45 and 56 kDa proteins are increased threefold as shown by densitometric analysis.

Fig. 7. Representative picture of fluorescent bands visualized by ultraviolet lights on FITC-labeled solubilized protein subjected to limited trypsin digestion. Solubilized proteins initially incubated with 200 mM PITC and dialyzed extensively were labeled with 30 mM FITC. The labeled proteins were subjected to limited trypsin digestion and subsequently analyzed by SDS-PAGE and ultraviolet light visualization of fluorescent bands. Equal amount (10 μg) of proteins were loaded on each lane. Lane 1 no trypsin digestion, Lane 2 with trypsin digestion.

cAMP- dependent protein kinase A. More recently these investigators have provided strong additional evidence for this hypothesis. They have purified the putative regulatory protein of the Na-H antiporter, determined its partial amino acid sequence and developed polyclonal antibodies against the synthetic peptide of this protein [17]. This polyclonal antibody was used to immunodeplete brush border membranes of the putative regulatory protein and these investigators have shown that in brush border membranes immunodepleted of this protein, PKA did not inhibit the Na-H antiporter. Coreconstitution of this protein component with brush border membranes depleted of same fraction restored the inhibitory effect of PKA on the Na-H antiporter. This result provides convincing evidence that the activity of the Na-H antiporter is regulated by a protein that can be dissociated from the antiporter and which represents the site of action of PKA.

We have previously shown that the activities of the Na-H antiporter and the Na^+/HCO_3^- cotransporter are regulated by the same signal transduction factors and vary in a parallel fashion [10, 11]. We therefore hypothesize that the inhibitory effect of cAMP-PKA on the Na^+/HCO_3^- cotransporter may be also abolished by limited protein digestion. In preliminary studies in basolateral membranes, we showed that limited protein digestion stimulated the ^{22}Na uptake in the presence of bicarbonate but not in the presence of gluconate indicating that it directly stimulates Na^+/HCO_3^- activity without altering diffusive Na^+ uptake [11]. A similar effect was also observed in proteoliposomes. We have previously reported that the CSU of protein kinase A inhibits Na^+/HCO_3^- activity in basolateral membranes and in proteoliposomes. Of great interest was the finding (Fig. 3) that in solubilized protein treated with trypsin, the effect of the catalytic subunit to inhibit the activity of the Na^+/HCO_3^- cotransporter was blunted. These two findings i.e., the stimulation of the baseline activity of the cotransporter and the blunting of the inhibitory effect of the CSU of protein kinase A on the cotransporter protein by limited trypsin digestion are similar to the findings reported by Weinman et al. on the Na-H antiporter [14]. The inhibitory effect of CSU of cAMP-PKA on trypsin-treated solubilized protein could be restored by addition of a fraction from size exclusion chromatography con-

taining proteins of molecular weight in the 42 kDa. This finding indicates that trypsin treatment did not abolish the inhibitory effect of CSU by irreversibly altering the cotransporter. The restoration of the inhibitory effect of CSU in trypsin treated membranes by a fraction identified from size exclusion chromatography suggests that the Na^+/HCO_3^-, like the Na-H antiporter, is regulated by a cofactor that can be dissociated from the cotransporter.

Alternatively, it could be argued that the effect of trypsin is mediated at best in part, through an effect on phosphatase activity. Phosphatase activity was detected in proteoliposomes in amount comparable to that reported in other tissues [12], and therefore it is possible that trypsin enhances the baseline activity of the cotransporter by activating phosphatases and thereby removing the phosphorylation of the cotransporter. Unfortunately, this hypothesis could not be proven because trypsin appeared to interfere with the assay for phosphatase activity. Notwithstanding this problem, the results suggest that the Na^+/HCO_3^- cotransporter activity level is partially phosphorylated in the basal state.

We attempted to identify this fraction by several approaches. SDS-PAGE analysis of trypsin treated solubilized protein did not consistently disclose the absence of specific protein. We have previously purified the Na^+/HCO_3^- cotransporter and have suggested that 56 kDa protein doublet represents an active component of the cotransporter protein [4]. We have also shown that fluorescein isothiocyanate (FITC) specifically interacts and labels the Na^+/HCO_3^- cotransporter. We identified three protein fractions which are of the 92, 56, and 42 kDa molecular weights which are thought to represent components of the Na^+/HCO_3^- cotransporter [13] with 56 and 42 kDa bands being consistently labeled by FITC. Of interest in the present studies was the finding that in trypsin treated solubilized protein labeled with FITC there was disappearance of the 42 kDa protein fraction with preservation of the 56 kDa. Of additional interest was the finding that the catalytic subunit of PKA phosphorylated several proteins with a threefold increase in the phosphorylation of 56 kDa and in the 42–45 kDa bands. The finding that the protein fractions are phosphorylated by PKA and the fraction containing 42–45 kDa restores the inhibitory effect of cAMP-PKA strongly suggest that this protein fraction is involved in the regulation of the Na^+/HCO_3^- cotransporter by PKA. Although these studies do not prove that the 42–45 kDa protein mediates the inhibitory effect of PKA, they do suggest that the Na^+/HCO_3^- cotransporter like the Na-H antiporter is regulated by a cofactor which could be dissociated from the cotransporter.

The presence of a regulatory protein mediating the effect of hormones and other substances of the Na^+-K^+-ATPase has also been described in detail [3, 7]. The Na-K-ATPase of the rat thick ascending limb has been shown to be regulated by 32 kDa protein which is phosphorylated by dopamine through cyclic AMP (DARPP-32, dopamine and cyclic AMP regulated phosphoprotein of molecular weight 32,000). This same regulatory protein has also been described and characterized in rat brain striatal slices [6]. It appears that the function of several transporters may be regulated by phosphorylation of a cofactor.

In summary, the present studies demonstrate that activity of the purified Na^+/HCO_3^- is inhibited by cyclic AMP-PKA system and this inhibition may be mediated through a component that can be dissociated from cotransporter. Further studies are needed to purify and characterize this cofactor.

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