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Rabbit ileal villus cell brush border Na^+/H^+ exchange is regulated by Ca^{2+}/calmodulin-dependent protein kinase II, a brush border membrane protein

(Na absorption/phosphorylation/plasma membrane vesicles/intestinal transport)

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ABSTRACT **Ileal brush border membranes contain an endogenous Ca^{2+}/calmodulin (CaM)-dependent protein kinase activity that modulates the activity of the apical membrane Na^+/H^+ exchanger. To further characterize this kinase, synapsin I, a substrate for Ca^{2+}/CaM-dependent protein kinases, was added to preparations of ileal brush border membranes. In the presence of Ca^{2+}/CaM, synapsin I was phosphorylated. Phosphopeptide mapping demonstrated that the addition of Ca^{2+}/CaM to brush border membranes stimulated the phosphorylation of sites in synapsin I specific for Ca^{2+}/CaM-dependent protein kinase II. Immunoblots containing brush border and microvillus membrane proteins were probed with an antibody that recognizes the 50-kDa subunit of rat brain Ca^{2+}/CaM-dependent protein kinase II. This antibody labeled major and minor species of 50 and 53 kDa, respectively, with more labeling of the brush border than the microvillus membranes. Right-side-out ileal villus cell brush border vesicles were prepared containing CaM, ATP, and 350 nM free Ca^{2+}. Na^+/H^+ exchange was inhibited by the presence of Ca^{2+}/CaM/ATP within the vesicles. A 21-amino acid peptide inhibitor of CaM kinase II was enclosed within some vesicle preparations by freeze–thaw. The effect on Na^+/H^+ exchange of Ca^{2+}/CaM/ATP was partially reversed by the inhibitor peptide. These studies demonstrate the presence of Ca^{2+}/CaM-dependent protein kinase II in rabbit ileal villus cell brush border membranes. Based on the effect of a specific inhibitor peptide of Ca^{2+}/CaM kinase II, it is concluded that this kinase inhibits brush border Na^+/H^+ exchange, which participates in the regulation of ileal Na^+ absorption.**

In the rabbit ileum, drugs and hormones that lower cellular free calcium ($[Ca^{2+}]_i$) stimulate Na^+ absorption, while those that increase $[Ca^{2+}]_i$ inhibit Na absorption (1, 2). The transport process regulated by Ca^{2+} is neutral NaCl absorption, which is believed to be made up of linked Na^+/H^+ and Cl^-/HCO_3^- exchange proteins (3–5). Previous studies with intact ileal mucosa suggested that the effects of $[Ca^{2+}]_i$ on basal Na^+ absorption were mediated by the Ca^{2+} binding protein calmodulin (CaM) (6). These studies were largely based on use of Ca^{2+}/CaM antagonists, which have multiple actions. Furthermore, enclosure of Ca^{2+}, CaM, and ATP into right-side-out brush border vesicles from ileal villus cells blocks Na^+/H^+ exchange (7, 8). In addition, an endogenous CaM kinase is present in ileal villus cell brush border membranes, which is active at basal levels of $[Ca^{2+}]_i$ (9). Thus, basal $[Ca^{2+}]_i$ activates an apical membrane CaM-dependent kinase, which inhibits the ileal villus cell brush border Na^+/H^+ exchanger and the NaCl absorptive process.

The identity of the brush border CaM kinase is not known. Two classes of CaM-dependent protein kinases have been described: those with narrow substrate specificity such as myosin light-chain kinase, CaM kinase I, and Ca^{2+}/CaM-dependent kinase III (elongation factor II kinase); and one class of kinases with broad substrate specificity. The latter is the multifunctional CaM kinase or Ca^{2+}/CaM kinase II family (10–13). CaM kinase II is a macromolecular complex composed of at least two major subunits of ≈50 and ≈60 kDa in various proportions.

In the present work, we show that brush border membranes from rabbit ileal villus cells contain a form of the multifunctional Ca^{2+}/CaM kinase and that this kinase is involved in modulating the effects of Ca^{2+} on basal ileal Na^+ absorption.

MATERIALS AND METHODS

Preparation of Rabbit Ileal Villus Cell Brush Border Membranes and Microvillus Membranes. Brush border membranes, consisting of membrane sheets, were prepared by Mg^{2+} precipitation starting with light scrapings from rabbit ileal mucosa as described (9). The mucosa was homogenized in 10 vol of 5 mM Hepes/5 mM EDTA, pH 7.5, containing 50 μg of phenylmethylsulfonyl fluoride per ml with a Polytron homogenizer (10 10-sec bursts with 20-sec cooling intervals). The homogenate was centrifuged at 1000 × *g* for 10 min and the supernatant was discarded. The pellet was washed three times with 5 mM Hepes/5 mM EDTA by centrifugation at 1000 × *g* for 10 min in a Sorvall SS-34 rotor. The pellet was resuspended in 5 mM Hepes/250 mM sorbitol and centrifuged at 25,000 × *g* in a Sorvall SS-34 rotor for 20 min. The pellet was homogenized at top speed for 1 min with a motor-driven Teflon glass homogenizer. $MgSO_4$ was added to a final concentration of 10 mM and the suspension was allowed to stand for 10 min. After centrifugation at 5000 × *g* for 10 min, the pellet was discarded, and the supernatant was centrifuged at 25,000 × *g* for 20 min. The final pellet was resuspended in 5 mM Hepes/250 mM sorbitol, pH 7.5, stored at −80°C and studied <1 week after preparation.

Brush border membranes, consisting predominantly of closed vesicles, were made as described (7, 8) starting with

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Abbreviations: $[Ca^{2+}]_i$, cellular free calcium concentration; CaM, calmodulin.

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light scrapings of ileal mucosa as in the above membrane preparation. Homogenization was at high speed in a Waring Blendor for 3 min in a solution containing 60 mM mannitol, 2.4 mM Tris·HCl (pH 7.1), 1 mM EGTA, along with the protease inhibitors phenylmethylsulfonyl fluoride (0.32 mM), 0.003 mM *N*-(α-rhamnopyranosyloxyhydroxyphosphinyl)-L-leucyl-L-tryptophan (phosphorhamidon), and 0.003 TIU of aprotinin per ml. All steps in the membrane preparation were performed at 4°C except where noted. The homogenate was treated with 10 mM $MgCl_2$ for 15 min and centrifuged at 12,000 × *g* for 15 min. After the pellet was discarded, the supernatant was centrifuged at 27,000 × *g* for 30 min. The pellet was resuspended in 60 mM mannitol, 5 mM EGTA, 10 mM Tris·HCl (pH 7.1). The 10 mM $MgCl_2$ precipitation steps were repeated, except this time at 25°C, and the membranes were resuspended in 300 mM mannitol, 5 mM D-gluconate (hemimagnesium salt), 20 mM Hepes/Tris, pH 7.5 and centrifuged at 30,000 × *g* for 40 min. The final pellet was resuspended in the same buffer by using a 25-gauge needle, stored in liquid nitrogen, and generally was studied <1 week after preparation.

Microvillus membranes, depleted of cytoskeletal core, were prepared following the thiocyanate method of Hopfer *et al.* (14), starting with light scrapings from ileal mucosa and using 0.41 M NaSCN. The final pellet was suspended in 5 mM Hepes containing 250 mM sorbitol (pH 7.5), stored at −80°C, and used within 1 week of preparation.

These three membrane preparations were compared by determining the final membrane enrichment in sucrase specific activity (15) compared to the homogenate specific activity. Brush border membrane sheets were purified 21-fold, brush border membrane vesicles were purified 15-fold, and microvillus membranes were purified 94-fold. The Mg precipitation method used to prepare brush border sheets and vesicles removes much, but not all, of the brush border cytoskeletal core. Thiocyanate, used to prepare the microvillus membrane, much more completely removes the cytoskeleton.

Phosphorylation of Synapsin I and Peptide Mapping. Brush border membrane sheets and synapsin I were incubated in a 100-μl reaction mixture for 120 sec at 4°C containing 5 μM [γ-^{32}P]ATP, 3 mM EGTA, 5 mM $MgCl_2$, 10% (wt/vol) sucrose, and 2.88 mM Ca^{2+} [free Ca^{2+} equal to 0.8 μM, calculated by computer-based program as described (15)] and 5 μM CaM when indicated. The reaction mixture contained 50–100 μg of membrane protein and 10 μg of purified bovine whole brain synapsin. The reaction was terminated by adding 50 μl of 0.1 M EDTA, 5% (wt/vol) SDS, 200 mM dithiothreitol, and 2.5 μg of Pyronin Y followed immediately by immersion in boiling water for 2.5 min. The phosphorylated proteins were separated on reducing SDS (0.1%)/5–15% polyacrylamide gradient gels according to Laemmli (16). After electrophoresis, the gels were stained with Coomassie brilliant blue, dried, and subjected to autoradiography with Kodak XAR-5 film.

To determine sites phosphorylated in synapsin I by the Ca^{2+}/CaM-dependent protein kinase present in the brush border membrane, phosphorylated synapsin I bands were excised from the SDS gels and subjected to limited proteolysis by *Staphylococcus aureus* V8 protease (3 μg per lane), as modified (17) from Cleveland *et al.* (18). The phosphopeptides generated by this digestion were separated by SDS/PAGE and identified by autoradiography. Under these conditions, previous studies have shown that phosphorylation of a synapsin peptide of 30 kDa at sites 2 and 3 is specific for Ca^{2+}/CaM-dependent protein kinase II (19). In the peptide mapping experiments, due to the presence of a heavily phosphorylated peptide of 77 kDa in the brush border membranes, which partially masked the synapsin I, it was necessary to keep the ratio of membrane protein to synapsin I at 4 or less. Consequently, in the phosphorylation mixture used for peptide mapping, the membrane protein was ≈10 μg per 100 μl and synapsin I was ≈5 μg.

Anti Ca^{2+}/CaM Kinase II Polyclonal Antibody. Antibodies to Ca^{2+}/CaM-dependent protein kinase II were prepared as described (20). A peptide containing amino acids 281–302 of the α subunit of the rat brain enzyme was synthesized and 3.5 mg was coupled to porcine thyroglobulin (33 mg) by incubation with glutaraldehyde in phosphate-buffered saline (pH 7.5) at 4°C followed by dialysis against phosphate buffer. For immunization, 0.5 ml of the conjugate containing ≈200 μg of the peptide was mixed with an equal amount of complete Freund's adjuvant and injected intradermally into an adult New Zealand rabbit. After booster injections in incomplete Freund's adjuvant, rabbit serum was screened for specific antibodies by immunoblot analysis using rat cortex homogenate as antigen. Labeled bands were detected by incubation with a goat anti-rabbit antibody coupled to alkaline phosphatase. The antisera identified a prominent 50-kDa species as well as several other bands. For the affinity purification of antibodies, 25 mg of peptide was coupled to 5 ml (swollen volume) of activated CH-Sepharose (Pharmacia). Antiserum (40 ml) was brought to 25 mM benzamidine and applied to the column. After the column was washed with 500 mM NaCl/20 mM benzamidine, antibody was eluted with 4.0 M $MgCl_2$ (pH 6.0). The eluate was dialyzed into 200 mM NaCl/10 mM Hepes, pH 7.4, and concentrated to 1 mg/μl in Aquacide. When incubated with Western blots of rat brain cortex homogenates, the affinity-purified antibody intensely labeled a 50-kDa band corresponding to the α subunit of the Ca^{2+}/CaM-dependent protein kinase II. Lighter labeling of a 60-kDa band corresponding to the β subunit of the kinase was sometimes observed. Incubation of this antibody with the peptide antigen eliminated labeling of the 50-kDa species detected by immunoblot.

Recognition of Brush Border Substrates by Ca^{2+}/CaM Kinase II Antibody. Ileal brush border membrane vesicles (100 μg per lane), microvillus membranes made by the thiocyanate technique (100 μg per lane), partially purified Ca^{2+}/CaM kinase II from rat brain cortex (0.1 μg per lane), and rat brain cortex homogenate (8 μg per lane) were solubilized in sample buffer (0.03 M EDTA/1.7% SDS/67 mM dithiothreitol/2.5 μg of pyronin Y) and subjected to SDS/polyacrylamide gel electrophoresis with an acrylamide concentration of 7.5%. Ca^{2+}/CaM-dependent protein kinase II was purified from the soluble fraction of rat cerebral cortex by sequential ion-exchange (DEAE-cellulose), gel permeation (Sephacryl S-400), and affinity (CaM-Affigel) chromatography as described (12). After SDS/PAGE, one sample of each condition and the molecular mass standards (29–205 kDa, Sigma, no. MW-SDS-200) were stained with Coomassie brilliant blue. The other sample was transferred electrophoretically to nitrocellulose membranes as described (21), soaked overnight in 50 mM Tris·HCl, pH 7.4/150 mM NaCl/0.05% (wt/vol) NaN_3/0.5% (wt/vol) Tween 20 (IR buffer), and then incubated for 2.5 hr at 20°C with 15 μl per 10 ml of rabbit preimmune serum or affinity-purified antibody to Ca^{2+}/CaM kinase II. The blots were washed three times in IR buffer plus 0.5% sodium deoxycholate and 0.5% Triton X-100 and further incubated 1 hr with 40 μl of goat anti-rabbit IgG coupled to alkaline phosphatase (Sigma no. A-8065) in 10 ml of IR buffer. The antibody–antigen complexes were visualized by incubation in 20 ml of 50 mM Na_2CO_3, pH 9.5/4 mM $MgCl_2$ plus 2 mg nitroblue tetrazolium and 200 μl of 5-bromo-4-chloroindolyl-phosphate (5 mg/ml) in *N*,*N*-dimethylformamide.

Ca^{2+}/CaM Kinase II Peptide Inhibitor. The inhibitor used was a peptide with the amino acid sequence of residues 281–302 of the α subunit of rat brain Ca^{2+}/CaM kinase II, with alanine substituted for threonine at position 286 (21). This peptide inhibits the catalytic activity of Ca^{2+}/CaM

kinase II with a K_i of ≈3 μM when assayed with synapsin I as substrate and it has been previously used to inhibit Ca^{2+}/CaM kinase II activity in intact nerve terminals (21).

Transport Studies: Na^+/H^+ Exchange in Villus Cell Brush Border Membrane Vesicles. Constituents necessary for regulation of Ca^{2+}/CaM-dependent protein kinase activity were incorporated into the brush border vesicles by freezing and thawing the vesicles as described (7, 8). Frozen aliquots of ileal brush borders were thawed and then homogenized with a motor-driven Teflon glass homogenizer at high speed in a membrane buffer consisting of 200 mM mannitol, 5 mM $Mg(gluconate)_2$, 2 mM EGTA, 40 mM Mopso/Tris, pH 6.5, at 4°C. The vesicles were resuspended in the membrane buffer, centrifuged at 120,000 × *g* for 60 min at 4°C, and resuspended in the same buffer at a concentration of 10 mg/ml by homogenization through a 25-gauge needle. Then 350-μl aliquots of the membranes along with indicated additions (total volume, 36 μl) were rapidly frozen in liquid nitrogen for 5 min and thawed slowly in an ice water slurry at 4°C for 1 hr. Final concentrations after additions were (*i*) none; (*ii*) 350 nM free Ca^{2+}, 5 μM CaM, 50 μM ATP, 5 mM creatine phosphate, and 28 units of creatine kinase per ml; (*iii*) 158 μM peptide inhibitor; and (*iv*) same as in *ii* plus 158 μm peptide inhibitor. All additions except creatine phosphate were made immediately before freezing the vesicles. Creatine phosphate was added immediately after the aliquot was removed from the ice water slurry. After thawing, the vesicles were kept at 30°C for 2 min and then at 4°C for an additional 15 min. Immediately thereafter, $^{22}Na^+$ uptake studies were performed. $^{22}Na^+$ uptake into vesicles at pH 6.5 was performed with external pH 8.0 (a) or 6.5 (b), with Na^+/H^+ exchange defined as the difference in Na^+ uptake in a minus b, as described for these vesicles (7, 8). The Na^+ transport buffer used in the acid inside pH gradient-stimulated Na^+ uptake studies contained (final concentration) 180 mM mannitol, 1.8 mM EGTA, 4.5 mM $Mg(gluconate)_2$, 1.0 mM NaCl (0.02 μCi of $^{22}NaCl$ per μl; 1 Ci = 37 GBq), 36 mM Tris/Mes such that the final combined pH of the transport and membrane buffers was 8.0. The stop solution contained 40 mM mannitol, 90 mM potassium gluconate, 20 mM Tris/Mes at pH 8.0. The Na^+ transport buffer used in experiments not involving a pH gradient (external pH 6.5) contained (final concentration) 180 mM mannitol, 1.8 mM EGTA, 4.5 mM $Mg(gluconate)_2$, 1.0 mM NaCl (0.04 μCi of $^{22}NaCl$ per ml), 36 mM Mopso/Tris such that the combined pH remained 6.5. The stop solution contained 40 mM mannitol, 90 mM potassium gluconate, 20 mM Mopso/Tris, pH 6.5.

Studies of the initial rates of $^{22}Na^+$ uptake were performed by mixing 15 μl of the membrane with 30 μl of transport buffer at 25°C. Quadruplicate samples were studied. At 3, 5, or 8 sec after mixing, the uptake was stopped by electronically timed injection of 1 ml of ice-cold stop solution. The reaction mixture was rapidly vacuum filtered through nitrocellulose filters (pore size, 0.45 μm) (Millipore) and rinsed with 6 ml of ice-cold stop solution. The filters were placed in 3 ml of scintillant (Liquiscint, National Diagnostics, Sommerville, NJ) and radioactivity was measured. Protein was assayed by the method of Bradford (3) (Bio-Rad). Initial rates of uptake were expressed in pmol per mg of protein per sec computed by linear regression analyses of data obtained in individual experiments, with means of quadruplicate samples for each time point used for calculation. In preliminary studies, it was established that all uptake studies were performed during periods of linear uptake.

RESULTS

Antibody to Brain Ca^{2+}/CaM Kinase II Recognizes Peptides in Villus Cell Brush Border Membranes. Brush border membrane sheets, microvillus membrane, rat brain cortex homogenate, and purified rat brain Ca^{2+}/CaM kinase II were separated on SDS/7.5% polyacrylamide gels and transferred to nitrocellulose membranes. These membranes were incubated with a polyclonal antibody to rat brain CaM kinase II. Labeling of a prominent 50-kDa and a lighter 53-kDa peptide was detected in the brush border membranes. The 50-kDa peptide had the same electrophoretic mobility as a species detected in a preparation of purified brain kinase and in rat brain cortex homogenate (Fig. 1). No labeling was seen in the presence of rabbit pre-immune serum (data not shown). When similar amounts of brush border membrane sheets and microvillus membranes, depleted of cytoskeletal proteins, were studied by Western blotting, both membrane preparations showed similar bands, although a much more intense signal was seen with the brush border membrane. Autoradiography of brush border membranes studied by *in vitro* phosphorylation in the presence and absence of Ca^{2+}/CaM as described (9) indicated that both the 50- and the 53-kDa species detected by immunoblot coincided with prominent phosphorylated brush border peptides, the phosphorylation of which was increased by Ca^{2+}/CaM (data not shown).

Ca^{2+}/CaM-Induced Phosphorylation of Synapsin I by Brush Border Membranes. The Ca^{2+}/CaM kinase in brush border membranes phosphorylated the exogenous protein kinase substrate synapsin I (Fig. 2). When brush border membranes and synapsin I were incubated in the presence of [γ-^{32}P]ATP, in the presence and in absence of exogenous Ca^{2+}/CaM, a phosphorylated doublet of 77/82-kDa was observed (compare lanes 3 and 4), which correspond to the synapsin I doublet shown by Coomassie brilliant blue staining of purified rat brain synapsin (compare lane 6, which is synapsin I plus brush border membrane, with lane 5, which is brush border membrane without added synapsin I). Phosphorylation of synapsin I was dependent on the presence of Ca^{2+} and CaM.

When the upper band of the phosphorylated synapsin doublet was cut from the gel and peptide mapping was carried out, a 30-kDa peptide fragment was found to be phosphorylated in a Ca^{2+}/CaM-dependent manner (Fig. 2*B*, lanes A and B). This 30-kDa peptide fragment contains sites 2 and 3 in synapsin I (22). In addition, an endogenous, highly phosphorylated 77-kDa phosphoprotein in the brush border preparation migrates very close to synapsin on the polyacryla-

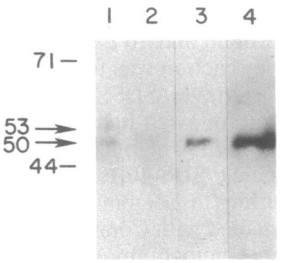

FIG. 1. Ca^{2+}/CaM kinase II antibody recognition of 50- and 53-kDa brush border membrane peptides. Ileal villus cell brush border membrane sheets (lane 1), microvillus membranes made by the Hopfer/thiocyanate technique (14) (lane 2), Ca^{2+}/CaM protein kinase II from rat brain (lane 3), and rat brain cortex homogenate (lane 4) were separated on SDS/7.5% polyacrylamide gels and transferred to nitrocellulose membranes, and the Western blots were incubated with the polyclonal antibody to rat brain Ca^{2+}/CaM kinase II. The polyclonal antibody labeled a peptide of 50 kDa in purified Ca^{2+}/CaM kinase II (lane 3) and in rat brain cortex homogenate (lane 4). In brush border membranes and microvillus membranes, this antibody labeled peptides of 50 and 53 kDa (lanes 1 and 2). While the same amount of microvillus membranes and brush border sheets were studied, more intense staining was seen in the brush border. Positions of molecular mass standards are on the left and arrows indicate the 53- and 50-kDa peptides, with purified CaM kinase II and rat brain cortex homogenate further identifying a peptide of 50 kDa.

mide gel. To be certain that phosphopeptides from this protein were not contaminating the synapsin peptides, this protein was also subjected to peptide mapping. The phosphopeptides contained in the 77-kDa species migrated differently from those obtained from exogenous synapsin I (lane C).

The Effects of Ca^{2+}/CaM Kinase II Inhibitor Peptide on Ca^{2+}/CaM/ATP-Induced Inhibition of Na^+/H^+ Exchange in Ileal Brush Border Vesicles. Brush border vesicles exhibit Na^+/H^+ exchange, defined as the difference between $^{22}Na^+$ uptake during the linear phase of Na^+ influx in the presence and absence of an acid inside pH gradient. As demonstrated previously (7, 8) and shown in Fig. 3, the presence of Ca^{2+}/CaM/ATP with an ATP regenerating system inhibits Na^+/H^+ exchange by ≈50%. To study the role of the Ca^{2+}/CaM-dependent protein kinase II in this effect, a 21-amino acid peptide inhibitor containing a modified Ca^{2+}/CaM kinase II autoregulatory domain was incorporated into the brush border vesicles by freezing and thawing. The inhibitor peptide (158 μM) did not alter Na^+/H^+ exchange in the absence of Ca^{2+}/CaM/ATP (Fig. 3). However, when the inhibitor peptide was frozen and thawed with the vesicles, the effect of Ca^{2+}/CaM/ATP to inhibit Na^+/H^+ exchange was significantly decreased, and in the presence of the peptide inhibitor, Ca^{2+}/CaM/ATP no longer significantly decreased Na^+/H^+ exchange. When expressed as percent inhibition of basal Na^+/H^+ exchange, Ca^{2+}/CaM/ATP caused a 46% ± 5% inhibition in the absence of the peptide inhibitor and 25% ± 9% inhibition in the presence of peptide inhibitor ($n = 3$; $P < 0.02$, comparing the extent of Ca^{2+}/CaM/ATP inhibition of Na^+/H^+ exchange in the absence and presence of the peptide inhibitor).

The effect of Ca^{2+}/CaM/ATP, the peptide inhibitor, and the combination of these was determined on Na^+-dependent D-glucose uptake at 90 sec and on equilibrium volumes at 75 min, using described techniques (8). There was no significant effect on glucose "overshoots" or equilibrium volume of Ca^{2+}/CaM/ATP alone, of the inhibitor peptide, or the combination (data not shown).

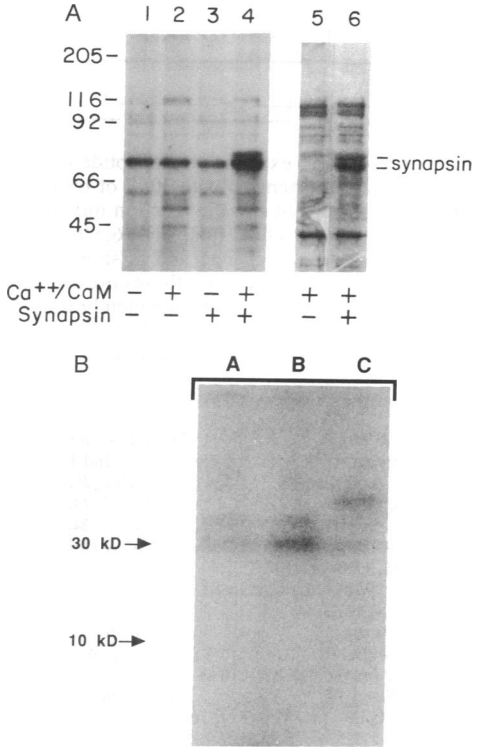

FIG. 2. (*A*) SDS/5–15% polyacrylamide gel of ileal villus cell brush border membranes showing Ca^{2+} and CaM-dependent phosphorylation of synapsin I by brush border membrane protein kinase. Lanes 1–4, autoradiograms; lanes 5 and 6, Coomassie brilliant blue-stained gels used for lanes 2 and 4, respectively. Each lane contains 80 μg of brush border membrane protein that was phosphorylated for 120 sec at 4°C. Proteins in lanes 2 and 4 were phosphorylated in the presence of 0.8 μM free Ca^{2+} and 5 μM exogenous CaM. Lanes 3 and 4 each contain 10 μg of synapsin. Bars indicate the position of the synapsin identified by Coomassie brilliant blue staining of purified rat brain synapsin I. Results shown were typical of experiments repeated three times with separate brush border membrane preparations. (*B*) Phosphorylation of synapsin I sites 2 and 3 (specific for Ca^{2+}/CaM kinase II) by intestinal brush border preparations. Labeled synapsin I bands (lanes A and B) or the 77-kDa band phosphorylated in the absence of synapsin I (lane C; equivalent to lane 2 in *A*) were excised from dried gels after autoradiography. The labeling of synapsin I in lane A was in the absence of, and that in lane B was in the presence of, Ca^{2+}/CaM. These excised bands were digested by *S. aureus* V8 protease and the products were separated on SDS/15% polyacrylamide gel, according to the method of Cleveland *et al.* (18). Labeled bands were detected by autoradiography. Under these conditions, synapsin I sites 2 and 3 are contained in a peptide of 30 kDa, while site 1 is contained in a 10-kDa species. Labeling of synapsin I sites 2 and 3 in the presence of Ca^{2+}/CaM is demonstrated in lanes A and B. Results shown are typical of experiments repeated twice with separate brush border membrane preparations.

DISCUSSION

Previous studies from this laboratory have suggested that a CaM-dependent protein kinase is involved in the regulation of Na^+ absorption by ileal villus absorptive cells (6–8). This has been observed in intact tissue by using the CaM inhibitor, W_{13}, which increases ileal active NaCl absorption, and in isolated brush border vesicles by the addition of CaM and Ca^{2+} (7, 8). An endogenous CaM-dependent protein kinase has been described in ileal brush border membranes (9).

However, a number of CaM-dependent protein kinases have been described and the identity of the rabbit ileal brush border kinase was unknown. CaM kinase II is a multifunctional protein kinase found in multiple tissues. A macromolecular Ca^{2+}/CaM kinase activity has been previously characterized in chicken intestinal membranes and is composed of a 50-kDa subunit (23). This enzyme has some of the properties of a CaM kinase II (e.g., size and autophosphorylation of a 50-kDa subunit). A number of isozymes of the CaM kinase II exist with variable ratios of the subunits among tissues. For example, the enzyme derived from some parts of the brain contains a minor 52-kDa subunit, that isolated from the liver contains a doublet of 52 and 54 kDa, while the enzyme from pancreas contains a single 52-kDa subunit (24). The physiologic implications of these various subunit compositions are unknown, since all forms of the enzymes have kinase activity.

Three findings suggest that the CaM kinase in the rabbit ileal villus cell brush border membrane is CaM kinase II. First, this activity phosphorylates synapsin I, a CaM kinase II substrate, at the same sites as does purified brain CaM kinase II. Second, a polyclonal antibody to purified brain CaM kinase II cross-reacts with brush border peptides of similar electrophoretic mobility to brain Ca^{2+}/CaM kinase II. Finally, the effect of Ca^{2+}/CaM/ATP on $^{22}Na^+$ uptake by brush border vesicles is reduced by a peptide inhibitor of CaM kinase II, which contains the autoregulatory domain of the kinase (21). In spite of a K_i of ≈3 μM for this peptide as a Ca^{2+}/CaM kinase II inhibitor in broken cell studies, higher concentrations have been used to study the role of CaM kinase II, including 3 mM in rat brain synaptosomes (21), and a 1 mM concentration of a similar inhibitor peptide was used in rat central nervous system pyramidal cells (25). Of note is that studies demonstrating a low K_i were done with purified

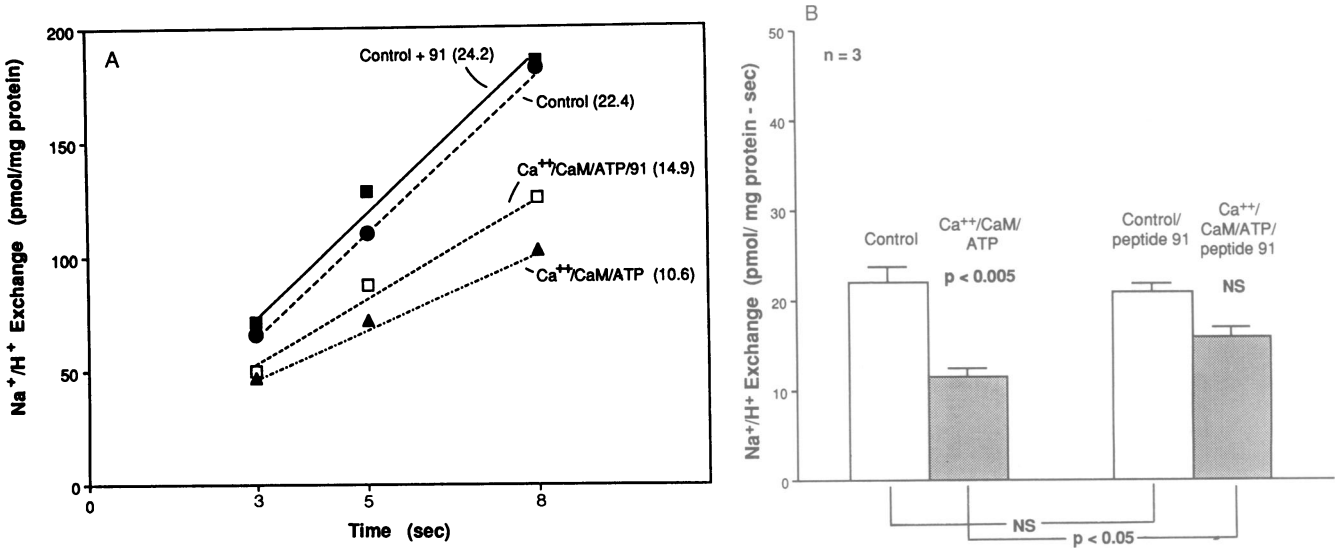

FIG. 3. Reversal of Ca^{2+}/CaM/ATP inhibition of ileal villus cell brush border membrane Na^+/H^+ exchange by a peptide inhibitor of CaM kinase II (peptide 91). $^{22}Na^+$ initial uptake studies were performed by mixing 15 μl of membrane suspension with 30 μl of transport buffer at 25°C. At 3, 5, and 8 sec after mixing the membrane and transport buffers, the Na^+ uptake was stopped and the reaction mixture was rapidly filtered through nitrocellulose filters. Na^+ uptake studies were performed (a) in the absence of a pH gradient (pH_i/pH_o, 6.5/6.5) and (b) in the presence of an acid inside pH gradient (pH_i/pH_o, 6.5/8.0). The difference a minus b is Na^+/H^+ exchange and is shown. (*A*) This is a representative single experiment, which was repeated on three separate membrane preparations. Values in parentheses are the slopes, which represent Na^+/H^+ exchange rates in pmol per mg of protein per sec. (*B*) Mean ± SE of three experiments on separate membrane preparations. *P* values above the bars represent comparison of the Ca^{2+}/CaM/ATP effect compared to controls without Ca^{2+}/CaM/ATP. *P* values below the bars represent comparison of the effect of peptide inhibitor on basal Na^+/H^+ exchange or on Na^+/H^+ exchange in the presence of Ca^{2+}/CaM/ATP (paired *t* test). NS, not significant.

CaM kinase II in broken cell studies in which access to the transport protein regulated was not an issue. While this peptide fails to inhibit cAMP-dependent protein kinase, protein kinase C, and CaM kinase III over the same range that it inhibits CaM kinase II, it does inhibit CaM kinase I. Thus, even though CaM kinase I is not present in ileal brush borders (based on the peptide map of synapsin I obtained in the presence of ileal brush border), we cannot be certain that there are not other brush border CaM kinases that are also inhibited by this peptide. In some respects, the brush border CaM kinase is different from the other CaM kinase isozymes described. It lacks a 58/60-kDa subunit and contains a minor 53-kDa subunit. Activation of this kinase in ileum inhibits brush border Na^+/H^+ exchange, whereas, in most tissues, phosphorylation of the Na^+/H^+ exchanger stimulates Na^+ absorption (26). Further study will be needed to determine whether this difference in regulation of Na^+/H^+ exchange is due to specific features of the intestinal kinase or the intestinal brush border Na^+/H^+ exchanger.

Whether CaM kinase II is present in the ileal villus cell microvillus membrane or in the brush border cytoskeletal core has not been totally resolved by these studies. Even though immunoblot analysis of equal amounts of brush border and microvillus membranes demonstrated much more labeling of lanes containing brush border membranes, supporting a cytoskeletal distribution, there are alternative explanations. For instance, the presence of some labeling in the cytoskeleton depleted microvillus membrane could mean that the CaM kinase II is found in both microvillus membrane and cytoskeletal core; and the high concentration of thiocyanate used may have removed attached microvillus membrane kinase. Immunocytochemical approaches are needed to determine whether CaM kinase II is located solely or just preferentially in the brush border cytoskeletal core.

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