CAMP-mediated Inhibition of the Renal Brush Border Membrane Na^+-H^+ Exchanger Requires a Dissociable Phosphoprotein Cofactor

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Abstract

Prior studies have suggested that protein kinase A (PKA)-mediated inhibition of the rabbit renal brush border membrane (BBM) Na^+-H^+ exchanger involves a regulatory protein that is distinct from the transporter. This putative regulatory protein was purified by column chromatography and SDS-PAGE, and a partial primary amino acid sequence was determined. An affinity-purified polyclonal antibody to a synthetic peptide representing a sequence of the protein recognized a polypeptide of 55 kD in BBM but not in basolateral membrane. The antibody immunoprecipitated a PKA substrate of a similar molecular mass from detergent-solubilized BBM proteins. When assayed after reconstitution, PKA in the presence of ATP and Mg^{2+} did not inhibit Na^+-H^+ exchange transport in a fraction of solubilized BBM proteins eluting from an anion exchange column between 0.2 and 0.4 M NaCl (fraction B). Coreconstitution of fraction B with the immunoprecipitated 55-kD protein restored the inhibitory effect of PKA (change = 42%, $P < 0.05$). By contrast, Na^+-H^+ exchange transport in total solubilized BBM proteins was inhibited 25% ($P < 0.05$) by PKA, ATP, and Mg^{2+}. This effect was abolished by immunodepletion of the cAMP regulatory protein (change = +5%, P = NS). These findings provide evidence that the regulation of renal BBM Na^+-H^+ exchange transport by PKA is affected by repletion and depletion of a specific protein. This suggests that PKA-mediated inhibition of the renal BBM Na^+-H^+ exchanger requires participation of a regulatory protein that is distinct from the transporter itself. (*J. Clin. Invest.* 1993. 92:1781–1786.) Key words: renal electrolyte transport • acid–base regulation • protein kinases • cAMP • rabbit kidney

Introduction

Parathyroid hormone (PTH)[1] decreases the secretion of hydrogen ions in the cells of the renal proximal convoluted tubule by inhibiting the activity of the brush border membrane (BBM) Na^+-H^+ exchanger (1–3). A major signal transduction pathway used by parathyroid hormone in the renal proximal tubules involves the generation of cAMP and the subsequent activation of a phosphorylation cascade mediated by cAMP-dependent protein kinase (PKA). The cellular events that follow the activation of PKA by PTH and result in a decrease in the activity of the BBM Na^+-H^+ exchanger have been the subject of extensive study. Hensley et al. (4) reported that PTH mediated the removal or retrieval of the transporter from its apical membrane location. Others have speculated that the activity of the BBM Na^+-H^+ exchanger might be modulated by direct phosphorylation by PKA. The complete structure of the renal BBM Na^+-H^+ exchanger has yet to be elucidated. A candidate cDNA, however, has been cloned recently and the proposed amino acid structure of this antiporter protein contains several consensus sequences for PKA (5).

Another mechanism by which PTH could inhibit the BBM Na^+-H^+ exchanger involves PKA-mediated phosphorylation of a regulatory component that is separable from the transporter (6–8). Several lines of evidence have been advanced to indicate that the activity of the Na^+-H^+ exchanger can be dissociated from its regulation by PKA (7, 8). Coreconstitution experiments confirm that PKA-mediated regulation of the activity of the Na^+-H^+ exchanger involved a protein with distinct chromatographic properties from the exchanger itself. This polypeptide is a necessary cofactor for the expression of the inhibitory effect of PKA on the activity of the BBM Na^+-H^+ exchanger. This putative regulatory cofactor, however, has not been purified to homogeneity.

The present experiments were undertaken to define further the function of the proposed regulatory protein. The experimental approach involved isolation of the putative polypeptide by anion exchange chromatography and slab gel electrophoresis, determination of its partial amino acid sequence, and generation of a monospecific antibody to a synthetic peptide representing a sequence in this regulatory protein. The antipeptide antibody was used to immunodeplete and immunoreplete total and selected chromatographic fractions of solubilized BBM proteins. These experiments provide the most compelling evidence to date that PKA-mediated inhibition of the BBM Na^+-H^+ exchanger involves participation of a regulatory cofactor that is distinct from the exchanger itself.

Methods

BBM were prepared from the rabbit kidney by a magnesium precipitation method and membrane proteins were extracted by mixing one part of the membrane preparation (5 mg/ml) with 1.25 parts of 8% octyl glucoside at pH 6.0 for 15 min at 0°C (2, 9). The membrane–detergent mixture was then centrifuged at 100,000 *g* for 30 min. The solubilized membrane proteins were applied to a Mono Q HR 5/5 (Pharmacia Fine Chemicals, Piscataway, NJ) anion exchange column and batch eluted sequentially with a two-step gradient using solutions

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1. *Abbreviations used in this paper:* BBM, brush border membrane; BLM, basolateral membrane; PKA, protein kinase A; PTH, parathyroid hormone.

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containing 50 mM Tris, 0.1 mM EDTA, 0.1 mM DTT, 2% octyl glucoside, and 0.2 M NaCl (fraction A) at a rate of 1 ml/min followed by the same solution containing 0.4 M NaCl (fraction B) at the same rate (8). Fraction B and aliquots of the unfractionated solubilized proteins were desalted on a Sephadex-G25 column.

Initial solubilized proteins or fraction B proteins were mixed with asolectin (35 mg/ml; crude soybean phospholipid; Associated Concentrates, Woodside, NY), which had been sonicated to translucency for 10 min at 22°C. Proteoliposomes were prepared by dialysis for 18 h at 4°C using 10–12-kD cutoff dialysis membranes. The dialysis buffer contained 250 mM mannitol, 50 mM MES/Tris, and 30 mM potassium gluconate (pH 6.0).

Sodium uptake in reconstituted proteoliposomes was determined by applying the reaction mixture to 1-ml Dowex 50 × 8 (Tris), 100-mesh columns, and eluting with vacuum suction with 1 ml of 300 mM mannitol (pH 8.0) at 0°C. The internal pH of the proteoliposomes (pH 6.0) was set by the dialysis solution. Except where indicated otherwise, the uptake solutions contained 1 mM $^{22}Na^+$ (sp act, 2.5 mCi/μg sodium), 1 μg/ml valinomycin, and either 250 mM mannitol, 50 mM Tris/MES, 30 mM potassium gluconate (pH 8.0), or 250 mM mannitol, 50 mM MES/Tris, and 30 mM potassium gluconate (pH 6.0). In prior published studies and in preliminary studies, the difference between $^{22}Na^+$ uptake in the presence or absence of a pH gradient was equal to the amiloride (2 mM)-inhibitable component of proton gradient–stimulated $^{22}Na^+$ uptake in controls and after phosphorylation of proteins with PKA (10). In addition, prior preliminary experiments indicated that neither the presence of glucose (5 mM) nor furosemide (10^{-3} M) affected the uptake of sodium under experimental conditions identical to those of the present studies. Accordingly, in the present experiments, the Na^+-H^+ exchange rate was taken as the proton gradient–stimulated component of $^{22}Na^+$ uptake under voltage-clamped conditions. The adequacy of the voltage clamp was confirmed previously using Dis-C_3-(5) (7).

The putative regulatory protein was purified initially from a fraction of solubilized renal BBM proteins eluting from an anion exchange column between 0.05 and 0.1 M NaCl using SDS-PAGE (11). The polypeptide of interest, one of the major substrates for PKA in this protein fraction, was excised from 10% polyacrylamide slab gels, electroeluted, and incubated with cyanogen bromide (0.5 mg) in 70% formic acid for 24 h at room temperature (7, 12). The solution was diluted 10-fold with water, dried, and redissolved in 0.1% SDS. The peptide digests were subjected to electrophoresis on a second 15% polyacrylamide slab gel and electrophoretically transferred to PDVF membranes for amino acid sequencing.

A synthetic peptide representing amino acids 2–10 of the amino-terminal end of a 31-kD fragment of the putative regulatory cofactor (see Fig. 1) was conjugated to keyhole limpet hemocyanin through a terminal cysteine residue using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester as the coupling reagent and emulsified in complete Freund's Adjuvant. Rabbits were immunized by intradermal injection with additional booster injections of the antigen in incomplete Freund's Adjuvant at regular intervals. The antiserum was partially purified by two precipitations with 50% saturated ammonium sulfate, and chromatography on DEAE Affi-Gel blue to remove residual serum albumin and trace serum protease activity. Affinity-purified antibodies were prepared by chromatography on Affi-Gel-10 cross-linked with the original synthetic peptide.

Western immunoblots were performed by the method of Towbin et al. (13). The proteins were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose. The nitrocellulose was blocked with 5% bovine serum albumin containing 0.1% Tween-20 for 6 h before reacting with the generated antiserum overnight at 4°C. The filters were washed briefly with phosphate-buffered saline (pH 7.5). Immune complexes were detected by an enhanced chemical luminescence system (Amersham Corp., Arlington Heights, IL).

To immunoprecipitate the putative regulatory polypeptide from solubilized renal BBM proteins, the monospecific antiserum was bound to protein A–Sepharose CL-4B beads (Pharmacia Fine Chemicals). The solubilized BBM proteins were mixed with the bound beads for 2 h at room temperature. The beads were removed by centrifugation and washed three times with phosphate-buffered saline. The immunopurified proteins were eluted from the beads and renatured using 3 M sodium thiocyanate (14, 15). The eluted sample was then desalted using a Sephadex G-25 column.

The effects of phosphorylation by PKA were examined by incubating the BBM proteins in solutions containing ATP (50 μM) and magnesium (200 μM) in the absence or presence of purified PKA for 2 min at 30°C. The catalytic subunit of PKA was purified by the method of Beavo et al. (16). Protein concentrations were estimated by the method of Lowry et al. (17). Results of transport experiments are presented as the mean of means±SEM of duplicate or triplicate determinations and analyzed using the paired *t* test or Peritz Analysis of Variance (18). Representative gels and autoradiographs are presented.

Results

Based on prior experiments, the putative target phosphoprotein accounting for PKA-mediated inhibition of the rabbit renal BBM Na^+-H^+ exchanger had an apparent molecular mass of ≥ 42 kD and its abundance was enriched in a fraction of solubilized BBM proteins that elutes from an anion exchange column between 0.05 and 0.1 M NaCl (7, 8, 19). This protein was excised from SDS-PAGE slab gels of this fraction of solubilized BBM proteins and electroeluted. Initially, it was not possible to obtain a primary amino acid sequence of the intact regulatory protein due, presumably, to a blocked amino-terminal end. It also was not possible to obtain pure fragments for sequence analysis using trypsin. Nonenzymatic cleavage of the polypeptide using cyanogen bromide, however, resulted in distinct cleavage products. Fig. 1 is the amino acid sequence of a 31-kD cyanogen bromide fragment of the putative regulatory protein. Database search indicated that the sequence of this peptide was unique. Some amino acid homology to the β-subunit of human transducin, a retinal GTP binding protein, was observed. The possible structural relation between these proteins is considered further in the discussion.

A synthetic peptide representing amino acids 2–10 was used to immunize rabbits and develop a polyclonal antibody. Fig. 2 is a Western immunoblot of rabbit renal BBM. The affinity-purified antibody recognized a protein band at ~ 55 kD (lane *1*). As shown in Fig. 2, lane *2*, preabsorption of the antibody with the peptide antigen abolished completely recognition of the indicated band. In addition, preimmune sera did not react with the 55-kD polypeptide in the renal BBM. Fig. 3 is a Western immunoblot of rabbit renal BBM, basolateral membranes (BLM), and two separate protein fractions obtained by anion exchange chromatography of octyl glucoside–solubilized BBM proteins. The antibody recognized a broad protein band at 55 kD in BBM but not in BLM. The antibody recognized a protein band of the same molecular mass and an additional band at a somewhat lower apparent molecular mass in a pro-

31-kD Fragment K K G P N G Y G F N L H S D K D A P W Q F I V
Transducin beta-2 chain A F F P N G Y A F T T G S D D A T C R L F D L

Figure 1. Amino acid sequence of the 31-kD phosphoprotein fragment of the putative regulatory cofactor. Also shown is the amino acid sequence of the β-subunit of transducin, a retinal GTP binding protein. Double dots denote amino acid identity; single dots indicate conservative amino acid substitutions.

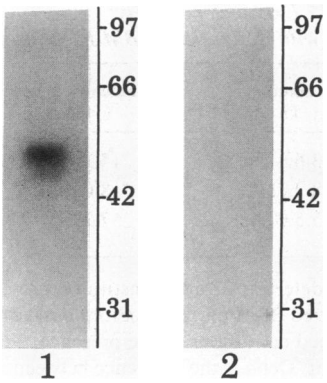

Figure 2. Representative Western immunoblot of rabbit renal BBM proteins using an affinity-purified antipeptide polyclonal antibody to the putative regulatory protein (lane *1*). Lane *2* is a Western immunoblot of the same BBM proteins in which the antibody was preabsorbed with its peptide antigen. Molecular mass standards (kD) are indicated.

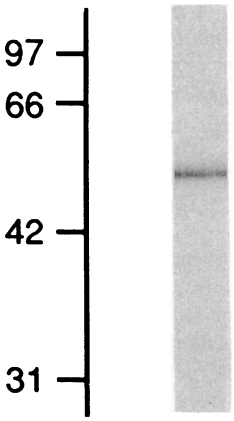

Figure 4. Representative protein stain of SDS gel of the immunoprecipitate obtained using solubilized rabbit renal BBM and the antipeptide antibody to the putative regulatory cofactor. Molecular mass standards (kD) are indicated.

tein fraction of solubilized rabbit renal BBM that elutes from an anion exchange column between 0.05 and 0.1 M NaCl (Fig. 3, lane *0.1 M NaCl*). This is the protein fraction from which the putative regulatory protein was purified initially. Preabsorption of the antibody with its peptide antigen prevented recognition of both bands in this protein fraction. Fraction B is the anionic protein fraction that elutes between 0.2 and 0.4 M NaCl. This protein fraction has been demonstrated previously to possess Na^+-H^+ exchange activity that is not regulated by PKA (8). No immunoreactive proteins were recognized in this protein fraction. The antibody also did not react with any proteins in crude membrane fractions of rabbit brain or skeletal muscle (data not shown). Fig. 4 is a representative protein stain of an SDS gel indicating that the antibody immunoprecipitated a single protein. Fig. 5 is a composite showing protein staining and a Western immunoblot (lanes *1* and *3*, respectively) of the protein precipitated by the antibody from solubilized rabbit renal BBM proteins. Lane *2* is an autoradiograph of the immunoprecipitated protein incubated in the phosphorylation solution containing γ-[^{32}P]ATP and PKA. By all three methods, the apparent molecular mass of the immunoprecipitated polypeptide is similar to that identified by Western immunoblot analysis of rabbit renal BBM. These results indicate also that the polypeptide recognized by the antibody is a substrate for PKA.

To assay the function of the protein recognized by the antipeptide antibody, a series of transport experiments were performed in reconstituted proteoliposomes. As summarized in Table 1 and Fig. 6, Na^+-H^+ exchange activity in fraction B proteins incubated with ATP and Mg^{+2} averaged 10.5±1.3 nmol × mg protein^{-1} × 2 min^{-1} in the absence of PKA and 10.3±1.1 in the presence of PKA (P = NS). These findings confirm results of prior studies indicating that PKA does not regulate the activity of the Na^+-H^+ exchanger in this protein fraction (8). Coreconstitution of fraction B proteins with the protein immunoprecipitated by the antibody resulted in a significantly lower rate of Na^+-H^+ exchange activity of 6.1±1.1 nmol × mg protein^{-1} × 2 min^{-1} after PKA-mediated phosphorylation. Denaturing of the putative regulatory factor by heat treatment resulted in loss of PKA-mediated inhibition of Na^+-H^+ exchange transport (change = −1±7%, P = NS). Additional studies were performed to demonstrate that the inhibitory effect of the regulatory factor required both ATP and PKA. In the presence of PKA but not ATP, the putative regulatory protein did not affect Na^+-H^+ exchange activity (change = +3±6%, P = NS). In the presence of ATP but not PKA, the putative regulatory protein also did not affect Na^+-H^+ exchange activity (change = +4±8%, P = NS). The amount of regulatory cofactor reconstituted could not be determined accurately due to the low amounts of protein recovered by immunoprecipitation. To demonstrate that the renatured cofactor affected Na^+-H^+ exchange transport in a concentration-dependent manner, the cofactor was diluted serially before reconstitution with fraction B. As compared with controls containing no regulatory cofactor, PKA in the presence of ATP, Mg^{+2}, and undiluted regulatory protein inhibited Na^+-H^+ exchange activity by 28%. Na^+-H^+ exchange activity was 23% lower than controls when the regulatory protein was diluted 1:10. Dilutions of the immunoprecipitated protein by 1:100 or 1:1,000 abolished the inhibitory effect of PKA on the rate of transport.

In separate experiments (Table 2 and Fig. 7), Na^+-H^+ exchange activity was assayed in total solubilized BBM proteins. PKA inhibited Na^+-H^+ exchange activity from 7.3±0.2 nmol

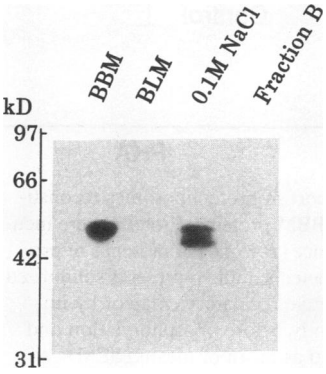

Figure 3. Representative Western immunoblots of rabbit renal BBM proteins, rabbit BLM proteins, octyl glucoside–extracted BBM proteins eluting from an anion exchange column between 0.05 and 0.1 M NaCl (lane *0.1 M NaCl*), or between 0.2 and 0.4 M NaCl (lane *Fraction B*). Molecular mass standards are indicated.

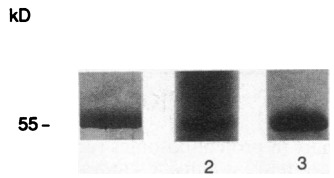

Figure 5. Representation of the polypeptide immunoprecipitated by the antipeptide antibody to the putative regulatory cofactor. Lane *1* is a representative silver stain for protein of the SDS-PAGE of the immunoprecipitated protein. Lane *2* is an SDS-PAGE autoradiograph of the immunoprecipitated protein phosphorylated by PKA. Lane *3* is a Western immunoblot of the immunoprecipitated protein using the affinity-purified polyclonal antibody. Molecular mass standards are indicated.

Table I. Effect of PKA on Na^+-H^+ Exchange Activity in Proteoliposomes Reconstituted with Fraction B

	Fraction B (−PKA)	Fraction B (+PKA)	Fraction B + RF (+PKA)
8/6	29.9±1.9	29.6±1.4	25.1±0.9*
6/6	19.4±1.6	19.3±1.7	19.0±1.8
Delta	10.5±1.3	10.3±1.1	6.1±1.1*

The uptake of 1 mM sodium was determined in reconstituted proteoliposomes prepared using fraction B of solubilized BBM proteins. Sodium uptake was measured under voltage clamped conditions in the presence (8/6) or absence (6/6) of pH gradient. Delta is the difference between uptake at 8/6 vs. 6/6 and is taken as a measure of Na^+-H^+ exchange. All protein samples were incubated in a phosphorylation solution containing ATP and Mg^{+2} in the absence (fraction B minus PKA) or presence of PKA (fraction B plus PKA) before reconstitution. Fraction B + RF samples contain fraction B proteins plus the polypeptide immunoprecipitated by the affinity-purified polyclonal antibody (RF). This protein sample was incubated in ATP, Mg^{2+}, and PKA before reconstitution. Values are mean of means±SEM for four preparations. Values are expressed as nmol × mg protein^{-1} × 2 min^{-1}.
* $P < 0.05$.

Table II. Effect of PKA on Na^+-H^+ Exchanger Activity in Proteoliposomes Reconstituted with Solubilized BBM Proteins

	Control (−PKA)	Control (+PKA)	Immunodepletion (+PKA)
8/6	15.8±1.8	13.6±1.9*	15.2±0.9
6/6	8.5±1.6	8.2±1.4	8.0±1.4
Delta	7.3±0.2	5.5±0.4*	7.2±0.7

The uptake of 1 mM sodium was determined in reconstituted proteoliposomes prepared from solubilized BBM protein. Sodium uptake was measured under voltage clamped conditions in the presence (8/6) or absence (6/6) of pH gradient. Delta is the difference between uptake at 8/6 vs. 6/6 and is taken as a measure of Na^+-H^+ exchange. All protein samples were incubated in a phosphorylation solution containing ATP and Mg^{2+} in the absence (control minus PKA) or presence of PKA (control plus PKA) before reconstitution. Immunodepletion was accomplished using the affinity-purified polyclonal antibody. This protein sample was incubated in ATP, Mg^{2+}, and PKA before reconstitution. Values are mean of means±SEM for four preparations. Values are expressed as nmol × mg protein^{-1} × min^{-1}.
* $P < 0.05$.

× mg protein^{-1} × 2 min^{-1} to 5.5±0.4 ($P < 0.5$). This finding is similar to prior reports from this laboratory (6). Immunoabsorption of the putative regulatory cofactor from total solubilized BBM proteins abolished the inhibitory effect of PKA. Western immunoblots confirmed a significant decrease of the 55-kD protein.

Discussion

Prior studies from this laboratory have suggested that PKA inhibits the activity of the renal BBM Na^+-H^+ exchanger and that this inhibitory effect requires participation of a regulatory cofactor that is distinct from the exchanger itself (1–3, 6–8). Evidence supporting this thesis was derived from experiments using solubilized BBM proteins subjected to limited proteolysis with trypsin or fractionation of renal BBM proteins by anion exchange chromatography. In both cases, Na^+-H^+ exchange transport was demonstrated but the activity of the transporter was no longer regulated by PKA. Coreconstitution of the trypsin treated proteins or the anion exchanger column fraction (fraction B) with selected other BBM protein fractions restored the inhibitory effect of PKA on the activity of the exchanger. Based on SDS-PAGE and autoradiography, the BBM protein fractions that restored the regulatory response of the Na^+-H^+ exchanger to PKA contained a PKA substrate polypeptide of ≥ 42 kD. In our prior studies, however, the regulatory cofactor

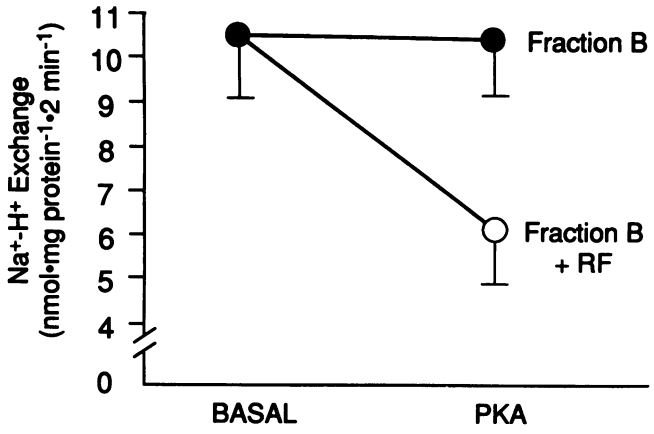

Figure 6. Na^+-H^+ exchange transport in proteoliposomes reconstituted with the anionic protein fraction B of solubilized rabbit renal BBM proteins. Proteins were incubated in ATP and Mg^{+2} in the absence (*BASAL*) or presence of protein kinase A (*PKA*). RF is the putative regulatory cofactor immunoprecipitated using the antibody that was coreconstituted with fraction B proteins. Results are expressed as mean of means±SEM.

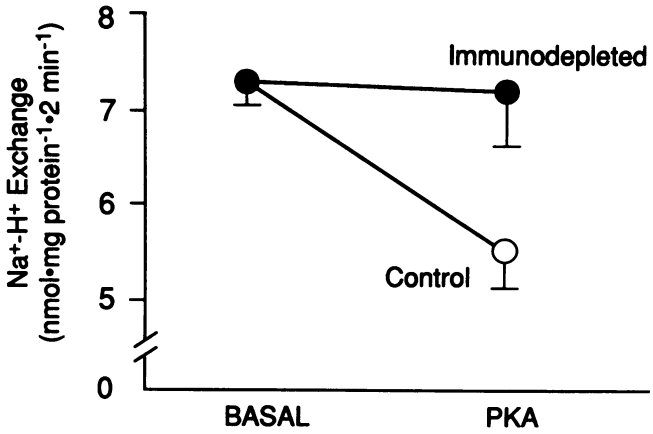

Figure 7. Na^+-H^+ exchange transport in proteoliposomes reconstituted with solubilized rabbit renal BBM proteins. Proteins were incubated in ATP and Mg^{+2} in the absence (*BASAL*) or presence of protein kinase A (*PKA*). Immunodepleted sample represents solubilized BBM proteins depleted of the putative regulatory cofactor by immunoprecipitation using the antibody before phosphorylation and reconstitution. Results are expressed as mean of means±SEM.

was not purified to homogeneity and, accordingly, questions remained as to the physiologic role of this polypeptide. The present experiments were designed to study this in greater detail.

The putative regulatory protein was partially purified using anion exchange chromatography. After separation of these proteins by SDS-PAGE, the phosphoprotein of interest was excised from the slab gels, electroeluted, and cleaved chemically using cyanogen bromide. A 23-amino acid sequence was obtained and confirmed several times from a 31-kD phosphopeptide fragment of the 42-kD protein. Search of protein data bases indicated that the peptide sequence was unique. The sequenced polypeptide bore no homology to any of the Na^+-H^+ exchangers cloned to date. This finding is consistent with prior functional data that indicate that the putative cofactor itself does not mediate proton gradient stimulated sodium uptake in reconstituted proteoliposomes (7). The polypeptide, however, showed an interesting homology to the β subunit of transducin, a retinal GTP binding protein. Amino acid identity between the 23-amino acid sequence of the putative regulatory protein and the β-subunit of transducin was 44% but included a stretch of 5 identical amino acids. If conservative substitutions were considered, the overall homology could be increased to 77%. While initially of interest, two-dimensional peptide maps of the 42-kD phosphoprotein from rabbit renal BBM did not resemble those of the subunits of any other known G proteins (S. Shenolikar and E. J. Weinman, unpublished observations). At the present time, the significance of the sequence homology with transducin is unknown.

An affinity-purified polyclonal antibody to a synthetic peptide representing nine amino acids of the putative regulatory cofactor was generated in rabbits. This antibody recognized a specific band on Western immunoblots of BBM. The ability of the antibody to recognize this band was blocked completely by preabsorption of the antibody with its peptide antigen. Preimmune sera contained no such reactive antibodies. These results demonstrate the specificity of the antibody. The antibody did not react with any proteins in rabbit renal BLM. The antibody also did not recognize any proteins in crude membrane fractions from rabbit skeletal muscle or brain. These latter findings are of interest given that the Na^+-H^+ exchanger in muscle and brain tissue is probably a different transporter than that found in the renal BBM and that Na^+-H^+ exchange transport in these tissues is not regulated by PKA. In addition, Na^+-H^+ exchange activity has not been demonstrated in BLM. By contrast, preliminary studies indicate that the antibody recognizes a single protein of $\sim$ 55 kD in BBM from rabbit small intestine and in BBM but not BLM from $LLCPK_1$ cells. BBM of small intestinal and $LLCPK_1$ cells demonstrate Na^+-H^+ exchange activity that is regulated by PKA in a manner analogous to that in the BBM of rabbit kidney proximal tubule cells. The antibody identified a protein of the same apparent molecular mass as in BBM in a detergent-solubilized fraction of BBM proteins eluting from an anion exchange column between 0.05 and 0.1 M NaCl. This is the fraction of solubilized BBM proteins that restored the inhibitory effect of PKA on Na^+-H^+ exchange activity in trypsinized BBM proteins and the fraction from which the regulatory protein was initially isolated (7). A minor band at a somewhat smaller molecular size was also observed. Recognition of both the major and minor bands in this protein fraction was prevented by preabsorption of the antibody with its peptide antigen. The antibody did not recognize proteins in an anionic fraction of BBM proteins (fraction B) that contains Na^+-H^+ exchange activity not regulated by PKA (8). Thus, to a first approximation, the antibody appears to detect a polypeptide in renal BBM and detergent-solubilized BBM fractions that correlates with the activity of the putative regulatory cofactor determined from functional studies. The protein identified by the antibody has an apparent molecular mass higher than that estimated earlier by PKA-mediated phosphorylation of BBM protein fractions. Whether proteolysis accounts for the generation of the minor immunoreactive band of slightly smaller molecular size and the 42-kD phosphoprotein isolated for protein sequencing has not been established at the present time.

Functional studies were undertaken to establish a physiologic role for the polypeptide recognized by the antibody. The present studies confirm our prior observations that the activity of the Na^+-H^+ exchanger in fraction B is not regulated by PKA (8). In addition, as noted above, the affinity-purified polyclonal antibody did not react with any proteins in fraction B. When fraction B was coreconstituted with the immunoprecipitated polypeptide recognized by the antibody, the inhibitory effect of PKA on the activity of the Na^+-H^+ exchange was restored. In the absence of ATP or PKA, the immunoreactive regulatory protein did not affect the rate of Na^+-H^+ exchange transport significantly. To extend these observations, fresh solubilized BBM proteins were depleted of the putative regulatory cofactor by immunoabsorption. In contrast with control samples, solubilized BBM proteins reacted with the antibody failed to demonstrate inhibition of Na^+-H^+ exchange activity in response to ATP, Mg^{2+}, and PKA. Western immunoblot analyses confirmed that the antibody removed or decreased the amount of the putative regulatory protein from the solubilized protein sample. Taken together, these findings provide evidence for a critical role of a specific BBM protein in mediating the regulation of the Na^+-H^+ exchange transporter by PKA under the specific conditions of the experiments.

It is important to emphasize that the conclusion that PKA-mediated inhibition of the Na^+-H^+ exchange requires a cofactor distinct from the transporter is based solely on in vitro studies using solubilized BBM proteins from the kidney of the rabbit. Prior studies using phosphopeptide and radioiodinated peptide maps have indicated that native rabbit renal BBM contains a PKA substrate protein that appears to be identical to the putative regulatory cofactor isolated by chromatographic methods (19). Nonetheless, the physiologic significance of this polypeptide in the intact cell remains to be established. One type of proof would be the demonstration of a relation between phosphorylation of the regulatory cofactor and the activity of the exchanger in intact cells or native membranes. In preliminary experiments, native rabbit renal BBM were phosphorylated maximally using γ-[^{32}P]ATP, Mg^{+2}, and purified PKA in the presence of protein phosphatase inhibitors. The putative regulatory cofactor was then immunoprecipitated after solubilization of the membrane proteins with detergent. By protein staining, a single protein of 55 kD was precipitated but radioactivity was barely detectable on autoradiography even after 8 d of exposure. Prior studies have indicated that native BBM contains both active protein kinases and protein phosphatases. These studies indicated also that dephosphorylation occurred rapidly (2–10 min) and was difficult to inhibit (8). It would be postulated that the failure to detect radioactivity in the immunoprecipitated sample may relate to dephosphorylation of the

target protein due to the time required for processing. By contrast, solubilized membrane preparations do not contain active protein phosphatases (8). Thus, phosphorylation of total solubilized proteins or the immunoprecipitated protein (as done in the present studies) permits maximal phosphorylation with little or no dephosphorylation. It is also possible that more potential phosphorylation sites are available in the solubilized form of the protein compared with when the protein is membrane bound. Whatever the correct explanation, these preliminary experiments indicate that definition of the biologic effect of the 55-kD protein will require a different experimental approach. In vivo studies of the hormonal control of the putative regulatory protein may require identification of an established kidney proximal tubule cell line that not only expresses significant levels of the protein but also demonstrates cAMP-mediated inhibition of the Na^+-H^+ exchange activity.

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