J. Membrane Biol. 109, 233–241 (1989)

The Journal of
Membrane Biology

Effect of Limited Trypsin Digestion on the Renal Na^+-H^+ Exchanger and Its Regulation by cAMP-Dependent Protein Kinase

E.J. Weinman, W.P. Dubinsky, Q. Dinh, D. Steplock, and S. Shenolikar
Departments of Internal Medicine, Pharmacology and Physiology, University of Texas Medical School, Houston, Texas 77025

Summary. The Na^+-H^+ exchanger from solubilized rabbit renal brush border membranes is inhibited by cAMP-dependent protein kinase (PKA) mediated protein phosphorylation. To characterize this inhibitory response and its sensitivity to limited proteolysis, the activity of the transporter was assayed after reconstitution of the proteins into artificial lipid vesicles. Limited trypsin digestion increased the basal rate of proton gradient-stimulated, amiloride-inhibitable sodium uptake in reconstituted proteoliposomes and blocked the inhibitory response to PKA-mediated protein phosphorylation. To determine if the inhibitory response to PKA-mediated protein phosphorylation could be restored to the trypsin-treated solubilized proteins, nontrypsinized solubilized brush border membrane proteins were separated by column chromatography. The addition of small molecular weight polypeptides, fractionated on Superose-12 FPLC (V_e = 0.7), to trypsinized solubilized brush border membrane proteins restored the inhibitory response to PKA-mediated protein phosphorylation. Similarly, the addition of the 0.1 M NaCl fraction from an anion exchange column, Mono Q-FPLC, also restored the inhibitory response to PKA. Both protein fractions contained a common 42–43 kDa protein which was preferentially phosphorylated by PKA.

These results indicate that limited trypsin digestion dissociates the activity of the renal Na^+-H^+ exchanger from its regulation by PKA. It is suggested that trypsin cleaves an inhibitory component of the transporter and that this component is the site of PKA-mediated regulation. Phosphoprotein analysis of fractions that restored PKA regulation raises the possibility that a polypeptide of 42–43 kDa is involved in the inhibition of the renal Na^+-H^+ exchanger by PKA-mediated protein phosphorylation.

Key Words cAMP-dependent protein kinase · Na^+H^+ exchanger · renal electrolyte transport

Introduction

Recent studies have indicated that the Na^+-H^+ exchanger in renal brush border membranes is inhibited by cAMP-dependent protein kinase (PKA) mediated protein phosphorylation [9]. The present experiments examine the effects of limited trypsin digestion on the activity of the renal brush border membrane Na^+-H^+ exchanger and the regulation of this transporter by PKA. The rationale for the experiments relates to the specificity of trypsin cleavage and the primary structural requirements for protein phosphorylation by PKA. PKA phosphorylates serine or threonine residues when present in the following sequence: arginine · arginine (lysine) · X · Y · serine (threonine) [6]. Thus, the basic residues recognized by PKA also provide a cleavage site for trypsin. Limited proteolysis with trypsin was performed on detergent-solubilized proteins from rabbit renal brush border membranes. Na^+-H^+ exchange activity of the solubilized proteins was assayed after reconstitution into artificial phospholipid vesicles [7]. The results indicate that limited proteolysis by trypsin increases the basal Na^+-H^+ exchange rate in reconstituted proteoliposomes and blocks the inhibitory effect of PKA-mediated protein phosphorylation on the activity of the transporter. When trypsinized proteins are co-reconstituted with specific protein fractions obtained from brush border membranes, the inhibitory response to PKA is restored.

Materials and Methods

Brush border membranes were prepared from rabbit kidney by magnesium aggregation and differential centrifugation [3]. The membrane vesicles were suspended in a solution containing (in mM): 254 mannitol, 10 Tris, 16 HEPES, and 10 $MgSO_4$ (pH 7.6). To prepare proteoliposomes, the membranes (one part) were mixed with 8% octyl glucoside (1.25 parts) at pH 8.0 for 15 min at 4°C as previously described [7]. The octyl glucoside was prepared in a solution containing 250 mM mannitol, 50 mM MES/Tris HCl (pH 7.4) [7]. The membrane-detergent mixture was centrifuged at 100,000 × g for 30 min and the supernatant containing the solubilized proteins used in the remaining studies. Where examined, PKA regulation of Na^+-H^+ exchange was determined by incubating the solubilized proteins in a solution containing 50 μM ATP, and 100 μM $MgCl_2$ (pH 7.4) in the absence or presence of catalytic subunit of PKA (40 mU/ml) for 5 min at 30°C. The supernatant containing the solubilized brush border

Table. Effect of limited trypsin digestion on the Na^+-H^+ exchanger as assayed in reconstituted proteoliposomes

	Control	Experimental	*P*
Total sodium uptake	13.8 ± 0.8	17.7 ± 0.9	<0.01
Amiloride-sensitive sodium uptake	6.3 ± 0.4	9.5 — 0.8	<0.01
Amiloride-insensitive sodium uptake	7.5 ± 0.5	8.2 ± 0.4	NS

Proton gradient-stimulated (pH_{in} = 6.0, pH_{out} = 8.0) uptake of 1 mM sodium in artificial proteoliposomes prepared from solubilized brush border membrane proteins (Control) and solubilized brush border membrane proteins incubated with trypsin (Experimental). Values are the mean of means ± SEM for 12 preparations. Uptake values are expressed as nmol · mg protein^{-1} · 2 min^{-1}. NS = not significant.

membrane proteins (1.6 parts) was then mixed with one part asolectin (35 mg/ml), which had been sonicated to translucency for 10 min at 22°C. The asolectin was prepared in a solution containing 250 mM mannitol, 50 mM MES/Tris HCl (pH 6.0). Proteoliposomes were prepared by dialyzing the mixture against a solution containing 250 mM mannitol and 50 mM MES/Tris (pH 6.0) for 18 hr at 4°C with one buffer change, using dialysis membranes with 6,000–8,000 Da mol wt cutoff.

Except where specifically noted otherwise, the uptake of sodium in proteoliposomes was determined under pH gradient conditions (pH_{in} = 6.0, pH_{out} = 8.0). The uptake solution contained (in mM): 1 sodium, 250 mannitol, and 50 Tris/Mes (pH 8.0). Studies were performed in the presence or absence of amiloride (2 mM). Sodium uptake was measured using Dowex 50 × 8 (Tris), 100 mesh columns. In some studies, proton gradient-stimulated sodium uptake was measured under voltage clamped conditions (K^+_{in} = K^+_{out} = 30 mM and valinomycin 1 μg/ml). Proton permeability was determined by measurement of the change in acridine orange fluorescence [3, 7].

500 μl of solubilized proteins (50 μg/ml) were incubated in varying concentrations of trypsin (Type XI: DPCC-treated trypsin from bovine pancreas, Sigma, St. Louis, MO) for increasing periods of time at 30°C. The trypsin reaction was stopped by adding soy bean trypsin inhibitor at two- to 10-fold molar excess to the concentration of trypsin. As will be discussed, it was not possible to use the soy bean trypsin inhibitor in studies examining the relation between trypsin and PKA. In these studies, the solubilized proteins were incubated in immobilized trypsin (insoluble trypsin from bovine pancreas attached to polyacrylamide, Sigma, St. Louis, Mo). The trypsin was then removed by rapid filtration through polyethylene discs (50 μm pore size).

To fractionate the membrane proteins by size, 20 mg of solubilized brush border membrane proteins were applied to a Superose-12 (Pharmacia, Upsala, Sweden) FPLC column and eluted with a solution containing 50 mM Tris, 0.1 mM EDTA, 0.1 mM DTT, and 2% octyl glucoside at a rate of 0.5 ml/min. To obtain protein fractions separated by charge, 20 mg of solubilized brush border membrane proteins were applied to a Mono Q HR 5/5 (Pharmacia, Upsala, Sweden) anion exchange column. Fractions were eluted with a step gradient using solutions containing 50 mM Tris, 0.1 mM EDTA, 0.1 mM DTT, 2% octyl glucoside, and 0 to 0.4 M NaCl at a rate of 1 ml/min. In one series of experiments, solubilized brush border membrane proteins from the size exclusion column were sequentially applied to the anion exchange column and eluted with 0.1 M NaCl. The ability of the protein fractions to restore regulation of trypsinized solubilized brush border membrane proteins was determined by mixing the separated protein fractions with trypsinized proteins. The protein mixture was then phosphorylated as previously described and Na^+-H^+ exchange activity assayed after reconstitution.

Substrate proteins for PKA were identified by substitution of γ-[^{32}P]-ATP for unlabeled ATP in the phosphorylating solution, electrophoresis on 10% polyacrylamide gels in the presence of SDS, and autoradiography as previously described [8, 9]. The catalytic subunit of PKA was prepared by the method of Beavo et al. [1]. Protein concentrations were measured after TCA (25% wt/vol) precipitation by the method of Lowry et al. [5]. Statistical analysis was performed using the *t* test for paired data. Results are expressed as means of means ± SEM.

Results

In preliminary experiments, the effect of varying time of incubation and the concentration of trypsin on the amiloride-sensitive component of proton gradient-stimulated sodium uptake was determined. High concentrations of trypsin (>100 μg/ml) and/or prolonged periods of incubation (>20 min) resulted in decreased rates of sodium uptake. Shorter periods of incubation (10 min) with enzyme concentrations of 25 to 50 μg/ml resulted in a consistent increase in sodium uptake. The latter experimental conditions were used in the remainder of the studies. In all experiments presented, pretreatment of the vesicles with soy bean trypsin inhibitor completely blocked the effect of trypsin on the Na^+-H^+ exchanger, but had no effect by itself.

The Table summarizes the effects of trypsin on sodium uptake under proton gradient conditions in reconstituted proteoliposomes. Total sodium uptake was significantly increased by trypsin treatment of the solubilized proteins from 13.8 ± 0.8 nmol · mg protein^{-1} · 2 min^{-1} in controls to 17.7 ± 0.9 ($P < 0.01$). The amiloride-insensitive component of sodium uptake was not significantly affected. Trypsin increased the amiloride-sensitive component of proton gradient-stimulated sodium uptake by 50.7 ± 9.3% ($P < 0.01$). Additional studies were performed in proteoliposomes prepared with solubilized proteins as noted above but in which proton gradient-stimulated sodium uptake was determined under voltage clamped conditions (K^+_{in} = K^+_{out} = 30 mM, valinomycin 1 μg/ml) (Fig. 1). The adequacy of the voltage clamp was confirmed in separate studies using the voltage-sensitive dye DiS-C_3-(5) [2]. Proteoliposomes were prepared by dialysis against a solution containing 190 mM mannitol, 50 mM MES/Tris, and 30 mM K gluconate (pH 6.0). The proteoliposomes were incubated in a solution containing either 190 mM mannitol, 30 mM K gluconate, 50 mM MES/Tris, 1 μg/ml valinomycin,

(1 μg/ml), and 2 μM Dis-C_3-(5) (pH 6.0) or 190 mM mannitol, 30 mM K gluconate, 50 mM Tris/MES, 1 μg/ml valinomycin, and 2 μM DiS-C_3-(5) (pH 8.0). The excitation and emission wave lengths were 620 and 669 nm, respectively. With the above external solutions, the fluoresence signal remained constant over time, indicating no change in the intravesicular voltage. The addition of NaCl to a final concentration of 1.5 mM also did not change the fluorescence signal. Imposition of K^+ gradients by deletion of K^+ from the external solution or by increasing the K^+ concentration in the external solution to 80 mM resulted in clear changes in the signal, indicative of a change in the vesicular voltage. Under voltage clamped conditions, trypsinization increased the amiloride-sensitive component of proton gradient-stimulated sodium uptake by 72.5 ± 16.4% ($P < 0.05$). The change in acridine orange fluorescence was used to determine the rates of proton efflux from the proteoliposomes. In the presence of N-methyl gluconate (50 mM) in the external solution, the rate constant for protein efflux averaged 0.024 ± 0.004 sec^{-1} in control proteoliposomes and 0.022 ± 0.003 in proteoliposomes that had been incubated in trypsin (P = NS). In the presence of sodium gluconate (50 mM) in the external solution, the rate constant for proton efflux was significantly higher in proteoliposomes containing trypsin-treated proteins (0.048 ± 0.002 sec^{-1}) than in control proteoliposomes (0.030 ± 0.004) ($P < 0.05$). To obtain an independent measure of sodium permeability, the uptake of 50 mM sodium was determined under proton gradient conditions. This concentration of sodium is much higher than the K_a for sodium in the reconstituted proteoliposomes [7]. Trypsin had no effect on sodium uptake under these experimental conditions (% change = 0 ± 4%, $n = 3$, P = NS). To rule out the possibility that the stimulation of the Na^+-H^+ exchanger by trypsin digestion of the solubilized proteins was the secondary consequence of an alteration in the efficiency of reconstitution, amiloride-sensitive protein gradient-stimulated uptake of sodium was determined in proteoliposomes incubated in trypsin after formation of the proteoliposomes. In these studies, proteoliposomes were formed at pH 7.0 and the trypsin reaction performed at pH 7.0 so as not to acid inactivate the trypsin. The uptake of sodium was measured from a solution at pH 8.0. As compared to controls not exposed to trypsin, trypsinization of the preformed proteoliposomes significantly increased the amiloride-sensitive component of proton gradient-stimulated sodium uptake by 34.4 ± 7.0% ($n = 4$, $P < 0.05$).

The relation between trypsinization of the solubilized proteins and the effect of protein phosphorylation mediated by PKA on Na^+-H^+ exchange was examined. Preliminary experiments indicated that soybean trypsin inhibitor by itself blocked the inhibitory effect of the catalytic subunit of PKA on the Na^+-H^+ exchanger. The inhibitor did not, however, block the action of PKA as assayed in a histone 1 phosphorylation assay but rather, appeared to interact with the solubilized proteins. The nature of the interaction was not explored further. It also was not possible to examine the effects of PKA in preformed proteoliposomes due to the marked inhibitory effect of soy bean phospholipid on PKA activity. To circumvent these experimental problems, solubilized membrane proteins were incubated with immobilized trypsin. Protein recovery after filtration of the trypsin beads was complete and the filtration process itself did not alter the rates of proton gradient-stimulated sodium transport. This procedure also eliminated any potential proteolysis of PKA during subsequent studies. After removal of trypsin, the solubilized proteins were incubated in a phosphorylating solution containing ATP and magnesium in the presence or absence of catalytic subunit of PKA. The proteins were then incorporated into proteoliposomes after overnight dialysis and the rates of sodium uptake determined under proton gradient conditions in the absence or presence of amiloride (Fig. 2). Trypsinization using insoluble trypsin significantly increased amiloride-sensitive sodium uptake from 6.1 ± 0.5 nmol · mg $protein^{-1}$ · 2 min^{-1} in controls to 7.4 ± 0.4 ($P < 0.05$). In control proteoliposomes, PKA-mediated

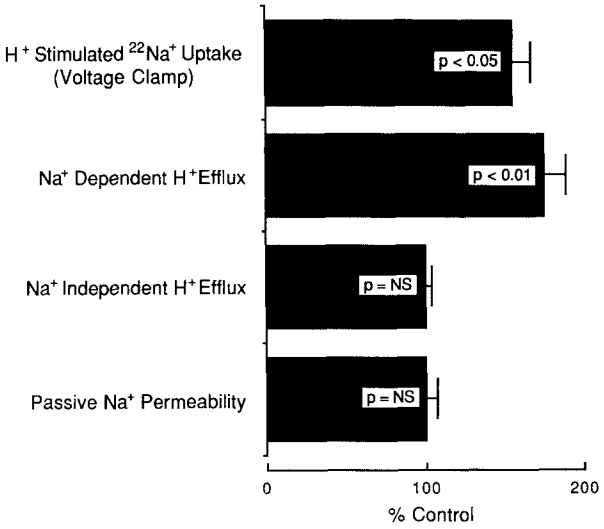

Fig. 1. The effect of trypsinization of solubilized brush border membrane proteins on selected transport functions as assayed in reconstituted proteoliposomes. *See* text for details of individual experimental protocols. Results are expressed as % control ± SEM. NS = not significant

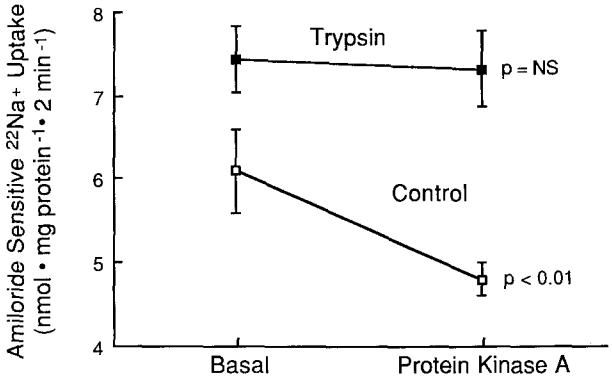

Fig. 2. The relation between the amiloride-sensitive component of proton gradient-stimulated sodium uptake, trypsin, and PKA-mediated protein phosphorylation. Solubilized brush border membrane proteins were incubated in a phosphorylating solution in the absence (*Basal*) or presence of the catalytic subunit of cAMP-dependent protein kinase (*Protein Kinase A*). Studies were performed in control solubilized proteins and in proteins incubated with immobilized trypsin prior to phosphorylation. Results are expressed as the mean of means ± SEM. NS = not significant

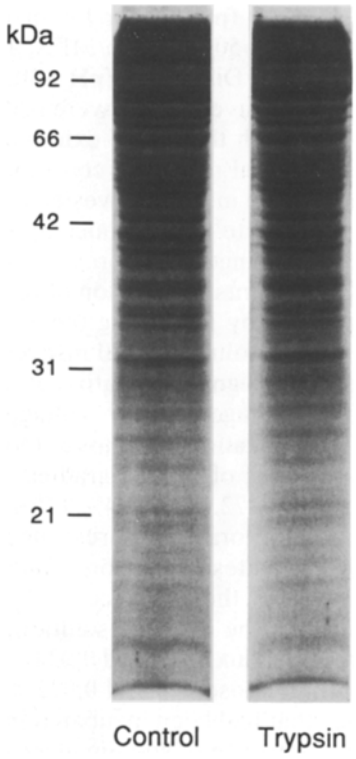

Fig. 3. Coomassie Blue stained SDS-PAGE gels of solubilized brush border membrane proteins before (*Control*) and after limited trypsin digestion with immobilized trypsin

protein phosphorylation significantly decreased the amiloride-sensitive component of proton gradient-stimulated sodium uptake by 19.1 ± 3.7%, ($n = 8$, $P < 0.01$). Trypsinization of the solubilized proteins completely blocked the inhibitory effect of PKA-mediated protein phosphorylation (Fig. 3).

Based on the evidence noted above, it is suggested that a trypsin-sensitive polypeptide may be involved in PKA-mediated regulation of the Na^+-H^+ exchanger. To gain additional insights into this relation, solubilized brush border membrane proteins were separated by Superose-12 FPLC. Fractions eluting at $V_e = 0.7$ (fraction S) contained several small molecular weight polypeptides. Solubilized rabbit brush border membrane proteins were trypsinized as previously indicated. To this sample, 10 to 20 μl of the Superose column fraction S was added. The proteins were then phosphorylated by PKA in the presence of ATP and magnesium, and reconstituted into proteoliposomes. In proteoliposomes containing trypsinized proteins without fraction S, proton gradient-stimulated, amiloride-inhibitable sodium uptake averaged 5.3 ± 0.5 nmol · mg protein^{-1} · 2 min^{-1} and 5.4 ± 0.5 in the absence of and after PKA-mediated protein phosphorylation, respectively ($n = 6$, P = NS). In proteoliposomes containing trypsinized proteins co-reconstituted with 10 to 20 μl of fraction S containing 3.2 μg of proteins, amiloride-sensitive sodium uptake averaged 5.6 ± 0.6 nmol · mg protein^{-1} · 2 min^{-1} in control conditions and was significantly decreased to 3.5 ± 0.5 after PKA-mediated protein phosphorylation ($n = 6$, $P < 0.05$). The addition of increased amounts of fraction S did not result in changes in either the basal rate of sodium uptake or the magnitude of the response to PKA. Figure 4 summarizes the effect of PKA-mediated protein phosphorylation on proton gradient-stimulated, amiloride-inhibitable sodium uptake expressed as percent of basal activity. Also shown are parallel studies using nontrypsinized proteins. PKA-mediated protein phosphorylation decreased the activity of the Na^+-H^+ exchanger in nontrypsinized control proteins by 36 ± 6% ($P < 0.05$). PKA did not inhibit sodium uptake in proteoliposomes containing trypsinized proteins (% change = −4 ± 10%, P = NS). The addition of fraction S to trypsinized proteins restored the inhibitory response to PKA (% change = 35 ± 5%, $P < 0.05$).

The reconstitution of fraction S alone in the absence of solubilized brush border membrane proteins was determined to rule out the possibility that this fraction was highly enriched in the Na^+-H^+ exchanger and that the inhibitory response to PKA-mediated protein phosphorylation was the consequence of addition of untrypsinized transporter. As compared to liposomes containing no added protein, the reconstitution of 20 μl of fraction S re-

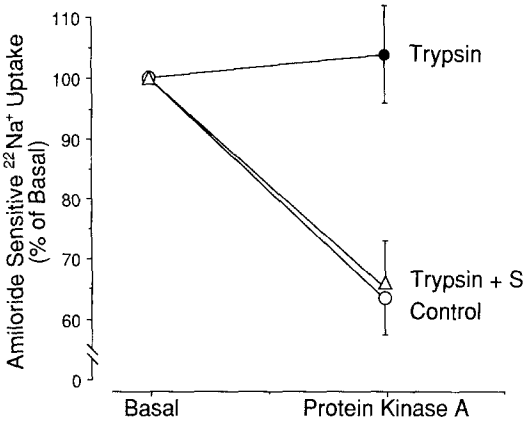

Fig. 4. The effect of PKA-mediated protein phosphorylation of solubilized brush border membrane proteins on proton gradient-stimulated, amiloride-inhibitable sodium uptake in reconstituted proteoliposomes. Proteins were incubated in a solution containing ATP and magnesium in the absence (*Basal*) or presence of the catalytic subunit of PKA. Results are expressed as % of basal (mean ± SEM). Shown are the effects of PKA-mediated protein phosphorylation on Na^+-H^+ exchange transport in control solubilized proteins, in proteins that underwent limited trypsin digestion prior to phosphorylation, and trypsinized proteins co-reconstituted with the V_e = 0.7 eluate from a size exclusion chromatographic column separation of solubilized brush border membrane proteins (fraction S)

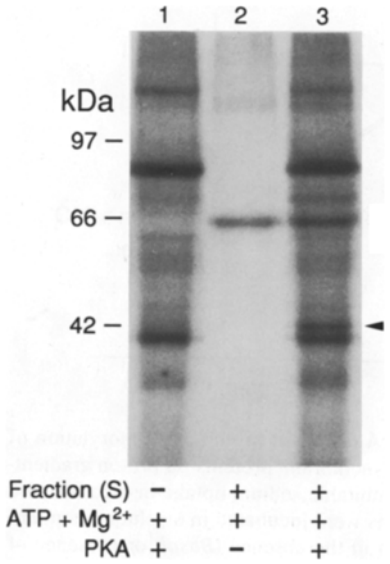

Fig. 5. Representative SDS-PAGE autoradiograph of the protein fraction of solubilized brush border membrane proteins eluted from a size exclusion column at V_e = 0.7 (fraction S). Lane *1* is the catalytic subunit of PKA. Fraction S was incubated with $-[^{32}P]$-ATP and magnesium in the absence (lane *2*) or presence (lane *3*) of PKA. Molecular weight standards are indicated. Arrow points to a 42–43 kDa polypeptide

sulted in no change in sodium uptake. Co-reconstitution of control nontrypsinized proteins and 20 μl of fraction S resulted in no change in basal Na^+-H^+ exchange transport activity or in the magnitude of the response to PKA-mediated protein phosphorylation.

Figure 5 is a representative SDS-PAGE autoradiograph of fraction S phosphorylated by PKA. Lane *1* is the catalytic subunit preparation incubated in ATP and magnesium. Fraction S was incubated in ATP and magnesium in the absence (lane *2*) or presence of PKA (lane *3*). By this analysis, only the 42–43 kDa polypeptide (arrow) appears unique PKA substrate present in fraction S. The phosphorylation of this 42–43 kDa polypeptide by PKA is blocked by the specific inhibitor of this protein kinase (*data not shown*).

In preliminary experiments, 0.1, 0.2, 0.3, and 0.4 M NaCl fractions from the anion exchange column were mixed with trypsinized solubilized proteins and phosphorylated by PKA. 80 μl of each fraction was examined, and samples were not normalized for the differing amounts of protein in these fractions. Under these conditions, only the 0.1 M fraction restored PKA regulation to trypsinized solubilized brush border membrane proteins. All additional studies were performed using the 0.1 M NaCl fraction (fraction MQ). In proteoliposomes containing trypsinized proteins but not the 0.1 M NaCl protein fraction, proton gradient-stimulated, amiloride-inhibitable sodium uptake averaged 5.2 ± 0.6 nmol · mg $protein^{-1}$ · 2 min^{-1} and 5.2 ± 0.4 in the absence of and after PKA-mediated protein phosphorylation, respectively (n = 6, P = NS). In proteoliposomes prepared with trypsinized proteins co-reconstituted with 20 μl of fraction MQ containing 5.8 μg of protein, amiloride-sensitive sodium uptake averaged 5.9 ± 0.7 nmol · mg $protein^{-1}$ · 2 min^{-1} in control conditions and was significantly decreased to 4.1 ± 0.4 after PKA-mediated protein phosphorylation ($P < 0.05$). The addition of increased amounts of fraction MQ did not result in changes in either the basal rate of sodium uptake or the magnitude of the response to PKA. Figure 6 summarizes the effect of PKA-mediated protein phosphorylation on proton gradient-stimulated, amiloride-inhibitable sodium uptake expressed as percent of basal activity. Also shown are parallel studies using nontrypsinized proteins. PKA-mediated protein phosphorylation decreased the activity of the Na^+-H^+ exchanger in nontrypsinized control proteins by 30 ± 9% ($P < 0.05$). PKA did not inhibit sodium uptake in proteoliposomes containing trypsinized proteins (% change = −4 ± 10%, P = NS). The addition of fraction MQ restored the inhibitory response to PKA (% change = 29 ± 7%, $P < 0.05$).

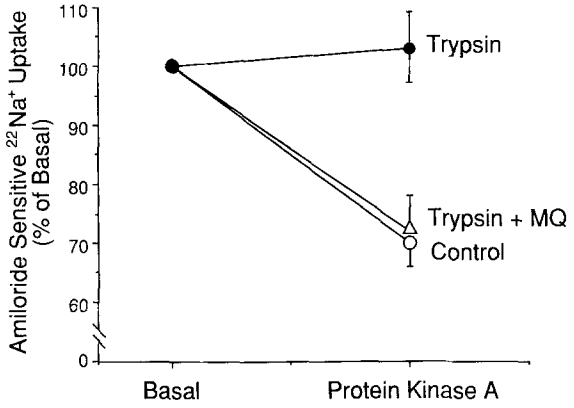

Fig. 6. The effect of PKA-mediated protein phosphorylation of solubilized brush border membrane proteins on proton gradient-stimulated, amiloride-inhibitable sodium uptake in reconstituted proteoliposomes. Proteins were incubated in a solution containing ATP and magnesium in the absence (*Basal*) or presence of the catalytic subunit of PKA. Results are expressed as % of basal (mean ± SEM). Shown are the effects of PKA-mediated protein phosphorylation on Na^+-H^+ exchange transport in control solubilized proteins, in proteins that underwent limited trypsin digestion prior to phosphorylation, and trypsinized proteins co-reconstituted with 0.1 M NaCl eluate from an anion exchange chromatographic separation of solubilized brush border membrane proteins (fraction MQ)

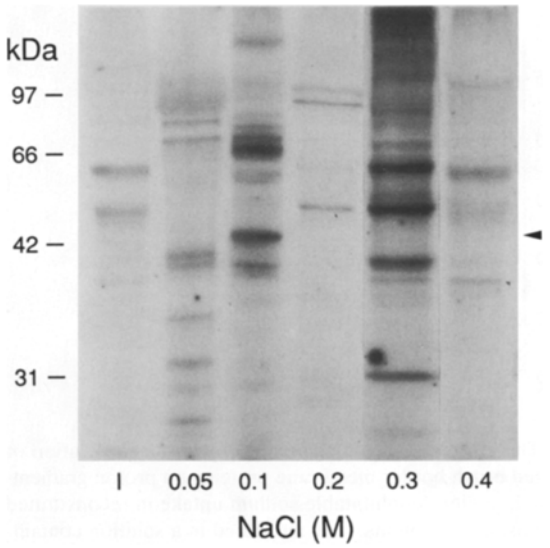

Fig. 7. Representative SDS-PAGE autoradiograph of solubilized brush border membrane protein fractions separated by anion exchange chromatography. *I* is the protein fraction not bound to the column. The protein fractions were incubated with $-[^{32}P]$-ATP, magnesium, and catalytic subunit of PKA. Molecular weight standards are indicated. Arrow points to a 42–43 kDa polypeptide

Fraction MQ (20 μl) did not affect the rate of Na^+-H^+ exchange activity when added to nontrypsinized proteins and did not increase sodium uptake when reconstituted into liposomes containing no other proteins.

Figure 7 is a representative SDS-PAGE autoradiograph of fractions eluted from the anion exchange column and phosphorylated by PKA. A 42–43 kDa protein is demonstrated in the 0.1 M NaCl fraction. The phosphorylation of the 42–43 kDa protein by PKA is blocked by the specific inhibitor of this kinase (*data not shown*). Figure 8 is a comparison of fraction S (lane *2*) and fraction MQ (lane *3*) indicating that both fractions contain the 42–43 kDa protein.

A final series of experiments employed a more purified protein fraction obtained by sequential chromatography on Superose-12 and Mono Q. The protein fraction from the size exclusion column (V_e = 0.7) was applied to the anion exchange column and eluted by 0.1 M NaCl (fraction S/MQ). Proton gradient-stimulated, amiloride-inhibitable sodium uptake in control nontrypsinized proteins was inhibited by PKA-mediated protein phosphorylation by 39 ± 6% (n = 8, $P < 0.01$) but was not inhibited when trypsinized proteins were used (% change = 0 ± 9%, P = NS) (Fig. 9). The addition of 20 μl of fraction S/MQ containing 0.5 μg of protein restored the inhibitory response to PKA (% change = 39 ± 9%, $P < 0.02$). The addition of increased amounts of fraction S/MQ did not result in changes in either the basal rate of sodium uptake or the magnitude of the response to PKA. Fraction S/MQ did not affect the rate of Na^+-H^+ exchange activity when added to nontrypsinized proteins and did not increase sodium uptake when reconstituted into liposomes containing no other proteins.

Discussion

Prior studies from this laboratory have indicated that proteoliposomes prepared from solubilized renal brush border membrane proteins demonstrate proton gradient-stimulated, amiloride-inhibitable sodium uptake [7]. The characteristics of sodium uptake in these reconstituted proteoliposomes bears a number of properties in common with the Na^+-H^+ exchanger observed in native renal brush border membrane vesicles [4]. Other published studies from our laboratories demonstrated that PKA-mediated protein phosphorylation of solubilized renal brush border membrane proteins decreased Na^+-H^+ exchange activity as assayed after the proteins are reconstituted into artificial lipid vesicles [8]. The inhibitory effect of PKA-mediated

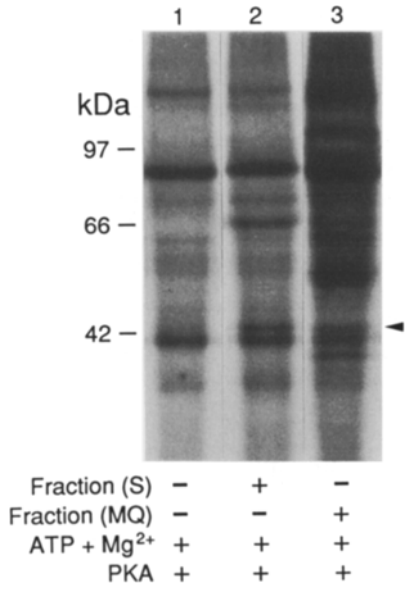

Fig. 8. Representative SDS-PAGE autoradiograph of fraction S (lane *2*) and fraction MQ (lane *3*) incubated with $-[^{32}P]$-ATP, magnesium, and catalytic subunit of PKA. Lane *1* is the catalytic subunit of PKA incubated with ATP and magnesium. Molecular weight standards are indicated. Arrow points to a 42–43 kDa polypeptide

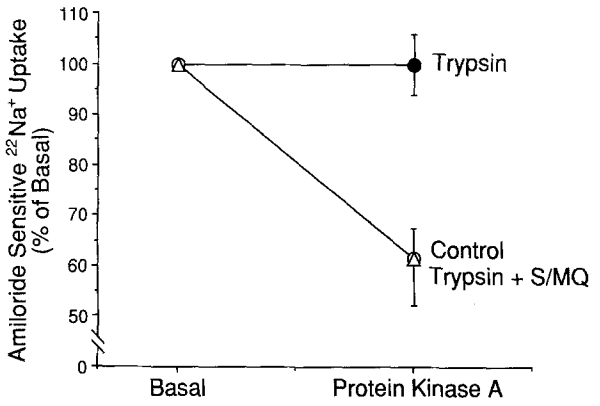

Fig. 9. The effect of PKA-mediated protein phosphorylation of solubilized brush border membrane proteins on proton gradient-stimulated, amiloride-inhibitable sodium uptake in reconstituted proteoliposomes. Proteins were incubated in a solution containing ATP and magnesium in the absence (*Basal*) or presence of the catalytic subunit of PKA. Results are expressed as % of basal (mean ± SEM). Shown are the effects of PKA-mediated protein phosphorylation on Na^+-H^+ exchange transport in control solubilized proteins, in proteins that underwent limited trypsin digestion prior to phosphorylation, and trypsinized proteins co-reconstituted with solubilized brush border membrane proteins separated sequentially by size exclusion (V_e = 0.7) and anion exchange (0.1 M NaCl) column chromatography (fraction S/MQ)

protein phosphorylation on the reconstituted Na^+-H^+ exchanger is consistent with prior studies in proximal tubules and in brush border membrane vesicles from the kidney [3, 9].

Studies examining the molecular basis for substrate recognition by protein kinases have indicated a role for basic residues in proximity to the amino acid phosphorylated by these kinases [6]. The optimal recognition sequence for PKA is arginine · arginine(lysine) · X · Y · serine(threonine). This sequence is particularly susceptible to proteolysis by trypsin. The present studies were designed to examine the relation between trypsin digestion of solubilized brush border membrane proteins and regulation of the Na^+-H^+ exchanger by PKA-mediated protein phosphorylation.

Incubation of solubilized brush border membrane proteins with trypsin resulted in a time- and trypsin concentration-dependent change in the activity of the reconstituted Na^+-H^+ exchanger. In general, extensive trypsin digestion resulted in a significant decrease or total loss of proton gradient-stimulated sodium uptake. This was associated with striking changes in the protein patterns on SDS-PAGE. By contrast, limited trypsin digestion resulted in no loss of Na^+-H^+ exchange activity. Under the specific experimental conditions used in the present studies, it was possible to demonstrate an increase in Na^+-H^+ exchange activity. Specifically, limited trypsin digestion was associated with an increase in proton gradient-stimulated, amiloride-inhibitable sodium uptake and in sodium-dependent proton efflux. Passive proton and sodium fluxes were not altered by limited trypsinization. An increase in proton gradient-stimulated sodium uptake was also demonstrated under chemically voltage clamped conditions. As shown in Fig. 3, the protein pattern was not significantly altered by trypsinization under experimental conditions associated with enhancement of the activity of the Na^+-H^+ exchanger.

To study the relation between trypsin digestion and regulation of the Na^+-H^+ exchanger by PKA, solubilized proteins were incubated in immobilized trypsin, and the reaction was stopped by rapid filtration of the digest. As compared to studies employing solubilized trypsin, the use of immobilized trypsin resulted in even more restricted proteolysis and lesser degrees of stimulation of the Na^+-H^+exchanger. As summarized in Fig. 2, incubation of nontrypsinized solubilized brush border membrane proteins in a phosphorylating solution containing ATP, magnesium, and catalytic subunit of PKA resulted in a significant decrease in Na^+-H^+ exchanger activity as assayed after the proteins were reconstituted into lipid vesicles. Prior limited tryp-

sin digestion of the solubilized proteins blocked the inhibitory response to PKA-mediated protein phosphorylation. These results are consistent with the interpretation that trypsin cleaved an inhibitory component of the Na^+-H^+ exchanger and that this component is a site for PKA-mediated regulation of the transporter.

To explore further the implications of the above findings, additional studies were performed to determine if specific protein fractions could restore the ability of PKA-mediated protein phosphorylation to regulate Na^+-H^+ exchanger activity of trypsinized solubilized brush border membrane proteins. Solubilized brush border membrane proteins were separated by size exclusion chromatography or by anion exchange chromatography. Specific protein fractions were added to trypsinized brush border membrane proteins. The mixture was then incubated in the phosphorylating solution and Na^+-H^+ exchange activity assayed after reconstitution. Mixing of trypsinized proteins with smaller molecular weight polypeptides eluted from the sizing column (V_e = 0.7) restored the inhibitory response of trypsinized proteins to PKA-mediated protein phosphorylation. In an analogous manner, addition of the 0.1 M NaCl eluate from the anion exchange column also restored the inhibitory response to PKA-mediated protein phosphorylation. Neither of these separated protein fractions altered the basal activity of the Na^+-H^+ exchanger and, in the concentrations used, did not demonstrate Na^+-H^+ exchange activity by themselves. These results suggest that both protein fractions contained a regulatory component of the Na^+-H^+ exchanger. The protein fraction eluted from the size exclusion column (Fig. 5) and the 0.1 M NaCl fraction from the Mono Q column (Fig. 7) both contained a 42–43 kDa PKA substrate. As noted previously, only these fractions restored the ability of PKA to regulate the activity of the Na^+-H^+ exchanger in trypsinized proteins. Figure 8, which is a side-by-side comparison of the size exclusion and anion exchange column fractions that restored PKA regulation of the Na^+-H^+ exchanger to trypsinized brush border membrane proteins, confirms that both protein fractions contain the 42–43 kDa PKA substrate polypeptide. Preliminary data indicates that radioiodinized peptide maps of trypsin and chymotrypsin digests of the 42–43 kDa protein obtained by size exclusion and anion exchange chromatography are identical. Phosphoprotein maps of V_8 protease digests of the 42–43 kDa protein are also the same. These data support the conclusion that both chromatography fractions contain the same 42–43 kDa PKA substrate polypeptide. It is to be specifically noted, however, that these results do not prove that the 42–43 kDa polypeptide is a regulatory component of the Na^+-H^+ exchanger. A more cautious interpretation would be that additional study of the relation between this polypeptide and regulation of renal Na^+-H^+ exchanger is warranted.

A final series of experiments were performed whereby solubilized brush border membrane proteins were separated sequentially by size followed by anion exchange chromatography. This resulting fraction was assayed for activity by mixing with trypsinized, solubilized brush border membrane proteins. As summarized in Fig. 9, this protein fraction restored the ability of PKA to regulate the Na^+-H^+ exchanger in trypsinized, solubilized brush border membrane proteins. This more highly purified protein fraction did not affect the basal rate of Na^+-H^+ exchange activity and, in the concentrations used, did not demonstrate Na^+-H^+ exchange activity by itself.

In summary, the results of these experiments suggest that the Na^+-H^+ exchanger contains an inhibitory component which, when cleaved by trypsin, results in enhanced Na^+-H^+ exchanger activity. This inhibitory component is rendered more active by PKA-mediated protein phosphorylation such that the activity of the transporter is decreased. Such a thesis could explain the dissociation between the activity of the Na^+-H^+ exchanger and its regulation by PKA-mediated protein phosphorylation by limited trypsin digestion. The data also suggests that a polypeptide of 42–43 kDa may be involved in the regulation of the Na^+-H^+ exchanger by PKA. It is acknowledged, however, that the present results do not unequivocally establish a link between regulation of the Na^+-H^+ exchanger by PKA and this 42–43 kDa phosphoprotein. These studies do, however, provide the basis for the further characterization of protein components that participate in the structure and function of the renal Na^+-H^+ exchanger.

The authors gratefully acknowledge the secretarial assistance of Ms. Ana Thannoun. This work was supported by grants from the National Institutes of Health (DK37319-01A1), Squibb Pharmaceutical Company, and Travenol Laboratories.

References

1. Beavo, J.A., Bechtel, P.J., Krebs, E.G. 1974. Preparation of homogenous cyclic AMP-dependent protein kinase(s) and its subunits from rabbit skeletal muscle. *Methods Enzymol.* **38**:299–308
2. Hoffman, J.F., Laris, P. 1974. Determination of membrane potentials in human and *Amphiuma* red cells by means of a fluorescent probe. *J. Physiol. (London)* **293**:519–552

3. Kahn, A.M., Dolson, G.M., Hise, M.K., Bennett, S.C., Weinman, E.J. 1985. Parathyroid hormone and dibutyryl cyclic AMP inhibit Na^+-H^+ countertransport in brush border membrane vesicles from a suspension of rabbit proximal tubule. *Am. J. Physiol.* **248:**F212–F218
4. Kinsella, J.L., Aronson, P.S. 1980. Properties of the Na^+-H^+ exchanger in renal microvillus membrane vesicles. *Am. J. Physiol.* **238:**F461–F469
5. Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. 1951. Protein measurements with the Folin phenol reagent. *J. Biol. Chem.* **193:**265–275
6. Shenolikar, S., Cohen, P. 1978. The substrate specificity of cyclic AMP dependent protein kinase: Amino-acid sequences at the phosphorylation sites of Herring protamine (Clupeine). *FEBS Lett.* **86:**92–98
7. Weinman, E.J., Shenolikar, S., Cragoe, E.J., Jr., Dubinsky, W.P., Solubilization and reconstitution of renal brush border Na^+-H^+ exchanger. *J. Membrane Biol.* **101:**1–9
8. Weinman, E.J., W. Dubinsky, S. Shenolikar 1988. Reconstitution of cAMP-dependent protein kinase regulated renal Na^+-H^+ exchanger. *J. Membrane Biol.* **101:**11–18
9. Weinman, E.J., Shenolikar, S., Kahn, A.M. 1987. cAMP-associated inhibition of Na^+-H^+ exchanger in rabbit kidney. *Am. J. Physiol.* **352:**F19–F25

Received 19 October 1988; revised 24 February 1989