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Regulation of Reconstituted Renal Na^+/H^+ Exchanger by Calcium-Dependent Protein Kinases

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Summary. Studies were performed to determine the effect of protein phosphorylation mediated by calcium-calmodulin-dependent multifunctional protein kinase II and calcium-phospholipid-dependent protein kinase on Na^+/H^+ exchange activity. Proteins from the apical membrane of the proximal tubule of the rabbit kidney were solubilized in octyl glucoside and incubated in phosphorylating solutions containing the protein kinase. $^{22}Na^+$ uptake was determined subsequently after reconstitution of the proteins into proteoliposomes. Calcium-calmodulin-dependent multifunction protein kinase II inhibited the amiloride-sensitive component of proton gradient-stimulated Na^+ uptake in a dose-dependent manner. The inhibitory effect of this kinase had an absolute requirement for calmodulin, Ca^{2+}, and ATP. Calcium-phospholipid-dependent protein kinase stimulated the amiloride-sensitive component of proton gradient-stimulated Na^+ uptake in a dose-dependent manner. The stimulating effect of this kinase had an absolute requirement for ATP, Ca^{2+}, and an active phorbol ester. These experiments indicate that Na^+/H^+ exchange activity of proteoliposomes reconstituted with proteins from renal brush-border membranes are inhibited by protein phosphorylation mediated by calcium-calmodulin-dependent multifunctional protein kinase II and stimulated by that mediated by calcium-calmodulin-dependent protein kinase.

Key Words renal electrolyte transport · sodium/hydrogen exchange transport · calcium-phospholipid-dependent protein kinase · calcium-calmodulin-dependent protein kinase · protein phosphorylation

Introduction

The Na^+/H^+ exchanger in the apical membrane of the proximal convoluted tubule mediates the reabsorption of bicarbonate [8, 13]. A number of recent studies have indicated that the activity of this transporter is under metabolic control [3, 11]. A particular focus of our laboratories has been the study of the relation between specific protein kinase-mediated protein phosphorylation and the activity of the renal brush-border membrane Na^+/H^+ exchanger [1, 6, 15, 17, 19]. Using brush-border membranes from the kidney of the rabbit, we have advanced evidence that protein phosphorylation mediated by cAMP-dependent protein kinase inhibits the Na^+/H^+ exchanger while that mediated by calcium-phospholipid-dependent protein kinase (protein kinase C) enhances its activity [17, 19]. Another Ca^{2+}-dependent protein kinase, calcium-calmodulin-dependent multifunctional protein kinase II, is present in the cytosol and plasma membranes of cells [7]. In some nonrenal tissues, this kinase has been suggested to modulate the transmembrane flux of electrolytes. The effect of calcium-calmodulin-dependent protein kinase II on renal electrolytes transport is not known. The specific effect of this kinase on the renal Na^+/H^+ countertransporter has not yet been studied.

The present study examines the effect of two Ca^{2+}-dependent protein kinases on the activity of the renal Na^+/H^+ exchanger. These investigations were facilitated by several recent observations from our laboratories. First, we have demonstrated that the Na^+/H^+ exchanger from renal brush-border membranes can be solubilized and its activity assayed in reconstituted proteoliposomes [18]. These reconstituted proteoliposomes demonstrate proton gradient-stimulated, electroneutral, amiloride-inhibitable uptake of Na^+. The proton gradient-stimulated uptake of Na^+ in these reconstituted proteoliposomes demonstrate characteristics of a mediated transport system that is similar, although not identical, to that present in native renal brush-border membranes. Second, we have demonstrated that cAMP-dependent protein kinase-mediated protein phosphorylation of the solubilized protein extract of renal brush-border membranes results in decreased Na^+/H^+ exchange activity when the proteins are reconstituted into artificial phospholipid vesicles [16]. These latter results indicate that the regulatory component(s) of the Na^+/H^+ exchanger remain in-

tact during the process of solubilization and reconstitution.

The experimental protocol of the present experiments follows closely that described for the study of the effect of cAMP-dependent protein kinase on the solubilized and reconstituted Na^+/H^+ exchanger [16]. Solubilized rabbit renal brush-border membrane proteins were incubated in phosphorylating solutions containing the specific protein kinase of interest. The activity of Na^+/H^+ exchanger was assayed after the proteins were reconstituted into artificial phospholipid vesicles. The results indicate that protein phosphorylation mediated by calcium-calmodulin-dependent protein kinase II inhibits the Na^+/H^+ exchanger while that mediated by protein kinase C stimulates this transport system.

Materials and Methods

Brush-border membranes were prepared from the kidney of the rabbit by a Mg^{2+} aggregation method as previously described [5]. The membranes were suspended in a solution containing (in mM) 254 mannitol, 10 Tris, 16 HEPES, and 10 $MgSO_4$ (pH 7.6). 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 7.6). 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 7.6 for 15 min at 4°C. The octyl glucoside was prepared in a solution containing (in mM) 274 mannitol, 10 Tris, and 16 HEPES (pH 7.6). In the final reaction mixture, the protein concentration was 2.2 mg/ml and the concentration of octyl glucoside was 4.4% [18].

The solubilized proteins were incubated at pH 7.4 for 5 min at 30°C in a solution containing ATP (50 μM), $MgCl_2$ (100 μM), and $CaCl_2$ (100 μM). For study of calcium-calmodulin-dependent protein kinase II, calmodulin (50 μM) plus purified calcium-calmodulin-dependent protein kinase II was added to the phosphorylating solution. For study of protein kinase C, TPA (12-0-tetradecanoyl phorbol-13-acetate) (1 μM) and partially purified protein kinase C were added. Where indicated, specific ingredients of the phosphorylating solutions were deleted or substituted or their concentrations altered.

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/Tris HCl (pH 6.0). The final concentration of the proteoliposome-detergent mixture was 0.5 mg/ml protein, 2.73% (wt/vol) octyl glucoside and 13.5 mg/ml asolectin. The mixture was then dialyzed against a solution containing (in mM) 250 mannitol, and 50 MES/Tris (pH 6.0) for 18 hr with one buffer change at 4°C using dialysis membranes with 6,000–8,000 dalton molecular weight cutoff [18].

The uptake of $^{22}Na^+$ in the proteoliposomes and liposomes was determined. Except where specifically indicated otherwise, the uptake solution contained (in mM), 1 Na, 250 mannitol, and 50 Tris/MES, (pH 8.0) (proton gradient conditions). Studies were performed in the absence or presence of amiloride (2 mM). 100 μl of the reaction mixture was applied to a Dowex 50 × 8 (Tris), 100 mesh column and eluted with 1 ml of 300 mM sucrose (pH 8.0) at 4°C. The eluent containing the vesicles was collected directly into scintillation fluid and the radioactivity determined. Proton permeability was determined using acridine orange as previously described [6, 18].

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 determined. SDS-PAGE analysis of the solubilized proteins was performed as previously described [16, 17, 19].

Calcium-calmodulin-dependent protein kinase II was prepared from rat forebrain by the method of Kelly and Shenolikar [7]. Extensive characterization of this protein kinase has previously been reported [7]. Activity of the purified kinase was determined using synapsin 1 as a specific substrate. Protein kinase C was partially purified by a modification of the method of Parker, Stabel and Waterfield [14]. Activity of protein kinase C was determined using a histone H_1 phosphorylation assay. Protein concentrations were determined by the method of Lowry et al. [9]. Statistical analysis was performed using Analysis of Variance.

Results

Calcium-Calmodulin-Dependent Protein Kinase II

Incubation of the solubilized proteins in a solution containing ATP (50 μM), Ca^{2+} (100 μM), Mg^{2+} (100 μM), and calmodulin (50 μM) resulted in $^{22}Na^+$ uptake in reconstituted proteoliposomes of 18.0 ± 1.0 nmol · 2 min^{-1} · mg $protein^{-1}$ in the absence of amiloride and 11.3 ± 1.0 in the presence of amiloride (2 mM). The addition of calcium-calmodulin-dependent protein kinase II to the phosphorylating solution decreased $^{22}Na^+$ uptake in a concentration-dependent manner but had no effect on the amiloride-insensitive component of Na^+ uptake or the uptake of $^{22}Na^+$ into liposomes. At the highest concentration of the kinase examined, $^{22}Na^+$ uptake averaged 15.5 ± 0.9 nmol · 2 min^{-1} · mg $protein^{-1}$ in the absence of amiloride ($P < 0.01$ *vs.* control) and 11.5 ± 1.0 in the presence of amiloride (P = NS *vs.* control). The amiloride-sensitive component of $^{22}Na^+$ uptake was decreased by 35 ± 7% ($P < 0.01$ *vs.* control). Figure 1 shows the relation between the concentration of calcium-calmodulin-dependent protein kinase II in the phosphorylating solution and the amiloride-sensitive component of proton gradient stimulated $^{22}Na^+$ uptake. The remaining studies were performed using a concentration of calcium-calmodulin-dependent protein kinase II of 30 mU/ml.

Additional studies were performed to determine if either the ATP or calmodulin concentration in the

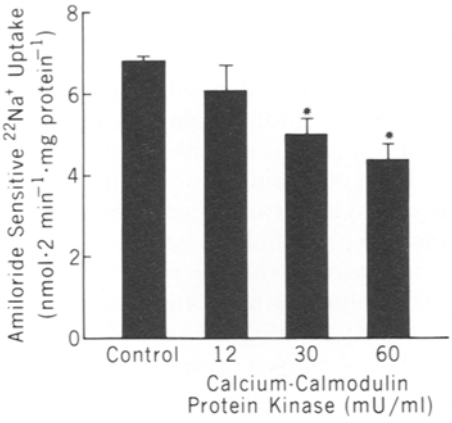

Fig. 1. Relation between amiloride-sensitive $^{22}Na^+$ uptake and calcium-calmodulin-dependent protein kinase in reconstituted proteoliposomes. The uptake of 1 mM Na^+ was determined under pH-gradient conditions (pH_i 6.0, pH_o 8.0) in the absence and presence of amiloride (2 mM). Values represent the mean ± SEM for six preparations. * indicates $P < 0.05$ as compared to controls

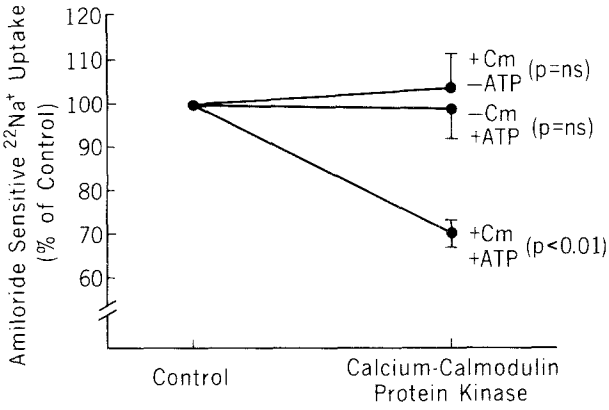

Fig. 2. The effect of calcium-calmodulin-dependent protein kinase II (30 mU/ml) on amiloride-sensitive $^{22}Na^+$ uptake in the presence or absence of calmodulin or ATP. Studies were performed under pH-gradient conditions. Values represent the mean ± SEM for six preparations

phosphorylating solution was rate limiting. Increases in the ATP concentrations up to 500 μM (1000 μM Mg^{2+}) resulted in no further decrease in the calcium-calmodulin-dependent protein kinase II-mediated decrease in Na^+/H^+ exchange activity. Increases in calmodulin concentrations up to 250 μM also did not affect further the magnitude of inhibition of transport by the kinase.

To rule out the possibility that calcium-calmodulin-dependent protein kinase II-mediated protein phosphorylation altered the integrity of the vesicles, the vesicular volume was determined by measurement of the uptake of rubidium under pH-gradient conditions and in the presence of equal concentrations of potassium (30 mM) on both sides of the membrane and valinomycin. Equilibrium was achieved by 30 min. The calculated intravesicular volumes averaged 1.75 ± 0.05 μl/mg lipid and 1.79 ± 0.02 in control proteoliposomes in the absence and presence of amiloride, respectively. In proteoliposomes containing phosphorylated proteins, the vesicular volumes averaged 1.73 ± 0.03 μl/mg lipid in the absence of amiloride and 1.77 ± 0.06 in the presence of amiloride.

The specificity of the response to calcium-calmodulin-dependent protein kinase II was determined in separate experiments by examining the effect of the kinase in the absence of either ATP or calmodulin in the phosphorylating solution. In the presence of both calmodulin and ATP, calcium-calmodulin-dependent protein kinase II resulted in a significant decrease in amiloride-sensitive $^{22}Na^+$ uptake in proteoliposomes of 30 ± 5% ($P < 0.01$) (Fig. 2). The omission of either calmodulin or ATP from the phosphorylating solution, in the absence of the kinase, had no affect on Na^+ uptake (*data not shown*). In the absence of ATP but in the presence of calmodulin, calcium-calmodulin-dependent protein kinase II did not significantly affect amiloride-sensitive $^{22}Na^+$ uptake. In the absence of calmodulin but in the presence of ATP, calcium-calmodulin-dependent protein kinase II did not significantly affect the uptake of Na^+. The Ca^{2+} dependency of the action of the kinase was examined by omitting Ca^{2+} and adding EGTA (5 mM) to the phosphorylating solution. In the nominal absence of Ca^{2+}, calcium-calmodulin-dependent protein kinase did not significantly inhibit the uptake of $^{22}Na^+$ (% change *vs.* control = 4 ± 8%, $n = 4$, P = NS).

To rule out the possibility that the calcium-calmodulin-dependent protein kinase II-mediated decrease in $^{22}Na^+$ uptake under proton-gradient conditions was the secondary consequence of alterations in permeability of the reconstituted proteoliposomes to either protons or Na^+, a series of additional experiments was performed. $^{22}Na^+$ uptake was determined under proton-gradient conditions and in the presence of valinomycin (1 μg/ml) and 30 mM potassium in the internal and external solutions. Under these presumed voltage-clamped conditions, calcium-calmodulin-dependent protein kinase II significantly inhibited the amiloride-sensitive component of $^{22}Na^+$ uptake from 7.2 ± 0.2 nmol · 2 min^{-1} · mg protein^{-1} to 5.4 ± 0.2 ($P < 0.05$, $n = 3$). The rate constant for passive dissipation of the proton gradient was measured using the change in acridine orange fluorescence. Under pH-gradient conditions and with 100 mM N-methylglucamine

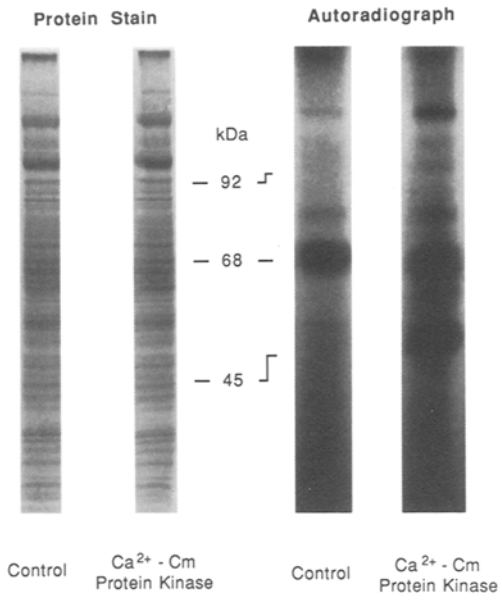

Fig. 3. Representative SDS-PAGE autoradiograms demonstrating substrate proteins in solubilized renal brush-border membranes of calcium-calmodulin multifunction kinase II. Solubilized proteins were incubated with a phosphorylation solution in the absence (control) or presence of calcium-calmodulin multifunction kinase II (30 mU/ml). Representative protein-staining patterns are shown on the left. Molecular weight standards are indicated

gluconate in the outside solution, the rate constant for dissipation of proton gradient averaged -0.046 ± 0.0006 sec^{-1} in control proteoliposomes and -0.046 ± 0.0006 in proteoliposomes containing proteins phosphorylated by calcium-calmodulin-dependent protein kinase II (P = NS, $n = 3$). The amiloride-insensitive component of $^{22}Na^+$ uptake provides an estimate of Na^+ permeability of the proteoliposomes. Amiloride-insensitive $^{22}Na^+$ uptake was not affected by the kinase. To confirm this finding, the uptake of $^{22}Na^+$ from an external solution containing 50 mM Na^+ was determined. This concentration of Na^+ is significantly higher than the K_a for Na^+ [8, 18]. Under these experimental conditions, calcium-calmodulin-dependent protein kinase II did not significantly affect Na^+ uptake (% change *vs.* control = $3 \pm 2\%$, P = NS, $n = 3$).

The rates of [^{32}P]-phosphate incorporation into protein was determined by incubating the solubilized proteins in γ-[^{32}P]-ATP, Mg^{2+}, Ca^{2+}, and calmodulin with and without calcium-calmodulin-dependent protein kinase II. In the absence of the kinase, incubation of the solubilized protein for 5 min resulted in rates of protein phosphorylation of 6 pmol of phosphate · min^{-1} · mg $protein^{-1}$. The rate of protein phosphorylation was increased threefold by calcium-calmodulin-dependent protein kinase II. The kinase-dependent increase in protein phosphorylation had an absolute requirement for Ca^{2+} and calmodulin. Additional studies were performed to determine if there was significant dephosphorylation of proteins after addition of phospholipid and overnight dialysis. Protein phosphorylation was compared after 5 min of calcium-calmodulin-dependent kinase phosphorylation and after incorporation of these proteins into phospholipid vesicles and formation of proteoliposomes by overnight dialysis. The amount of [^{32}P]-phosphate per mg protein in the proteoliposomes was 98% of that obtained at 5 min of incubation of the solubilized proteins in the phosphorylating solution.

SDS-PAGE analysis of the protein patterns of solubilized proteins, after the addition of asolectin, and after overnight dialysis were nearly identical. Comparable studies on solubilized proteins exposed to calcium-calmodulin-dependent protein kinase II were also identical to one another and to those obtained in control experiments. Figure 3 is an SDS-PAGE autoradiogram of solubilized brush-border membrane proteins incubated with the phosphorylating solution in the absence (control) or presence of calcium-calmodulin multifunction kinase II. Comparison of the autoradiograms indicates that calcium-calmodulin multifunction kinase II mediates the phosphorylation of a number of selected proteins.

Protein Kinase C

Solubilized brush-border membrane proteins were incubated in a phosphorylation solution containing ATP (50 μM), Ca^{2+} (100 μM), Mg^{2+} (100 μM), an active phorbol ester (1 μM), and protein kinase C. In the absence of the kinase, $^{22}Na^+$ uptake averaged 16.9 ± 1.0 nmol · 2 min^{-1} · mg $protein^{-1}$ in the absence of amiloride and 9.8 ± 0.6 in the presence of amiloride. Protein kinase C increased the uptake of $^{22}Na^+$ in a concentration-dependent manner but had no effect on the amiloride-insensitive component of Na^+ uptake or on the uptake of Na^+ in liposomes. At the highest concentration of protein kinase C examined, Na^+ uptake averaged 20.1 ± 1.4 nmol · 2 min^{-1} · mg $protein^{-1}$ in the absence of amiloride ($P < 0.02$ vs. control) and 10.2 ± 0.8 in the presence of amiloride (P = NS *vs.* control). Amiloride-sensitive $^{22}Na^+$ uptake was $30 \pm 4\%$ higher ($P < 0.01$). Figure 4 is the dose-response relation between protein kinase C and the amiloride-sensitive component of $^{22}Na^+$ uptake under proton-gradient conditions. In the remaining studies, protein kinase C was used in a concentration of 0.6 mU/ml. Increases in the ATP (and Mg^{2+}) con-

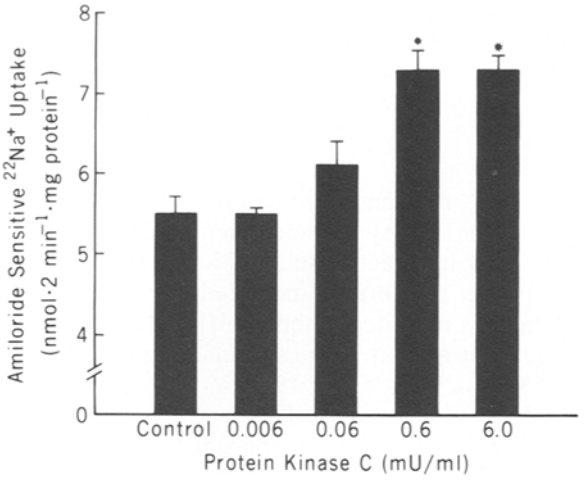

Fig. 4. Relation between amiloride-sensitive $^{22}Na^+$ uptake and protein kinase C under pH-gradient conditions. Values represent the mean ± SEM for six preparations. * indicates $P < 0.05$ as compared to controls

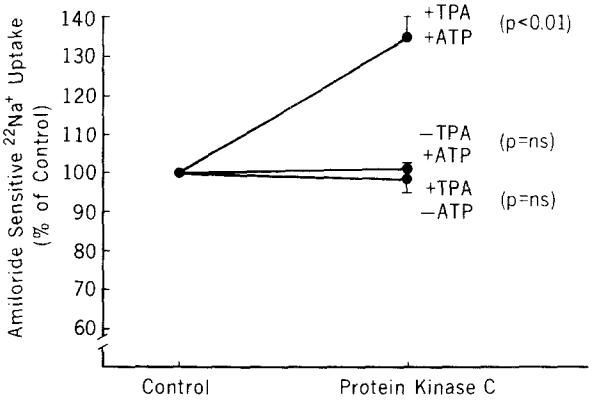

Fig. 5. Effect of protein kinase C (0.6 mU/ml) on amiloride-sensitive $^{22}Na^+$ uptake in the presence or absence of TPA or ATP. Studies were performed under pH-gradient conditions. Values represent the mean ± SEM for six preparations

centrations up to 500 μM (1000 μM) or the concentration of phorbol ester to 100 μM resulted in no further protein kinaes C-mediated increase in Na^+ uptake. In separate experiments, phosphatidylserine (200 μg/ml) and diolein (20 μg/ml) was substituted for TPA in the phosphorylation solution. Na^+ uptake was increased to the same magnitude as observed with TPA.

To rule out the possibility that protein kinase C-mediated protein phosphorylation altered the integrity of the vesicles, the vesicular volume was determined as previously indicated. The calculated intravesicular volumes averaged 1.80 ± 0.04 μl/mg lipid and 1.91 ± 0.07 in control proteoliposomes in the absence and presence of amiloride, respectively. In proteoliposomes containing phosphorylated proteins, the vesicular volumes averaged 1.87 ± 0.09 μl/mg lipid in the absence of amiloride and 1.82 ± 0.06 in the presence of amiloride.

The specificity of the response to protein kinase C was determined in separate studies. As shown in Fig. 5, in the presence of ATP and TPA, protein kinase C significantly increased the amiloride-sensitive component of Na^+ uptake from 6.9 ± 0.5 nmol · 2 min^{-1} · mg protein^{-1} to 9.2 ± 1.1 ($P < 0.01$). The omission of ATP or TPA from the phosphorylation solution, in the absence of protein kinase C, did not affect Na^+ uptake (*data not shown*). In the absence of TPA but in the presence of ATP, protein kinase C did not significantly affect Na^+ uptake in the reconstituted proteoliposomes. In the presence of TPA but in the absence of ATP, PKC failed to affect the uptake of Na^+. The substitution of α-4-phorbol-12,13-didecanoate, an inactive phorbol ester, for TPA was studied in separate experiments. In contrast to TPA, protein kinase C did not stimulate proton-gradient-stimulated Na^+ uptake when the phosphorylating solution contained the inactive phorbol ester (% change = −7 ± 3%, P = NS, n = 4). The Ca^{2+} dependency of protein kinase C was examined by omitting Ca^{2+} and adding EGTA (5 mM) to the phosphorylating solution. In the nominal absence of Ca^{2+}, protein kinase C-dependent protein phosphorylation did not significantly affect the uptake $^{22}Na^+$ (% change *vs.* control = −5 ± 5%, P = NS, n = 3).

To rule out the possibility that the effect of protein kinase C was the indirect consequence of altered permeability of the proteoliposomes to protons or Na^+, additional studies were performed. The effect of protein kinase C-mediated protein phosphorylation was determined in proteoliposomes under proton-gradient conditions in the presence of a voltage clamp (K_{in} = K_{out} = 30 mM, valinomycin 1 μg/ml). Under these conditions, protein kinase C significantly increased amiloride-sensitive Na^+ uptake by 38 ± 9% ($P < 0.05$, n = 5). Protein kinase C did not affect the rate constant for passive dissipation of the proton gradient (% change from control = 1.5 ± 3%, P = NS, n = 4) or the uptake of 50 mM Na^+ (% change from control = −8 ± 6%, P = NS, n = 5).

The rates of [^{32}P]-phosphate incorporation into protein was determined by incubating the solubilized protein in γ-[^{32}P]-ATP, Mg^{2+}, Ca^{2+}, TPA and with and without protein kinase C. In the absence of the kinase, incubation of the solubilized protein for 5 min resulted in rates of protein phosphorylation of 7 pmol of phosphate · min^{-1} · mg protein^{-1}. The rate of protein phosphorylation was increased fourfold by protein kinase C. The kinase-dependent increase

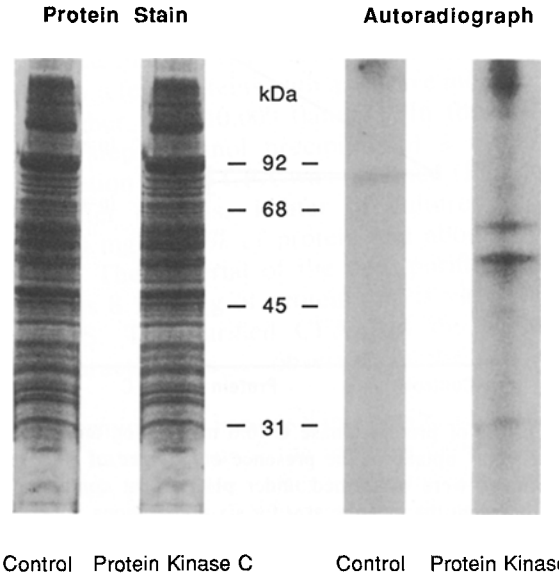

Fig. 6. Representative SDS-PAGE autoradiograms demonstrating substrate proteins in solubilized renal brush-broder membranes of protein kinase C. Solubilized proteins were incubated with a phosphorylation solution containing ATP, Mg^{2+}, phosphatidylserine, diolein, and protein kinase C (0.6 mU/ml) in the nominal absence of calcium (0 Ca^{2+}, 5 mM EGTA) (control) or in the absence of EGTA and the presence of calcium (100 μM). Representative protein-staining patterns are shown on the left. Molecular weight standards are indicated

in protein phosphorylation had an absolute requirement for Ca^{2+} and TPA. Additional studies were performed to determine if there was significant dephosphorylation of proteins after addition of phospholipid and overnight dialysis. Protein phosphorylation was compared after 5 min of protein kinase C phosphorylation and after incorporation of these proteins into phospholipid vesicles and formation of proteoliposomes by overnight dialysis. The amount of [^{32}P]-phosphate per mg protein in the proteoliposome was 108% of that obtained at 5 min of incubation of the solubilized proteins in the phosphorylating solution.

SDS-PAGE analysis of the protein patterns of solubilized proteins, after the addition of asolectin, and after overnight dialysis were nearly identical. Comparable studies on solubilized protein exposed to protein kinase C were also identical to one another and to those obtained in control experiments. Figure 6 is a SDS-PAGE autoradiogram of solubilized brush-border membrane proteins incubated with a phosphorylation solution containing ATP (50 μM), Mg^{2+} (100 μM), phosphatidylserine (200 μg/ml), diolein (20 μg/ml), EGTA (5 mM) and protein kinase C (control) or with the same solution in which EGTA was omitted and Ca^{2+} (100 μM) was added. Comparison of the autoradiograms indicates that protein kinase C mediates the phosphorylation of a number of selected proteins.

Discussion

The Na^+/H^+ exchanger in the proximal convoluted tubule of the kidney is under metabolic control [3, 11]. Studies in intact tissues as well as in isolated brush-border membranes indicated that the Na^+/H^+ exchanger is inhibited when cAMP-dependent protein kinase is activated and stimulated when protein kinase C is activated [17, 19]. Calcium-calmodulin-dependent multifunctional protein kinase II is present in the cytosol and plasma membranes of cells and has been suggested to be involved in transmembrane electrolyte fluxes [7]. Despite some studies which suggest a role for this kinase in selected aspects of renal function, the effect of calcium-calmodulin-dependent protein kinase II on renal electrolyte transport, in general, and on the Na^+/H^+ exchanger, specifically, has not yet been determined [1, 15, 20]. The present studies were designed to examine the effect of two Ca^{2+}-dependent protein kinases, calcium-calmodulin-dependent protein kinase II and protein kinase C, on the renal brush-border Na^+-H^+ exchanger. Performance of these studies was facilitated by the recent demonstration in our laboratories that the renal brush-border Na^+/H^+ exchanger could be solubilized and its activity assayed after reconstitution of the proteins into artificial phospholipid vesicles; that the soluble protein extract exhibits limited endogenous protein kinase or protein phosphatase activity; and that the regulatory component(s) of the Na^+/H^+ exchange complex, at least that component responsive to cAMP-dependent protein kinase, remained active during the process of solubilization and reconstitution [16, 18].

The experimental protocol was to incubate the solubilized proteins in a phosphorylation solution containing the kinase of interest. The proteins were then reconstituted into proteoliposomes and Na^+/H^+ exchange activity assayed. This methodology obviates the need for incorporation of the kinase into the artificial proteoliposomes and concerns about the availability of protein substrates. It is possible, however, that the efficiency of reconstitution is altered in response to the specific experimental maneuvers. Analysis of the protein patterns by SDS-PAGE, however, did not indicate any differences in the proteins incorporated into the proteoliposomes. The transport studies indicate that protein phosphorylation mediated by calcium-calmodulin-dependent protein kinase II inhibits the activity of the solubilized and reconstituted renal

brush-border Na^+-H^+ exchanger. The inhibitory effect of this kinase has an absolute requirement for ATP, Ca^{2+}, and calmodulin and cannot be explained by alterations in the permeability of the reconstituted proteoliposomes to protons or Na^+. Protein kinase C-mediated protein phosphorylation stimulates the activity of the solubilized and reconstituted renal brush-border membrane Na^+/H^+ exchanger. The stimulatory effect of this kinase has an absolute requirement for ATP, Ca^{2+}, and an active phorbol ester, and cannot be explained by alterations in the permeability of the reconstituted proteoliposomes to protons or Na^+.

The effect of protein kinase C-mediated protein phosphorylation to increase the activity of the renal Na^+/H^+ exchanger confirms and extends recent studies from our laboratories using native renal brush-border membranes [17]. The degree of stimulation in the reconstituted system is approximately twofold greater than that observed in natural membranes. The stimulatory effect of protein kinase C on the Na^+/H^+ exchanger appears to provide an explanation for the findings of Mellas and Hammerman [12] that exposure of suspended proximal tubules to phorbol esters resulted in intracellular alkalinization. The present studies, in conjunction with our recent studies demonstrating cAMP-dependent protein kinase inhibition of the reconstituted Na^+/H^+ exchanger, provide further support for the conclusion that the characteristics of the solubilized and reconstituted Na^+-H^+ exchanger are similar to those observed in natural brush-border membranes [16]. To our knowledge, the inhibitory effect of protein phosphorylation mediated by calcium-calmodulin-dependent protein kinase II on the renal Na^+/H^+ exchanger has not previously been reported.

Protein phosphorylation mediated by either calcium-calmodulin-dependent multifunction protein kinase II or protein kinase C is associated with a unique profile of substrate proteins in solubilized rabbit brush-border membranes (Figs. 3 and 6). These phosphorylation patterns differ from that mediated by cAMP-dependent protein kinase as previously reported [16]. The two calcium-dependent protein kinases appear to have a more limited number of substrates as compared to cAMP-dependent protein kinase. As analyzed by one-dimensional SDS-PAGE, there appear to be proteins which are substrates for more than one of the protein kinases examined. While the demonstration of proteins, which are common substrates for protein kinases which affect the activity of the Na^+/H^+ exchanger, is of interest, confirmation will require studies employing other techniques. The nature and physiologic significance of the substrate proteins for cAMP-dependent protein kinase, calcium-calmodulin multifunction protein kinase II, and protein kinase C is unknown at the present time. Moreover, it has not yet been determined whether or not the Na^+/H^+ exchanger itself is a phosphoprotein.

Extrapolation of the results of the present studies using solubilized proteins to an understanding of how the Na^+/H^+ exchanger is regulated in intact proximal tubule cells must await additional study. Nonetheless, it is of interest to speculate that activation of one or more of these kinases in the cells of the proximal tubule modulates the rate of proton-linked Na^+ reabsorption. As an example of possible linkages between protein kinase activation and renal Na^+/H^+ exchange activity, it is instructive to consider, briefly, the effects of parathyroid hormone on the proximal tubule. Parathyroid hormone inhibits bicarbonate reabsorption in the proximal tubule and decreases the activity of the Na^+/H^+ exchanger [6, 10]. Parathyroid hormone stimulates cAMP production by proximal tubule cells. Traditional thought has been that cAMP activates cAMP-dependent protein kinase which, in turn, inhibits the Na^+/H^+ exchanger in the apical membrane. Parathyroid hormone, however, also alters the cytosolic concentration of Ca^{2+} [1, 2, 4]. We have previously reported the parathyroid hormone and cAMP decreases the cytosolic concentration of Ca^{2+} in the cells of the proximal convoluted tubule [1]. More recently, an extensive series of studies by Hruska et al. [2, 4] indicate that parathyroid hormone increases the cytosolic concentration of Ca^{2+} in these cells. While the reasons for the differences in the effect of parathyroid hormone in the above studies are not known with certainty, the fact that the concentration of Ca^{2+} is altered by parathyroid hormone raises the possibility that one or more of the Ca^{2+}-dependent protein kinases are also involved in the expression of the effect of parathyroid hormone and/or cAMP on the renal brush-border Na^+/H^+ exchanger. It should be expressly noted, however, that the full physiologic significance of the present in-vitro observations is unknown and several uncertainties exist. For example, while both of the protein kinases examined affected the Na^+/H^+ exchanger, it is not known whether the concentrations of the kinases employed are physiologically relevant. In the intact cell, by contrast to the in-vitro conditions employed in the present studies, there is the possibility that the protein kinases directly or indirectly modulate the activity of other protein kinases. As previously indicated, data in intact renal proximal convoluted tubules support the conclusions from in-vitro studies using the solubilized Na^+/H^+ exchanger that activation of cAMP-dependent protein kinase inhibits the Na^+/H^+ exchanger while activation of protein kinase C

stimulates the activity of this transporter. The effect of protein phosphorylation mediated by calcium-calmodulin-dependent multifunction protein kinase II on Na^+/H^+ exchange activity in intact proximal tubule cells, however, has not been reported. By contrast to cAMP-dependent protein kinase and protein kinase C, study of the effect of calcium-calmodulin-dependent protein kinase II in intact cells is rendered difficult by the absence of specific stimulators or inhibitors of this kinase.

The present studies, in conjunction with our prior studies, indicate that the activity of solubilized Na^+/H^+ can be regulated by protein phosphorylation mediated by specific protein kinases. An important corollary is that the regulatory components of Na^+/H^+ exchanger remain active during the process of solubilization and are expressed after the proteins are reconstituted. The ability to solubilize the transporter and its regulators and to assay their activity after reconstitution should facilitate attempts to purify the transporter itself and/or its regulatory subunits.

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