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Reconstitution of cAMP-Dependent Protein Kinase Regulated Renal Na^+-H^+ Exchanger

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Summary. Studies were performed to determine if the Na^+-H^+ exchanger, solubilized from renal brush border membranes from the rabbit and assayed in reconstituted artificial proteoliposomes, could be regulated by cAMP-dependent protein kinase. Octyl glucoside solubilized renal apical membrane proteins from the rabbit kidney were phosphorylated by incubation with ATP and highly purified catalytic subunit of cAMP-dependent kinase. $^{22}Na^+$ uptake was determined subsequently after reconstitution of the proteins into proteoliposomes. cAMP-dependent protein kinase resulted in sustained protein phosphorylation and a concentration-dependent decrease in the amiloride-sensitive component of pH gradient-stimulated sodium uptake. The inhibitory effect of cAMP-dependent protein kinase demonstrated an absolute requirement for ATP and was blocked by the specific protein inhibitor of this kinase. cAMP-dependent protein kinase also inhibited $^{22}Na^+$ uptake in the absence of a pH gradient (pH_{in} 6.0, pH_{out} 6.0) and the inhibitory effect was blocked by the specific inhibitor of the kinase. Solubilized membrane proteins exhibited little endogenous protein kinase or protein phosphatase activity.

These studies indicate that Na^+-H^+ exchange activity of proteoliposomes reconstituted with proteins from renal brush border membranes is inhibited by phosphorylation of selected proteins by cAMP-dependent protein kinase. These findings also indicate that the regulatory components of the Na^+-H^+ exchanger remain active during the process of solubilization and reconstitution of renal apical membrane proteins.

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

Introduction

The reabsorption of bicarbonate in the proximal convoluted tubule of the kidney is mediated, in large measure, by the Na^+-H^+ exchanger present in the apical membranes of the cells of this nephron segment [9, 12]. Recent studies from our laboratory have indicated that renal brush border membranes can be solubilized with detergents and electroneutral, proton gradient stimulated, amiloride inhibitable sodium uptake demonstrated when the solubilized proteins are reconstituted into artificial phospholipid vesicles [16]. Studies in intact cells, as well as studies in isolated natural brush border membrane vesicles, have indicated that cAMP inhibits bicarbonate reabsorption in the proximal tubule and the activity of the Na^+-H^+ exchanger in the apical membrane of the cells in this nephron segment [3, 4, 8, 11, 14, 17]. As will be discussed later, studies attempting to correlate the relation between cAMP, cAMP-dependent protein kinase (the major cellular target for cAMP), and the activity of the Na^+-H^+ exchanger in natural membranes are rendered difficult by a variety of experimental considerations [17]. The present experiments were designed with two specific aims. First, we wished to determine if the activity of the solubilized Na^+-H^+ exchanger could be regulated by cAMP-dependent protein kinase. Such experiments are important to establish whether or not regulatory components of the transporter remained active during the process of solubilization and reconstitution of the brush border membrane proteins. Second, we wished to establish that cAMP-dependent protein kinase inhibits the Na^+-H^+ exchanger by a process that involves the selective phosphorylation of one or more membrane proteins. Due to some unique properties of the solubilized protein preparation, namely the limited protein kinase and protein phosphatase activity, it was possible to bypass a number of the experimental problems inherent in comparable studies in natural renal brush border membrane [17]. The data indicates that Na^+-H^+ exchange activity solubilized from rabbit renal apical membranes can be inhibited by cAMP-dependent protein kinase-mediated protein phosphorylation in an analogous manner to that observed with natural membranes.

Materials and Methods

Brush border membranes were prepared from the kidney of the rabbit by a magnesium aggregation method as previously de-

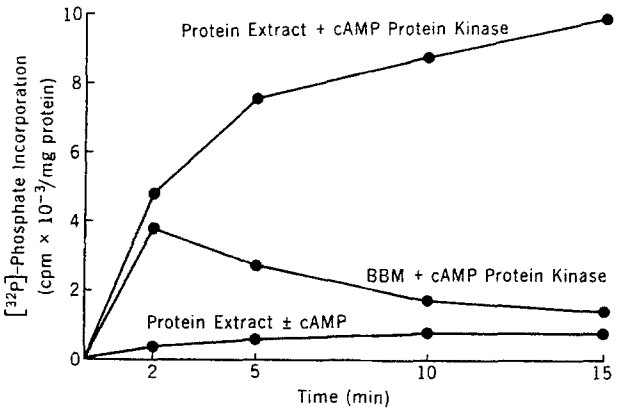

Fig. 1. [^{32}P]-phosphate incorporation into solubilized proteins from renal brush border membranes (*Protein Extract*) or natural renal brush border membranes (*BBM*) following incubation with γ-[^{32}P]-ATP and the catalytic subunit of cAMP-dependent protein kinase (*cAMP Protein Kinase*) (15 mU/ml) or cAMP (10^{-4} M)

scribed [7]. The membranes were suspended in a solution containing (in mM) 254 mannitol, 10 Tris, 16 HEPES, and 10 $MgSO_4$ (pH 7.5). The $MgSO_4$ was removed by washing and resuspending the brush border pellet to a final concentration of 5 mg/ml in a solution containing (in mM) 274 mannitol, 10 Tris, and 16 HEPES (pH 7.5). Brush border membrane proteins were extracted by mixing one part of the membrane preparation with 1.25 parts of 8% (wt/vol) octyl glucoside at pH 7.5 for 15 min at 4°C. The octyl glucoside was prepared in a solution containing (in mM) 75 mannitol, 10 Tris/base, 16 HEPES (pH 7.5). The membrane-detergent mixture was centrifuged at 100,000 × *g* for 30 min. The supernatant, which contains the Na^+-H^+ exchanger, had a final protein concentration of 2.2 mg/ml and octyl glucoside concentration of 4.4%.

The solubilized proteins were incubated in a phosphorylating solution containing ATP (50 μM), magnesium (100 μM), and highly purified catalytic subunit of cAMP-dependent protein kinase. The phosphorylation reaction was performed at pH 7.5 for 5 min at 30°C. The reaction was stopped by the addition of cold asolectin and placing the mixture on ice. In some experiments, ATP was omitted or the thermostable inhibitor of cAMP-dependent protein kinase added to the phosphorylation solution.

After completion of phosphorylation, the membrane-detergent mixture was centrifuged at 100,000 × *g* for 30 min. The supernatant (1.6 parts) was then mixed with one part of asolectin (35 mg/ml) which had been sonicated to translucency for 10 min at 22°C. The asolectin was prepared in a solution containing (in mM) 250 mannitol, 50 mM MES/base (pH 6.0). The final concentration of the proteoliposome-detergent mixture was 0.5 mg/ml protein, 2.73% (wt/vol) octyl glucose and 13.5 mg/ml asolectin. The mixture was then dialyzed against a solution containing (in mM) 250 mannitol, and 50 mM MES/Tris (pH 6.0) for 18 hr with one buffer change at 4°C using dialysis membranes with 6,000–8,000 dalton mol wt cutoff.

The uptake of $^{22}Na^+$ in the proteoliposomes and liposomes was determined in the absence and presence of 2 mM amiloride. The uptake solution contained (in mM) 1 Na, 250 mannitol, and 50 Tris/MES (pH 8.0) in studies performed under proton gradient condition. Where indicated, the pH of the uptake solution was adjusted to pH 6.0 to study the uptake of $^{22}Na^+$ in the absence of a proton gradient of the sodium content increased to 50 mM to study sodium permeability. 100 μl of the reaction mixture was applied to a Dowex 50 × 8 (Tris), 100-mesh column and eluted with vacuum suction with 1 ml of 300 mM mannitol (pH 8.0) at 4°C. The eluent containing the vesicles was collected directly into scintillation fluid and the radioactivity determined. The rate constant for dissipation of the proton gradient was determined using acridine orange fluorescence as previously described [8, 17].

In studies examining the incorporation of [^{32}P]-phosphate into proteins, radioactive γ-[^{32}P]-phosphate-ATP was substituted for unlabeled ATP. The proteins were then precipitated in 25% (wt/vol) TCA in the presence of 1 mM unlabeled ATP, and the protein-bound [^{32}P]-phosphate was determined. The characterization of the phosphoproteins was determined using SDS-PAGE as previously described [15, 17]. The phosphorylation reaction was stopped by the addition of 60 mM Tris-glycine buffer (pH 8.3) containing 3% SDS (wt/vol) and 30% glycerol (wt/vol). Samples were placed in a boiling water bath for 2 min and cooled on ice. Protein samples (80 μg) were then loaded on 7 to 16% gradient polyacrylamide slab gels. Electrophoresis was performed for 4 hr at 40 mA/gel. The proteins were stained with Coomassie Blue or silver, dried, and exposed to x-ray film using cassettes containing tungsten phosphate intensifier screens at −70°C.

The catalytic subunit of cAMP-dependent protein kinase was prepared by the method of Beavo et al. and its activity assayed by the phosphorylation of histone H2B [1]. By this method, over 85% of the recovered protein is represented by a single polypeptide of 40,000 daltons. The inhibitor of cAMP-dependent protein kinase was purified as described by Whitehouse and Walsh [18] and added to the phosphorylation solution to a final concentration of 1 μg/ml. This concentration of inhibitor was demonstrated to inhibit the phosphorylation of histone H_2B by 10 units of the catalytic subunit of cAMP-dependent protein kinase by over 95%. Proteins were measured by the method of Lowry et al. [10]. Statistical analysis was performed using Peritz Analysis of Variance [6].

Results

The incorporation of [^{32}P]-phosphate into solubilized membrane proteins after incubation in γ-[^{32}P]-ATP was determined in initial experiments. In addition, phosphorylation of proteins in natural brush border membranes was measured by exposing brush border membranes to radioactive ATP. These latter experiments were performed in hypotonic solutions to permit access of ATP and the catalytic subunit of cAMP-dependent protein kinase to the inside of the membrane, as previously detailed [15, 17]. Figure 1 is a representative experiment and summarizes the time course of [^{32}P]-phosphate incorporation. Exposure of natural brush border membranes to the catalytic subunit of cAMP-dependent protein kinase (15 mU/ml) resulted in rapid phosphorylation of membrane proteins. Phosphorylation was maximal at 2 min of incubation after which there was a progressive decrease in phosphate incorporation. A similar time course of phos-

phorylation is observed when natural membranes are incubated in cAMP (10^{-4} M). These patterns are similar to those previously reported by this laboratory [17]. By contrast, incubation of the solubilized proteins with ATP and the catalytic subunit of cAMP-dependent protein kinase (15 mU/ml) resulted in rapid and progressive phosphorylation over a 15-min time interval. Incubation of the solubilized proteins in radioactive ATP alone resulted in little incorporation of [^{32}P]-phosphate into proteins. The addition of cAMP (10^{-4} M) to the phosphorylating solution did not significantly stimulate protein phosphorylation. These findings would suggest that, in contrast to the intact membrane proteins, the solubilized proteins contain limited endogenous protein kinase and protein phosphatase activity.

Additional studies were performed to determine if protein dephosphorylation could be demonstrated after addition of asolectin and overnight dialysis. Incubation of the solubilized proteins in ATP, magnesium and the catalytic subunit of cAMP-dependent protein kinase (15 mU/ml) for 5 min resulted in a 8.5- ± 1.7-fold increase in protein phosphorylation as compared to rates obtained in the absence of the kinase ($n = 4$). The rate of kinase-stimulated protein phosphorylation was 86.6 ± 18.7 pmol of phosphate incorporated · mg protein^{-1} · min^{-1}. After addition of phospholipid and overnight dialysis, [^{32}P]-phosphate counts · mg protein^{-1} averaged 92 ± 6% of values obtained after 5 min of incubation of the solubilized protein in ATP, magnesium, and cAMP-dependent protein kinase. Thus, there was no significant dephosphorylation after formation of proteoliposomes and overnight dialysis. Figure 2 is a representative SDS-PAGE autoradiogram of solubilized proteins incubated in γ-[^{32}P]-phosphate-ATP without and with the catalytic subunit of cAMP-dependent protein kinase (15 mU/ml). Autoradiograms shown are after 5 min of phosphorylation, after addition of ascolectin, and after dialysis. There are only a limited number of phosphoproteins detected when the solubilized membranes are incubated in γ-[^{32}P]-ATP in the absence of the catalytic subunit of cAMP-dependent protein kinase; a finding that confirms that the solubilized preparation has only limited endogenous protein kinase activity. The catalytic subunit of cAMP-dependent protein kinase promoted the phosphorylation of approximately 15 proteins. Protein phosphorylation was inhibited by over 95% by the kinase inhibitor and resulted in autoradiogram not different than that observed in the control autoradiograms shown in Fig. 2 (*data not shown*).

To determine if cAMP-dependent protein kinase affected the Na^+-H^+ exchanger present in the solubilized proteins from the renal brush border, the

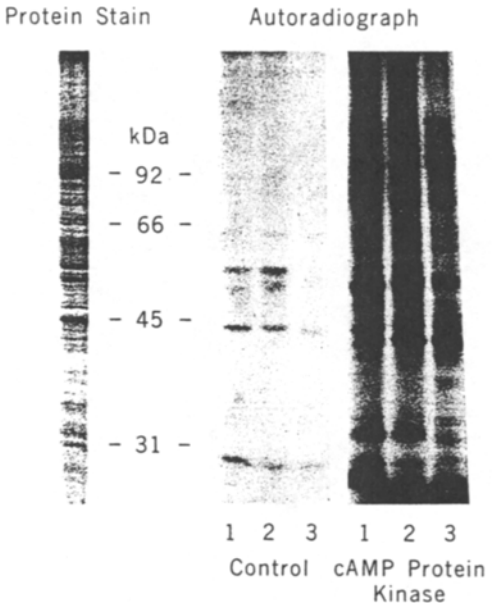

Fig. 2. SDS-PAGE autoradiographs of solubilized proteins incubated for 5 min in γ-[^{32}P]-ATP in the absence or presence of the catalytic subunit of cAMP-dependent protein kinase. Autoradiographs were obtained immediately after phosphorylation (*1*), after addition of asolectin (*2*), and after overnight dialysis (*3*). A representative protein-staining pattern is shown on the left. Molecular weight standards are indicated

soluble extract was incubated for 5 min with ATP without or with varying concentrations of the catalytic subunit of cAMP-dependent protein kinase. After phosphorylation, asolectin was added and the sample dialysed overnight to produce proteoliposomes. The uptake of 1 mM $^{22}Na^+$ was determined under pH gradient conditions (pH_{in} 6.0, pH_{out} 8.0). Under control conditions, $^{22}Na^+$ uptake averaged 15.1 ± 0.8 nmol · 2 min^{-1} · mg protein^{-1} in the absence of amiloride and 7.4 ± 0.7 in the presence of amiloride. The catalytic subunit cAMP-dependent protein kinase did not affect the uptake of sodium in liposomes or the amiloride-insensitive component of sodium uptake in proteoliposomes. Figure 3 is a graph of the amiloride-inhibitable component of $^{22}Na^+$ uptake, expressed as nmol · 2 min^{-1} · mg protein^{-1}, as a function of the concentration of the protein kinase catalytic subunit. There was a concentration-dependent inhibition of amiloride-sensitive sodium uptake. Maximum inhibition was observed when the solubilized proteins were incubated with a concentration of cAMP-dependent protein kinase of 15 mU/ml or greater. At concentrations of 15 mU/ml or greater, sodium uptake averaged 12.8 ± 0.9 nmol · 2 min^{-1} · mg protein^{-1} in the absence of amiloride ($P < 0.05$ *vs.* control) and 7.7 ± 0.6 nmol · 2 min^{-1} · mg protein^{-1} in the pres-

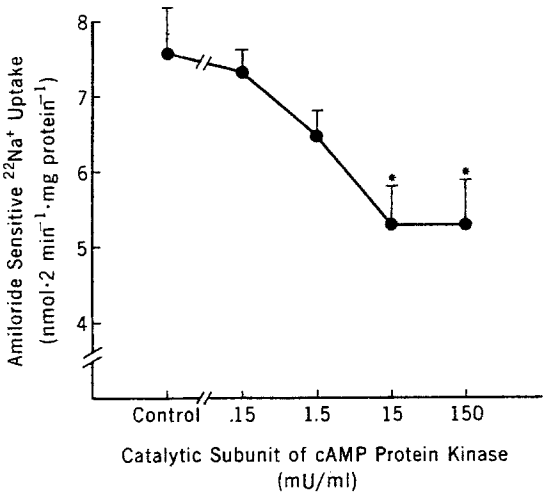

Fig. 3. Dose response relation between amiloride-sensitive $^{22}Na^+$ uptake and the concentration of catalytic subunit of cAMP-dependent protein kinase. Solubilized proteins from renal brush border membranes were incubated with ATP, magnesium, and the catalytic subunit. The proteins were then reconstituted into proteoliposomes. The internal pH was 6.0; the external pH was 8.0. The uptake solution contained 1 mM Na^+. Values represent mean ± SEM for eight preparations. *Indicates $P \leq 0.05$ as compare to control

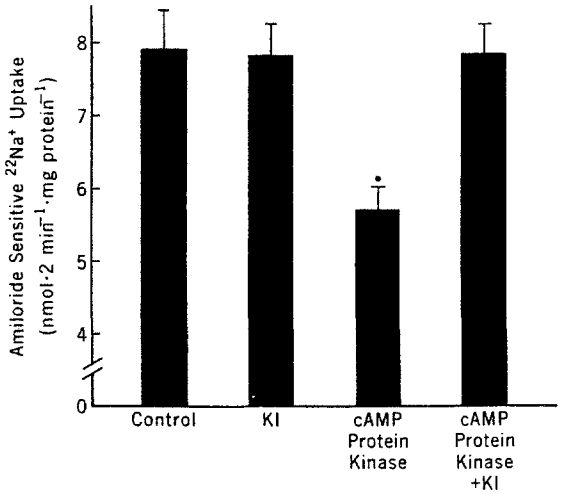

Fig. 4. Amiloride-sensitive sodium uptake in proteoliposome prepared with solubilized protein extracts from renal brush border membranes (*Control*), or solubilized extracts exposed to either the inhibitor of cAMP-dependent protein kinase (*KI*), the catalytic subunit of cAMP-dependent protein kinase (*cAMP Protein Kinase*), or both cAMP protein kinase and KI. All incubations were performed in the presence of ATP and magnesium, after which the proteins were reconstituted into proteoliposomes. The uptake of $^{22}Na^+$ was determined under proton gradient conditions (pH_{in} 6.0, pH_{out} 8.0). Values represent mean ± SEM for seven preparations. *Indicates $P \leq 0.05$ *vs.* control, KI, and *vs.* cAMP protein kinase + KI

ence of amiloride (P = NS *vs.* Control). At this concentration, amiloride-sensitive uptake was inhibited by 31 ± 5% ($P < 0.01$ compared to controls). In all the remaining experiments, the catalytic subunit of cAMP-dependent protein kinase was used in a concentration of 15 mU/ml.

In order to estimate the intravesicular volume and to exclude the possibility that cAMP-mediated protein phosphorylation affected the integrity of the proteoliposomes, the uptake of rubidium was determined in proteoliposomes under conditions whereby a proton gradient was present, $K_{in} = K_{out}$ = 30 mM, and valinomycin was present. Equilibrium was attained by 30 min. The calculated vesicular volumes averaged 1.51 ± 0.05 μl/mg lipid and 1.53 ± 0.04 in control proteoliposomes in the absence and presence of amiloride, respectively. In proteoliposomes containing phosphorylated proteins, the vesicular volumes were 1.49 ± 0.07 μl/mg lipid in the absence of amiloride and 1.48 ± 0.05 in the presence of amiloride. Figure 4 summarizes studies performed under proton gradient conditions, with and without amiloride, to determine the specificity of the response to the catalytic subunit of cAMP-dependent protein kinase. As compared to controls, the catalytic subunit of cAMP-dependent protein kinase inhibited the amiloride-sensitive uptake of sodium from 7.9 ± 0.6 nmol · 2 min^{-1} · mg protein^{-1} to 5.7 ± 0.4 ($P < 0.01$). The thermostable protein inhibitor of cAMP-dependent protein kinase itself did not significantly affect the uptake of sodium; a finding that confirms the absence of active cAMP-dependent protein kinase activity in the soluble protein extract. The protein kinase inhibitor did significantly block the inhibitory effect of the catalytic subunit of cAMP-dependent protein kinase. As summarized in Fig. 5, ATP itself had no effect on sodium uptake. In the absence of ATP, the catalytic subunit of cAMP-dependent protein kinase did not inhibit sodium uptake.

An additional series of studies were performed to rule out the possibility that the decrease in sodium uptake consequent to protein phosphorylation mediated by cAMP-dependent protein kinase was the secondary consequence of a change in the permeability of proteoliposomes to hydrogen or sodium ions. Sodium uptake was determined in the presence of 30 mM potassium inside and outside the vesicles and valinomycin (10 μg/ml). Under these presumed voltage clamped conditions, amiloride-sensitive sodium uptake averaged 6.7 ± 0.4 nmol · 2 min^{-1} · mg protein in control proteoliposomes and 5.2 ± 0.2 in proteoliposomes containing proteins phosphorylated by cAMP-dependent protein kinase ($P < 0.05$, $n = 3$). Sodium uptake was also mea-

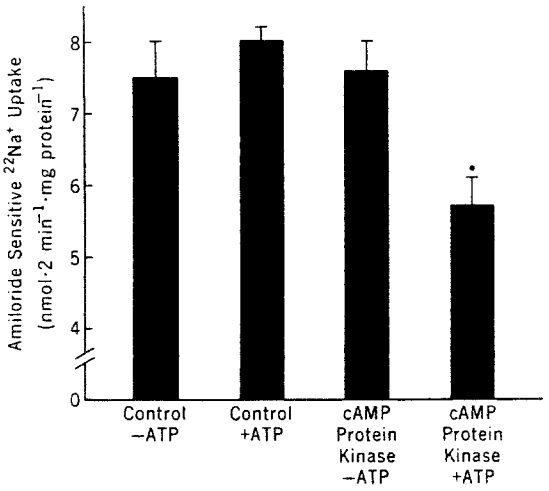

Fig. 5. Effect of cAMP-dependent protein kinase (*cAMP Protein Kinase*) on the uptake of 1 mM Na^+ into proteoliposomes under pH gradient conditions (pH_{in} 6.0, pH_{out} 8.0) in the presence and absence of ATP. Values represent mean ± SEM for four preparations. *Indicates $P \leq 0.05$ *vs.* control-ATP, *vs.* control + ATP, and *vs.* cAMP protein kinase-ATP

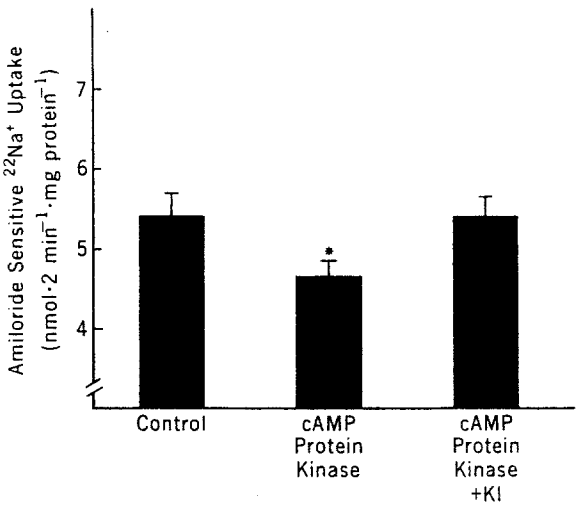

Fig. 6. Effect of cAMP-dependent protein kinase on amiloride-sensitive $^{22}Na^+$ uptake in the absence of pH gradient. Inside and outside pH was 6.0. Values represent mean ± SEM for five preparations. *Indicates $P \leq 0.05$ *vs.* control, and *vs.* cAMP protein kinase + KI

sured in the absence of a pH gradient where the internal and external pH was 6.0. Prior studies have indicated a reduced rate of sodium uptake under such condition as compared to proton gradient conditions [17]. Nonetheless, there was still a significant amiloride inhibitable component of sodium uptake when determined under conditions where the inside and outside pH was 6.0. These findings were confirmed in the present experiments. As shown in Fig. 6, the catalytic subunit of cAMP-dependent protein kinase (15 mu/ml) significantly inhibited the amiloride-sensitive uptake of sodium, and this inhibitory effect was blocked by the inhibitor of cAMP-dependent protein kinase. In separate experiments, the solubilized proteins were incubated in ATP and magnesium, as previously described, in the absence or presence of the catalytic subunit of cAMP-dependent protein kinase. Proteoliposomes were made using these proteins, and the rate constant for dissipation of the proton gradient (pH_{in} 6.0, pH_{out} 8.0) was determined using acridine orange fluorescence. The outside solution contained 100 mM N-methylglucamine gluconate. There was no difference in the rate constant between control and experimental proteoliposomes, which averaged 0.045 ± 0.004 sec^{-1} *vs.* 0.046 ± 0.004 sec^{-1}, respectively (P = NS, n = 3). The amiloride-insensitive component of sodium uptake provides an estimate of the permeability of the proteoliposomes to sodium. To provide an additional estimate of sodium permeability, the uptake of 50 mM sodium was determined under pH gradient conditions. This concentration of sodium is well above the K_a for sodium [16]. Protein phosphorylation mediated by cAMP-dependent protein kinase did not affect sodium uptake under these experimental condition (% change from control = −3.5 ± 1.9%, P = NS).

Discussion

Recent studies from our laboratory have demonstrated the feasibility of solubilizing renal apical membrane proteins and reconstituting these proteins into artificial phospholipid vesicles. These studies indicated that the reconstituted proteoliposomes demonstrate electroneutral, proton gradient stimulated, amiloride inhibitable sodium uptake [16]. The K_a for sodium was 4 mM. The present report examines cAMP regulation of the Na^+-H^+ exchanger using solubilized brush border membrane proteins. One purpose of the present experiments was to determine if, in the process of solubilizing the membranes, the regulatory component(s) of the Na^+-H^+ exchanger remained active.

A second aim of the present experiments was to clarify some of the uncertainties of our prior studies examining the relation between cAMP and the Na^+-H^+ exchanger in natural membranes [17]. Studies from a number of laboratories, including our own, have established that cAMP inhibits sodium and bicarbonate reabsoption in the proximal convolution of the kidney and that this inhibition occurs via a cAMP-associated inhibition of the Na^+-H^+ ex-

changer in the brush border membranes of the cells of this nephron segment [3, 4, 8, 11, 14, 17]. In a recent study from our laboratories using isolated brush border membranes, we advanced evidence that cAMP associated inhibition of the Na^+-H^+ exchanger involved cAMP-dependent protein kinase and required phosphorylation of one or more brush border membrane proteins [17]. Attempts to correlate the changes in transport with phosphorylation of specific membrane proteins, however, were rendered difficult by a number of factors. First, despite experimental conditions designed to optimize the change in Na^+-H^+ exchange activity, the inhibition of proton gradient stimulated, amiloride-inhibitable sodium uptake in brush border membrane vesicles incubated in ATP and cAMP or the catalytic subunit of cAMP-dependent protein kinase was only 15%. This value is nearly at the limits of resolution of the methodology. Second, even in the absence of specific stimulation of brush border membrane cAMP-dependent protein kinase, renal brush border membranes exhibit significant protein kinase activity. In addition, natural brush border membranes exhibit active protein phosphatase activity. The presence of high background rates of phosphorylation by protein kinases and rapid dephosphorylation of phosphoproteins by protein phosphatases made it difficult to correlate the changes in transport with changes in the phosphorylation state of specific proteins. While the conclusion that cAMP-associated inhibition of Na^+-H^+ exchange activity in isolated renal brush border membrane vesicles involved participation of cAMP-dependent protein kinase proved valid, the above considerations introduced a degree of uncertainty about the precise relation between protein phosphorylation and Na^+-H^+ transport activity.

In our prior studies using reconstituted proteoliposomes, we observed that the extracted proteins could be frozen for several weeks without loss of activity [16]. In the present studies on regulation of the Na^+-H^+ exchanger, it was observed that the catalytic subunit of cAMP-dependent protein kinase consistently inhibited proton gradient stimulated sodium uptake when fresh protein extracts were employed but yielded erratic findings when frozen extracts were used. To date, we have no explanation for this phenomena but might conjecture that some proteolysis or denaturation occurs during freezing and thawing and that a regulatory component of the Na^+-H^+ exchange complex is rendered partially inactive. In the studies reported in this communication, only fresh detergent extracts of brush border membrane proteins were employed.

In the process of determining the optimal conditions to study the regulation of the solubilized Na^+-H^+ exchanger, a number of observations were made which bear directly on the present experiments. The catalytic subunit of cAMP-dependent protein kinase, which itself does not require cAMP for activity, was used in the present experiments. As shown in Fig. 1 and in confirmation of our prior observations, the catalytic subunit of cAMP-dependent protein kinase resulted in rapid phosphorylation of natural membranes followed by rapid dephosphorylation [17]. By contrast, exposure of the solubilized membrane proteins to the catalytic subunit of cAMP-dependent protein kinase resulted in rapid and sustained protein phosphorylation. Solubilized membrane proteins incubated with ATP, with or without cAMP, demonstrated very little endogenous protein phosphorylation. Thus, in contrast to natural membranes, the solubilized extracts demonstrated significantly reduced protein kinase and protein phosphatase activity. The reasons for the reduced protein kinase activity in the solubilized protein extract as compared to natural brush border membrane is not known with certainty. It is suggested that the endogenous kinases are either poorly extracted from the membranes by the detergent or, more likely, inactivated during the process of solubilization. While little endogenous protein kinase or phosphatase activity could be demonstrated in the solubilized extracts of brush border membrane proteins, it was important to rule out the possibility that residual enzyme activity might be selectively preserved or even reactivated after the proteins had been reconstituted with phospholipid and dialysed. Protein phosphorylation studies, however, indicated that the amount of phosphate incorporated in proteins after 5 min of phosphorylation was not affected by subsequent addition of asolectin and extensive dialysis. The amount of protein bound [^{32}P]-phosphate incorporated remained significantly higher when phosphorylations were performed in the presence of the catalytic subunit as compared to controls even after overnight dialysis. The autoradiograms (Fig. 2) confirm that, in the absence of added catalytic subunit of cAMP-dependent protein kinase, very few phosphoprotein bands are discerned. The presence of exogenous catalytic subunit of cAMP-dependent protein kinase resulted in phosphorylation of at least 15 proteins. The pattern of phosphorylation remained virtually constant when examined immediately after completion of phosphorylation, after addition of asolectin, and after 16 hr of dialysis. Collectively, these findings indicate that the solubilized extracts possess only limited endogenous protein kinase or protein phosphatase activity. This fortuitous set of findings offered the possibility of studying protein kinase spe-

cific phosphorylation of solubilized brush border membrane proteins and bypassing a number of the experimental uncertainties noted in our prior studies using natural membranes.

The experimental approach employed was to incubate the solubilized proteins for 5 min at 30°C in ATP, magnesium, various concentration of the catalytic subunit of cAMP-dependent protein kinase and/or the specific inhibitor of this protein kinase. The proteins were then incorporated into proteoliposomes after removal of the detergent by overnight dialysis. This protocol circumvents the need for incorporation of the protein kinase into the artificial membranes and concerns about the adequate availability of the protein substrates. Incubation of the solubilized proteins in ATP alone had no effect on proton gradient stimulated sodium uptake. As shown in Fig. 3, incubation of the solubilized extract of brush border membrane proteins with the catalytic subunit of cAMP-dependent protein kinase resulted in a concentration-dependent decrease in proton-gradient stimulated, amiloride-inhibitable sodium uptake. At the highest concentrations examined, the inhibition of Na^+-H^+ exchange activity was 30%; a value twice as great as that observed in comparable experiments in natural membranes. As shown in Figs. 4 and 5, the inhibitory effect of the catalytic subunit of cAMP-dependent protein kinase on Na^+-H^+ exchange activity was blocked by the inhibitor of cAMP-dependent protein kinase. The kinase inhibitor itself had no effect on sodium uptake under the conditions of study. In the absence of ATP, the catalytic subunit of cAMP-dependent protein kinase did not affect sodium uptake. A final set of studies was performed to exclude the possibility that the decrease in sodium uptake was a secondary consequence of the inability of the proteoliposomes to sustain the imposed proton gradient. First, under pH gradient condition and in the presence of a voltage clamp, the catalytic subunit of cAMP-dependent protein kinase inhibited sodium uptake. Second, as previously demonstrated, there is a significant amiloride-inhibitable component of sodium uptake when the inside and outside pH was 6.0. As shown in Fig. 6, incubation of solubilized proteins with the catalytic subunit of cAMP-dependent protein kinase resulted in a significant decrease in amiloride-inhibitable sodium uptake when studied in the absence of a pH gradient. Finally, the rate constant for dissipation of the proton gradient was not affected by the catalytic subunit of cAMP-dependent protein kinase. These experiments, then, would indicate that cAMP-dependent protein kinase-mediated phosphorylation is associated with inhibition of the Na^+-H^+ exchanger from the renal apical membrane.

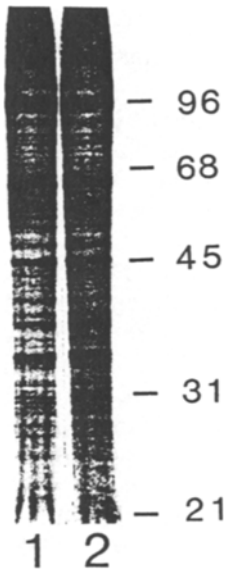

Fig. 7. SDS-PAGE analysis of proteins contained in reconstituted proteoliposomes prepared from solubilized brush border membrane proteins incubated in the phosphorylation solution in the absence (lane *1*) or presence (lane *2*) of the catalytic subunit of cAMP-dependent protein kinase. Protein bands are stained with silver. Molecular weight standards are indicated

As noted earlier, the protocol used in the present studies maximizes the interaction of substrate proteins with the kinase. The methodology, however, introduces the consideration that protein phosphorylation alters the efficiency of reconstitution. Until a specific marker of the transport protein(s) in question is available, this question cannot be addressed directly. However, the following speculations and data may be germane. First, the efficiency of reconstitution cannot be a nonspecific effect of phosphorylation *per se*. Preliminary evidence indicates that protein phosphorylation mediated by calcium-calmodulin dependent protein kinase inhibits the reconstituted Na^+-H^+ exchanger while that mediated by protein kinase C stimulates activity. Second, protein phosphorylation in natural membranes occurs on extramembraneous domains of the protein. The hydrophobic sequences of the protein, presumably, are not natural substrates. Accordingly, it may be suggested that the efficiency of reconstitution would be more dependent on the hydrophobic domains of the protein(s); sites not natural substrates for the kinase. Finally, we have extensively analyzed the one-dimensional SDS-PAGE protein patterns of reconstituted proteoliposomes prepared with proteins, which were incubated in ATP without and with the catalytic subunit of cAMP-dependent protein kinase. As shown in Fig. 7, the protein patterns were identical by this analy-

sis in all samples. Thus, to the limits of resolution of SDS-PAGE, the proteins present in the proteoliposomes are not affected by protein phosphorylation medated by cAMP-dependent protein kinase. Despite all of the above considerations, however, the possibility that protein phosphorylation alters the efficiency of reconstitution cannot be totally excluded.

The representative autoradiogram shown in Fig. 2, indicates that the catalytic subunit of cAMP-dependent protein kinase mediates the selective phosphorylation of approximately 15 proteins in the soluble extract. The absence of significant background phosphorylation permits a clearer elucidation of substrate proteins of this kinase than was possible in natural membranes. It is worth noting that the number of substrate proteins approximated that observed in natural membranes and emphasizes the broad specificity of substrates for cAMP-dependent protein kinase present in the renal apical membrane [15, 17]. The number of substrate proteins of cAMP-dependent protein kinase in renal brush border membranes of the rabbit kidney appears to be higher than that reported in the chick, dog, or rat [2, 5, 13]. It is uncertain at present, however, whether these discrepancies represent species differences or are the result of differences in experimental protocols. The explicit relation between a specific phosphoprotein(s) and altered activity of the Na^+-H^+ exchanger remains to be demonstrated. It should be feasible, in future studies, to fractionate the proteins in the soluble extract of brush border membranes and study protein kinase regulation of partially purified protein fractions. Such an experimental strategy may ultimately permit the definition of specific phosphoprotein(s) intimately involved with regulation of the Na^+-H^+ exchanger.

In summary, the present studies indicate that Na^+-H^+ exchange activity of solubilized and reconstituted renal brush border membrane proteins is inhibited by phosphorylation of selected proteins by cAMP-dependent protein kinase. These findings confirm and extend our previous observations on this regulatory process in natural renal brush border membranes [17]. In addition, the use of octyl glucoside extracted proteins for the study of protein kinase mediation of Na^+-H^+ exchanger activity is facilitated by virtue of inactivation of brush border membrane protein kinases and protein phosphatases. This methodology should also permit a detailed analysis of the relations between the Na^+-H^+ exchanger and other protein kinases.

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