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Role of Calcium and Calmodulin in the Regulation of the Rabbit Ileal Brush-Border Membrane Na^+/H^+ Antiporter

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Summary. In rabbit ileum, Ca^{2+}/calmodulin (CaM) appears to be involved in physiologically inhibiting the linked NaCl absorptive process, since inhibitors of Ca^{2+}/CaM stimulate linked Na^+ and Cl^- absorption. The role of Ca^{2+}/CaM-dependent phosphorylation in regulation of the brush-border Na^+/H^+ antiporter, which is believed to be part of the neutral linked NaCl absorptive process, was studied using purified brush-border membrane vesicles, which contain both the Na^+/H^+ antiporter and Ca^{2+}/CaM-dependent protein kinase(s) and its phosphoprotein substrates. Rabbit ileal villus cell brush-border membrane vesicles were prepared by Mg precipitation and depleted of ATP. Using a freeze-thaw technique, the ATP-depleted vesicles were loaded with Ca^{2+}, CaM, ATP and an ATP-regenerating system consisting of creatine kinase and creatine phosphate. The combination of Ca^{2+}/CaM and ATP inhibited Na^+/H^+ exchange by 45 ± 13%. This effect was specific since Ca^{2+}/CaM and ATP did not alter diffusive Na^+ uptake, Na^+-dependent glucose entry, or Na^+ or glucose equilibrium volumes. The inhibition of the Na^+/H^+ exchanger by Ca^{2+}/CaM/ATP was due to an effect on the V_{max} and not on the K_m for Na^+. In the presence of CaM and ATP, Ca^{2+} caused a concentration-dependent inhibition of Na^+ uptake, with an effect 50% of maximum occurring at 120 nM. This Ca^{2+} concentration dependence was similar to the Ca^{2+} concentration dependence of Ca^{2+}/CaM-dependent phosphorylation of specific proteins in the vesicles. The Ca^{2+}/CaM/ATP-inhibition of Na^+/H^+ exchange was reversed by W_{13}, a Ca^{2+}/CaM antagonist, but not by a hydrophobic control, W_{12}, or by H-7, a protein kinase C antagonist. We conclude that Ca^{2+}, acting through CaM, regulates ileal brush-border Na^+/H^+ exchange, and that this may be involved in the regulation of neutral linked NaCl absorption.

Key Words calcium · calmodulin · absorption · ileum · brush-border vesicle · phosphorylation

Introduction

Sodium absorption in rabbit ileal villus epithelial cells normally occurs through both solute-coupled generally electrogenic transporters and an electrically neutral Cl^--dependent process. This Cl^--dependent process is thought to consist of two coupled neutral exchangers, Na^+/H^+ and Cl^-/HCO_3^- [16, 17, 20, 21]. In addition, ileal brush-border membrane vesicles contain endogenous calmodulin (CaM), Ca^{2+}/CaM-dependent protein kinase(s) and its substrates [4].

In rabbit ileal microvillus membrane vesicles, the phenothiazine promethazine inhibits the Ca^{2+}/CaM-dependent phosphorylation of specific membrane proteins in a concentration-dependent manner, and stimulates NaCl absorption in intact ileum with similar concentration dependence [4]. We have recently shown, using rabbit ileal brush-border membrane vesicles, that the combination of Ca^{2+}, CaM and ATP inhibits the Na^+/H^+ exchange and that a hydrolyzable form of ATP is required for inhibition [23]. Since protein phosphorylation is known to regulate at least two major classes of transport proteins, ion pumps and voltage-dependent ion channels [14, 18, 19], this effect of Ca^{2+}/CaM and ATP could represent regulation by phosphorylation of another type of transport protein, the Na^+/H^+ antiporter. It is worth noting that Fan and Powell [11] initially demonstrated that Ca^{2+}/CaM inhibited Na^+ uptake in ileal brush-border membrane vesicles, but failed to demonstrate that the effect required ATP; an effect we suspect was due to the likely high values of ATP endogenously found in some small intestinal brush-border membrane preparations [25].

In the current study, we examined the roles of Ca^{2+} and calmodulin in the regulation of the Na^+/H^+ antiporter. Specifically defined were: (*i*) the effect of Ca^{2+}/CaM/ATP on the K_m and the V_{max} of the Na^+/H^+ exchanger; (*ii*) the specificity of the Ca^{2+}/CaM/ATP effect for the Na^+/H^+ exchanger compared with other Na^+-dependent processes; (*iii*)

the Ca^{2+} concentration required to inhibit the Na^+/H^+ antiporter relative to the Ca^{2+} concentration previously established for regulation of the Ca^{2+}/CaM-dependent phosphorylation of membrane proteins in ileal brush-border membrane vesicles; and (*iv*) mediation by calmodulin-dependent kinase or protein kinase C.

Materials and Methods

Brush-Border Membrane Preparation

Rabbit ileal brush-border vesicles were prepared by differential centrifugation and Mg precipitation as described [7]. New Zealand white male rabbits (2–3 kg) were sacrificed by i.v. injection of sodium pentobarbital. The distal one-third of the small intestine was removed, rinsed with iced 154 mM NaCl containing 0.5 mM phenylmethylsulfonylfluoride (PMSF) and 0.003 TIU/ml aprotinin, and then gently scraped to provide mostly villus cells. The collected cells were homogenized at high speed in a Waring blender for 3 min in a solution containing (in mM): 60 mannitol, 2.4 Tris(hydroxymethyl)-aminomethane HCl, pH 7.1, 1 EGTA, along with the protease inhibitors 0.32 PMSF, 0.003 N-(alpha-rhamnopyranosyloxyhydroxyphosphinyl)-L-leucine-tryptophan (phosphorhamidone) and 0.003 TIU/ml aprotinin. The homogenization and subsequent procedures 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 (in mM): 60 mannitol, 5 EGTA, 10 Tris, pH 7.1. The 10 mM $MgCl_2$ precipitation steps were repeated and the membranes resuspended in (in mM): 300 mannitol, 5 Mg(gluconate)$_2$, 20 HEPES/Tris at pH 7.4 (when two buffers are separated by (/), the second buffer is used to adjust the pH) and centrifuged at 30,000 × *g* for 40 min. The final pellet was resuspended in the same buffer using a 25-gauge needle. The membrane was stored in liquid nitrogen, and generally used less than one week after preparation.

Freeze-Thaw

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 in a solution containing the constituents. Frozen aliquots of ileal brush-border vesicles were thawed, after which the vesicles were homogenized in a glass-Teflon homogenizer at high speed in a membrane buffer consisting of (in mM): 200 mannitol, 5 Mg(gluconate)$_2$, 2 EGTA, 40 Mopso/Tris, pH 6.5, at 4°C. The vesicles were resuspended in the membrane buffer to a concentration of 10 mg/ml. 350 μl aliquots of the membranes along with 36 μl of additives then were rapidly frozen in liquid nitrogen for 5 min, and thawed slowly in an ice water slurry at 4°C for one hr. All additions except creatine phosphate were made immediately prior to 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, $^{22}Na^+$ or D-[^{3}H]glucose uptake studies were performed.

Transport Studies

^{22}Na and [^{3}H]glucose initial rate uptake studies were performed by mixing 15 μl of the membrane suspension with 30 μl of transport buffer at 25°C. Three, 5, or 8 sec after mixing the membrane and transport buffers, the uptake was stopped by electronically timed injection of 1 ml of ice-cold stop solution. Equilibrium uptake values were determined after a 90-min incubation at 25°C. The reaction mixture was rapidly vacuum filtered through nitrocellulose filters, 0.45 μm pore size (Millipore Corp., Bedford, MA) and rinsed with 6 ml of ice-cold stop solution. The filters were dissolved in 3 ml of scintillant (Liquiscint, National Diagnostics, Sommerville, NJ) and radioactivity measured in a liquid scintillation spectrometer. Protein was assayed using a Coomassie Brilliant blue based assay (Biorad Inc., Rockville Center, NY). Initial uptake rates were expressed in pmol/mg protein-sec computed by linear regression analyses of data obtained in individual experiments. In preliminary studies, it was established that all uptake studies were performed during periods of linear uptake, and that zero-time *y* intercepts were not significantly different from zero, indicating no significant binding of the transported material to the vesicles.

The Na^+ transport buffer used in the acid inside pH gradient-stimulated Na^+ uptake studies contained (in mM final concentration): 180 mannitol, 1.8 EGTA, 4.5 Mg(gluconate)$_2$, 1.0 NaCl (0.02 μCi/μl $^{22}NaCl$), 36 Tris/Mes such that the final combined pH of the transport and membrane buffers was 8.0. The stop solution contained (in mM): 40 mannitol, 90 K gluconate, 20 Tris/Mes at pH 8.0. The sodium transport buffer used in experiments not involving a pH gradient contained (in mM final concentration), 180 mannitol, 1.8 EGTA, 4.5 Mg(gluconate)$_2$, 1.0 NaCl (0.04 μCi/μl $^{22}NaCl$), 36 Mopso/Tris such that the combined pH remained 6.5. The stop solution contained (in mM): 40 mannitol, 90 K gluconate, 20 Mopso/Tris, pH 6.5.

In several experiments, the Na^+ concentration dependence of Na^+ uptake was determined both in the presence of an acid inside pH gradient (pH 6.5 in/pH 8.0 out) and in the absence of that gradient. Each of these experiments was performed both under control conditions and in the presence of Ca^{2+} (0.85 μM free Ca^{2+}), exogenous calmodulin (5 μM), creatine phosphate (10 μM), creatine kinase (26 U/ml) and ATP (5 mM). Initial rates of Na^+ uptake were determined at concentrations of Na gluconate between 2 and 24 mM. For analysis, precise Na^+ concentrations in the combinations of transport and membrane buffers were determined by atomic absorption spectrometry.

Determination of the K_m and V_{max} was by Eadie-Hostee analyses [24] of the Na^+ concentration dependence of Na^+/H^+ exchange with this exchange being defined as the difference between initial rates of Na^+ uptake in the presence and absence of an acid inside pH gradient. All Na^+ concentrations were studied in the presence and absence of Ca^{2+}/CaM and ATP on the same vesicle preparation on the same day.

The glucose uptake transport buffer contained (in mM): 20 mannitol, 2 EGTA, 5 Mg(gluconate)$_2$, 40 Mopso/Tris, pH 6.5, 90 NaCl, 0.15 D-[^{3}H]glucose (0.03 μCi/μl). The stop solution was the same as that used in the Na^+ transport experiments with no pH gradient.

Experiments were performed to determine the dependence on the free Ca^{2+} concentration of the effect of Ca^{2+}/CaM and ATP on the initial rates of Na^+ uptake in the presence of an acid inside pH gradient. This was done in experiments identical to those described above, but with free Ca^{2+} set at 70, 100, 200, 300, 850 and 1500 mM by altering the amount of total Ca(gluconate)$_2$ while keeping the EGTA, ATP and Mg concentrations fixed,

using a computer program based on the dissociation constants as described by Bartfai [1] and confirmed using a Ca^{2+} minielectrode [6]. At none of these free-Ca^{2+} concentrations was there an effect on Na^+ uptake in the absence of an acid inside pH gradient ($pH_{out} = pH_{in} = 6.5$). The concentration of Ca^{2+} causing an inhibitory effect 50% of maximum was determined by Woolf-Hanes analyses using the mean percent inhibition of Na^+ uptake at each free-Ca^{2+} concentration [24]. Each of these studies was done both in the presence and absence of Ca^{2+}, with Na^+ uptake in the absence of Ca^{2+} taken as zero inhibition.

It was also determined whether the effect of Ca^{2+}/CaM and ATP on Na^+ uptake in both the presence and absence of an acid inside pH gradient was altered by incubation, during freeze thaw, with the naphthalenesulfonamides W_{13} (45 μM) and W_{12} (45 μM), a concentration at which W_{13} was shown previously to be the more potent Ca^{2+}/CaM-dependent protein kinase inhibitor in brush-border membranes made from rabbit ileal villus cells [6, 10]. In addition, studies were performed with the isoquinolenesulfonamide H-7 (60 μM), which had previously been shown to alter ileal villus cell cytosol protein kinase C activity but not Ca^{2+}/CaM-dependent phosphorylation of ileal villus cell brush-border peptides [8].

We have previously established, in these ileal absorptive cell brush-border membrane vesicles under the freeze-thaw conditions described above [7, 23] that: (*i*) Na^+/H^+ exchange is equivalently defined as either the difference between Na^+ uptake with an acid inside pH gradient ($6.5_{in}/8.0_{out}$) and uptake with the same pH inside and outside the vesicles ($pH_{in} = pH_{out} = 6.5$), or the difference between Na^+ uptake with an acid inside pH gradient (pH_{in} 6.5/pH_{out} 8.0) with and without a 5-min incubation with 1 mM dimethylamiloride immediately prior to the transport measurement. (*ii*) Dimethylamiloride did not alter Na^+ uptake when the pH both inside and outside the vesicles was 6.5. (*iii*) Extravesicular Na^+ drives H^+ efflux from these vesicles, and this efflux is inhibitable by dimethylamiloride (the acridine orange fluorescence quenching technique was used to measure H^+ efflux) [16]. (*iv*) When the vesicles were freeze thawed as previously described, without and with 26 U/ml creatine kinase, 10 mM creatine phosphate, and 5 mM exogenous ATP, they contained 0.4 μM ATP and 47 μM ATP, respectively [23].

Unless specifically stated, all reagents were obtained from Sigma Chemical, St. Louis, MO. Radioisotopes were obtained from New England Nuclear, Boston, MA.

Results are expressed as the mean ± SEM of data from separate vesicle preparations. The data in each experiment are means of quadruplicate determinations. Statistical comparisons of uptake rates were done by determining the initial rates of Na^+ uptake in individual experiments. Statistical analyses were by Student's paired or unpaired *t* tests.

Results

Effect of Ca^{2+}/CaM/ATP on Ileal Brush-Border Na^+/H^+ Exchange

Initial rates of Na^+ uptake were determined in the presence of an acid inside pH gradient (pH_{in} 6.5/pH_{out} 8.0) [Fig. 1*A*] and in the absence of an acid inside pH gradient ($pH_{in} = pH_{out} = 6.5$) [Fig. 1*B*]. As previously established, the difference in the initial rates of Na^+ uptake of Fig. 1*A* minus 1*B* represents Na^+/H^+ exchange in these frozen and thawed vesicles [23], with initial rates of Na^+ uptake in the presence of an acid inside pH gradient exceeding that in the absence of an acid inside pH gradient by three to fivefold. Na^+ uptake with and without a pH gradient was linear for at least 10 sec for all conditions studied. In the presence of an acid inside pH gradient, Ca^{2+}/CaM/ATP decreased initial rates of Na^+ uptake by 20 ± 3%, (control 18.5 ± 1.8 *vs.* Ca^{2+}/CaM/ATP 12.3 ± 2.6 pmol/mg protein-sec, $n = 4$, $P < 0.02$) (Fig. 1*A*, right panel). In the absence of an acid inside pH gradient, Ca^{2+}/CaM/ATP did not significantly alter initial rates of Na^+ uptake [Fig. 1*B*, right panel], (control 6.1 ± 0.7 *vs.* Ca^{2+}/CaM/ATP 7.9 ± 1.9 pmol/mg protein-sec, $n = 4$, NS). As shown in Fig. 1*C*, right panel, Ca^{2+}/CaM/ATP inhibited Na^+/H^+ exchange by 45 ± 13%, (control 12.4 ± 1.8 *vs.* Ca^{2+}/CaM/ATP 6.8 ± 2.2 pmol/mg protein-sec, $n = 4$, $P < 0.05$). These data were obtained by determining mean ± SEM of initial uptake rates determined by linear regression analysis in each experiment individually. The lines in Fig. 1, right two panels, were drawn by linear regression analyses using all experimental time points, while the data for a single representative experiment are shown in Fig. 1, left two panels.

In contrast to the Ca^{2+}/CaM/ATP-inhibition of Na^+ uptake with an acid inside pH gradient and of Na^+/H^+ exchange, Ca^{2+}/CaM/ATP had no effect on Na^+ equilibrium volumes, either in the presence or absence of an acid inside pH gradient. Equilibrium concentrations were reached 90 min after vesicle exposure to Na^+ at 25°C. Na^+ equilibrium values in the presence of an acid inside pH gradient were 1.67 ± 0.25 and 1.28 ± 0.21 μl/mg protein in the absence and presence of Ca^{2+}/CaM/ATP, respectively, $n = 7$, NS. Na^+ equilibrium values in the absence of an acid inside pH gradient were 1.52 ± 0.20 and 1.55 ± 0.16 in the absence and presence of Ca^{2+}/CaM/ATP, respectively, $n = 5$, NS.

Kinetic analysis of initial rates of Na^+ entry at varying Na^+ concentrations, both in the presence and absence of an acid inside pH gradient, was performed to determine whether the Ca^{2+}/CaM/ATP effect on Na^+/H^+ exchange was achieved through inhibition of the V_{max} and/or through altering the Na^+ concentration causing an effect 50% of maximum on Na^+/H^+ exchange. As shown in Fig. 2, the maximum rate of Na^+/H^+ exchange was decreased from 141 pmol/mg protein-sec to 89 pmol/mg protein-sec in the absence and presence of Ca^{2+}/CaM/ATP, respectively. In contrast, the K_m was similar, being 5 and 4 mM in the absence and presence of Ca^{2+}/CaM/ATP, respectively, as calculated by Eadie-Hofstee linearization [Fig. 2 insert].

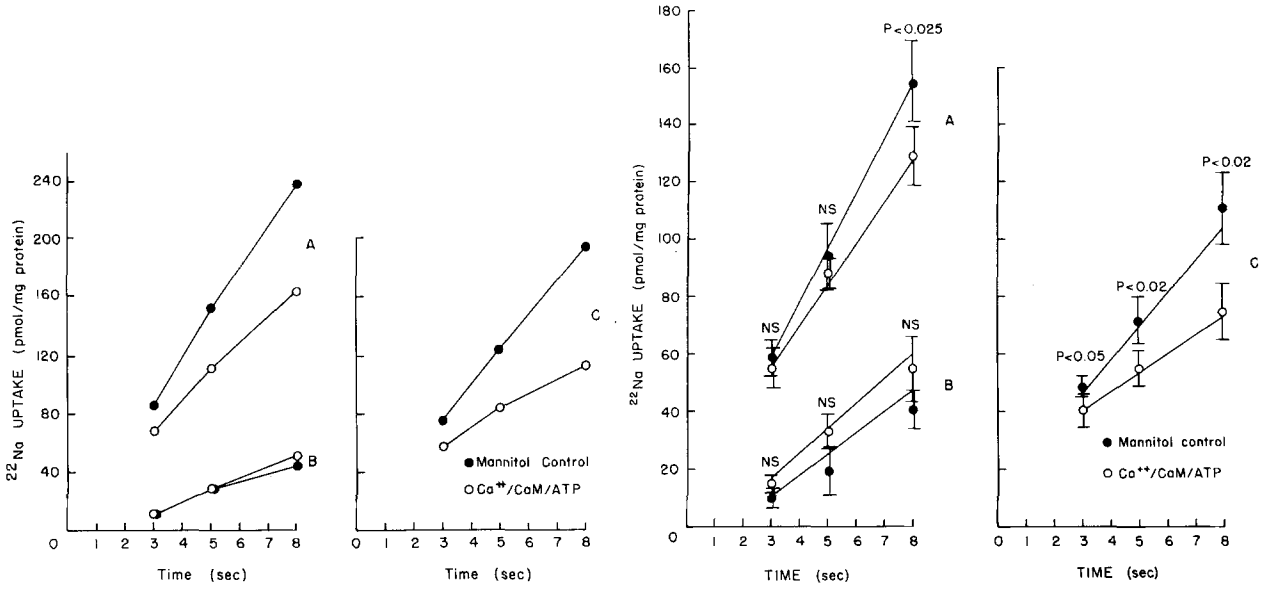

Fig. 1. Effects of Ca^{2+}/CaM/ATP on initial rates of Na^+ uptake in ileal brush-border membrane vesicles. In the left two panels, data from a single experiment are shown. In the right two panels, the drawn lines are derived from the linear regression analysis from all data points performed on four vesicle preparations using $^{22}Na^+$ uptake 3, 5 and 8 sec. Initial rates of Na^+ uptake were determined (*A*) in the presence (pH_{in} 6.5/pH_{out} 8.0) and (*B*) in the absence of an acid inside pH gradient (pH_{in} = pH_{out} = 6.5), and (*C*) Na^+/H^+ exchange determined as the difference of *A* minus *B*. 350 μl of the brush-border vesicle suspension containing 3.5 mg of protein along with 36 μl containing either 0.85 μM free Ca^{2+} [0.31 mM Ca(gluconate)$_2$], 5 μM exogenous calmodulin, 5 mM ATP, 10 mM creatine phosphate (Tris salt) and 10 U creatine kinase (0) or 45 mM mannitol (●) underwent a single cycle of freezing and 75 min of gradual thawing in an ice-water slurry followed by 2 min of incubation at 30°C. (*A*) Na^+ uptake with an acid inside pH gradient. 15 μl of membrane was mixed with 30 μl of incubation solution containing (in mM): 180 mannitol, 36 Tris/Mes, 1.8 EGTA, 4.5 Mg(gluconate)$_2$, 1.0 NaCl (0.02 μCi/μl ^{22}NaCl), such that the final combined pH was 8.0. (*B*) Na^+ uptake in the absence of a pH gradient. 15 μl of the above membrane was mixed with 30 μl of incubation solution containing (in mM): 180 mannitol, 36 Mopso/Tris, 1.8 EGTA, 4.5 Mg(gluconate)$_2$, 1.0 NaCl, (0.02 μCi/μl ^{22}NaCl), such that the final combined pH remained 6.5. Three, 5 and 8 sec later the uptake was stopped by electronically timed injection of a solution containing either (in mM): (*A*) 40 mannitol, 90 K gluconate, 20 Tris/Mes pH 8.0 or (*B*) 40 mannitol, 90 K gluconate, 20 Mopso/Tris pH 6.5. The points shown are the mean ± SEM of individual time points from each experiment. *P* values indicate comparison of mean ± SEM of the individual points for individual experiments (paired *t* test)

Effect of Ca^{2+}/CaM/ATP on Na^+-Dependent Glucose Uptake

Brush-border vesicles were studied in paired experiments examining the effects of Ca^{2+}/CaM/ATP on both the rate of Na^+-dependent glucose uptake, determined during a period of linear uptake (up to 8 sec), and on glucose equilibrium volumes, taken after 90 min incubation at 25°C. As shown in Table 1, Ca^{2+}/CaM/ATP did not cause a significant change in either Na^+-stimulated glucose uptake or glucose equilibrium volumes. In addition, the presence of Ca^{2+}/CaM/ATP had no effect on the zero-time *y* intercepts of Na^+-dependent glucose uptake. The values of these intercepts were not significantly different from zero in either condition, indicating the absence of glucose binding to the vesicles (the zero-time *y* intercept was 2.4 ± 6.5 *vs.* −0.1 ± 2.8 pmol/mg protein in control and Ca^{2+}/CaM/ATP exposed vesicles, respectively, n = 3, NS).

Ca^{2+} Concentration Dependence of Ca^{2+}/CaM/ATP-Dependent Inhibition of Na^+ Uptake

The Ca^{2+} concentration dependence of the Ca^{2+}/CaM/ATP inhibition of the initial rates of Na^+ uptake was determined in the presence of an acid inside pH gradient (pH_{in} 6.5/pH_{out} 8.0). Free Ca^{2+} was controlled by varying the amounts of total Ca^{2+} and keeping constant the concentrations of EGTA, ATP and Mg. The free Ca^{2+} was calculated according to a computer program that considered ATP, Mg, EGTA, total Ca^{2+} and ionic strength [1]. In separate experiments, it was established that none of the free-Ca^{2+} concentrations studied affected Na^+ uptake in the absence of a pH gradient (pH_{in} = pH_{out} = 6.5) (*data not shown*). Shown in Fig. 3, 70 nM free Ca^{2+} did not significantly inhibit Na^+ uptake, while all higher free-Ca^{2+} concentrations caused inhibition. The Ca^{2+} effect became maximal at 300 nM,

Table 1. Effect of Ca^{2+}/CaM/ATP on Na^+-dependent glucose uptake and glucose equilibrium volumes[a]

Group	Na^+-dependent glucose uptake (pmol/mg protein-sec)	Glucose equilibrium volumes (μl/mg protein)
Control $n = 3$	8.9 ± 1.2	0.75 ± 0.08
Ca^{2+}/CaM/ATP $n = 3$	9.9 ± 1.7	0.75 ± 0.12
P	NS	NS

[a] Brush-border membrane vesicles were resuspended in (in mM): 180 mannitol, 36 Mopso/Tris, pH 6.5, 4.5 $Mg(gluconate)_2$ and 1.8 EGTA and either 45 mM mannitol or 0.85 μM free Ca^{2+}, 5 μM calmodulin, 5 mM ATP and 26 U/ml creatine kinase, after which they were frozen in liquid nitrogen and thawed over 75 min in an ice-water slurry. This was followed by incubation at 30°C for 2 min. 10 mM creatine phosphate was added just prior to the final 17 min of the incubation. 15 μl of membrane buffer was mixed with 30 μl of transport buffer containing (in mM): 20 mannitol, 40 Mopso/Tris pH 6.5, 5 $Mg(gluconate)_2$, 2 EGTA, 90 NaCl, 0.15 D-glucose, D-[^{3}H]glucose (0.03 μCi/μl), and the uptake was stopped after 3, 5 or 8 sec by electronically timed injection of stop solution. Data shown are the slopes from this Na^+ uptake determined in each of n experiments. The 90-min equilibrium incubation was performed at 25°C. Results are ± SEM. P values refer to comparison of control and Ca^{2+}/CaM/ATP-treated vesicles.

with the free-Ca^{2+} concentration causing an effect 50% of maximum being 120 nM, as calculated by Woolf-Hanes analysis. The Ca^{2+}/CaM/ATP-induced inhibition of Na^+ uptake declined at 1500 nM free Ca^{2+} to a submaximal but still significant value.

The Role of Calmodulin in Inhibition of Na^+/H^+ Exchange-Inhibitor Studies

In order to determine whether Ca^{2+}/ATP was acting through calmodulin or through protein kinase C to inhibit ileal brush-border Na^+/H^+ exchange, inhibitor studies were performed using drugs under conditions in which they altered either Ca^{2+}/CaM-dependent phosphorylation or protein kinase C activity in the same ileal villus cells in which the transport effects were determined. We have previously reported that the isoquinolenesulfonamide H-7 (60 μM) inhibits ileal villus cell cytosol protein kinase C activity without significantly affecting Ca^{2+}/CaM-dependent phosphorylation of ileal villus cell brush-border membranes [8]. In addition, it was determined that the naphthalenesulfonamide, W_{13} (45 μM) but not its hydrophobic control W_{12} (45 μM) inhibits Ca^{2+}/CaM-dependent phosphorylation of ileal microvillus membrane substrates and that neither W_{13} nor W_{12} alters ileal villus cell cytosol

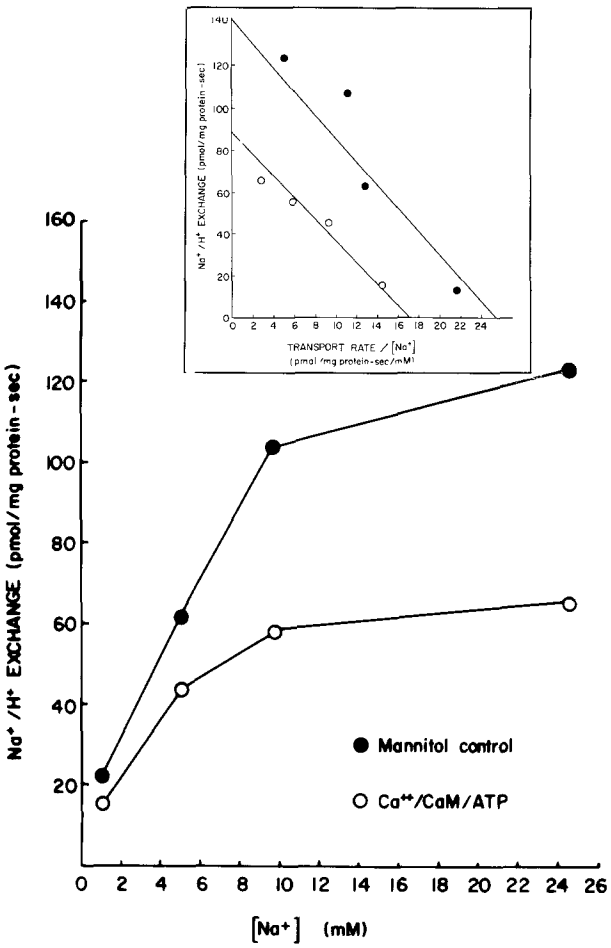

Fig. 2. Na^+ concentration dependence of Na^+/H^+ exchange in the presence and absence of Ca^{2+}/calmodulin/ATP. Experiments identical to those described in Fig. 1, with the following exceptions, were performed to measure Na^+/H^+ exchange in the presence of 45 mM mannitol or 0.85 μM free Ca^{2+}, 5 μM exogenous calmodulin, 5 mM ATP, 10 mM creatine phosphate (Tris salt) and 26 U/ml creatine kinase. In these studies, Na^+ uptake in the presence and absence of an acid inside pH gradient was determined only at 8 sec after mixing membrane and transport buffers, and varying Na^+ concentrations were used in the transport buffer with the actual concentrations determined by atomic absorption spectrometry. Final Na^+ concentrations were 1.1, 4.9, 9.6 and 24.6 mM. Data shown are from a single experiment, which was repeated on a separate vesicle preparation. Insert shows Eadie-Hofstee linearization of the Na^+/H^+ exchange

protein kinase C activity [6; *unpublished observations*]. In these studies, 60 μM H-7, 45 μM W_{13} or 45 μM W_{12} was added to the brush-border vesicles immediately prior to freeze thaw. The effects of these inhibitors on pH gradient-stimulated Na^+ uptake were determined. In addition, the effect of Ca^{2+}/CaM/ATP compared to the mannitol control was determined on pH gradient-stimulated Na^+ uptake in the presence of these drugs. As shown in Table 2, H-7, W_{12} and W_{13} all caused nonsignificant

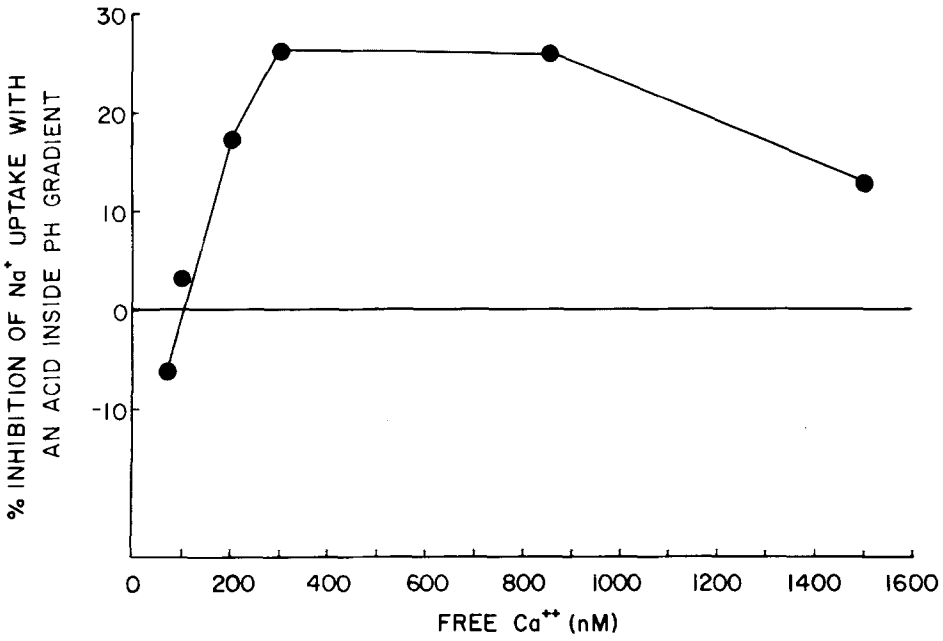

Fig. 3. Ca^{2+} concentration dependence of Ca^{2+}/calmodulin/ATP-dependent inhibition of Na^+ uptake with an acid inside pH gradient. Initial rates of Na^+ uptake were determined in the presence of an acid inside pH gradient (pH_{in} 6.5/pH_{out} 8.0), using identical conditions as described in the legend for Fig. 1*A*, with free-Ca^{2+} concentrations of intravesicular contents set with 1.8 mM EGTA using $Ca(gluconate)_2$, at 70, 100, 200, 300, 850 and 1500 nM in the presence of (in mM): 180 mannitol, 36 Mopso/Tris pH 6.5, 4.5 $Mg(gluconate)_2$, plus 5 μM exogenous calmodulin, 5 mM ATP, 10 mM creatine phosphate (Tris salt) and 26 U/ml creatine kinase and compared to Na^+ uptake in the absence of Ca^{2+} (no added calcium and 1.8 mM EGTA). Following freezing and thawing, 15 μl of membrane buffer was electronically mixed with 30 μl of transport buffer containing (in mM final concentration): 180 mannitol, 36 Tris/Mes, 1.8 EGTA, 4.5 $Mg(gluconate)_2$, 1.0 NaCl (0.02 μCi/μl ^{22}NaCl), such that the final combined pH was 8.0 and Na^+ uptake determined 3, 5 and 8 sec after mixing as described in the legend to Fig. 1. Results are means of 3–5 experiments using separate vesicle preparations at each free-Ca^{2+} concentration. In each experiment, the effect of Ca^{2+}/CaM/ATP on Na^+ uptake with an acid inside pH gradient was determined by comparison with a control in which the membrane buffer had zero free Ca^{2+}, the latter being considered as zero inhibition of Na^+ uptake

decreases in pH gradient-stimulated Na^+ uptake. In the presence of H-7 and W_{12}, Ca^{2+}/CaM/ATP significantly decreased pH gradient-stimulated Na^+ uptake, while in the presence of W_{13}, the Ca^{2+}/CaM/ATP effect to decrease Na^+ uptake was abolished. In separate studies, W_{13} and W_{12} (both 45 μM) did not significantly alter Na^+ uptake at pH 6.5 in the absence of a pH gradient (*data not shown*).

Another approach to confirm the role of calmodulin was to compare the effect of Ca^{2+}/CaM/ATP to that of Ca^{2+}/ATP on Na^+ uptake in identical experiments, but with exogenous CaM omitted. The presence of exogenous CaM caused a slightly, but not significantly, greater inhibition of Na^+ uptake with an acid inside pH gradient, which is attributed to the presence of endogenous calmodulin (control Na^+ uptake performed and determined as in Fig. 1*A* was 27.8 ± 2.5 pmol/mg protein-sec, $n = 4$; Ca^{2+}/ATP performed as in Fig. 1*A* but with exogenous CaM omitted was 24.6 ± 2.3, $n = 4$; Ca^{2+}/CaM/ATP 22.0 ± 2.1, $n = 4$). We attribute the partial CaM effect in the absence of added CaM to the endogenous CaM present in these vesicles [6], which we have been unable to deplete [6].

Discussion

This study characterizes the role of calmodulin in the calcium-dependent regulation of the rabbit ileal brush-border membrane Na^+/H^+ antiporter. We previously demonstrated a technique to place compounds having molecular weights up to at least M_r 70,000 into rabbit ileal brush-border membrane vesicles using a rapid freeze followed by slow thaw [7]. In that these vesicles form predominantly right side out, this freeze-thaw technique was necessary to place into the vesicles the factors needed for the activation of the endogenous calmodulin-dependent protein kinase and the phosphorylation of its substrates. In chicken small intestinal brush-border vesicles the Ca^{2+}/CaM-dependent protein kinase has been identified and appears to be a form of the Ca^{2+}/CaM-dependent protein kinase II [2]. The K_m

for ATP for this kinase is 10–20 μM. In addition, we demonstrated that Ca^{2+} and calmodulin inhibit Na^+/H^+ exchange by a process that requires a hydrolyzable form of ATP [23]. This is consistent with regulation of Na^+/H^+ exchange by a mechanism involving phosphorylation, and based on the data presented here it likely is carried out through the endogenous Ca^{2+}/calmodulin-dependent protein kinase present in this membrane [2, 6].

The inhibitory effect of Ca^{2+} and CaM on the Na^+/H^+ exchanger appears to be specific. As shown in this study, using identical conditions to those used for studying Na^+/H^+ exchange, Ca^{2+}/CaM/ATP failed to alter Na^+ uptake in the absence of an acid inside pH gradient, Na^+-dependent glucose uptake, binding of glucose or Na^+ to the vesicles, and equilibrium uptake of glucose and Na^+. Thus the Ca^{2+}/CaM/ATP effect appears to be specific and not dependent on changes in vesicle size or general vesicle permeability.

The fact that Ca^{2+}/CaM/ATP altered the V_{max} and not the K_m is not surprising, since other studies that have examined the acute and chronic regulation of Na^+/H^+ exchange have generally shown a change in V_{max} rather than a change in the affinity of the carrier for Na^+ [15, 22], although regulation of Na^+/H^+ exchange by a change in K_m has been reported [5]. Whether this change in V_{max} results from a change in the number of exchangers or a change in their turnover rate has not been established in any study. However, in this study, changes in Na^+ uptake caused by Ca^{2+}/CaM/ATP occur very quickly in an environment that contains no significant amounts of organelles besides the brush border. This strongly indicates that removal of exchangers to other organelles is not involved in this effect and is not in support of a mechanism via endocytosis/exocytosis.

The results presented here on the brush-border membrane confirm our previous suggestion that neutral NaCl absorption, of which Na^+/H^+ exchange is believed to be a component [16], is regulated by Ca^{2+}/CaM [10, 22]. The intact tissue studies depended on inhibitor-based pharmacologic studies, as do these vesicles studies. The naphthalenesulfonamide W_{13} is an inhibitor of Ca^{2+}/CaM-dependent kinase and when used at concentrations at which it is a CaM inhibitor in other systems, it inhibited the Ca^{2+}/CaM-induced phosphorylation of specific ileal villus cell brush-border membrane peptides [6]. At this concentration, W_{13} also reversed the Ca^{2+}/CaM/ATP inhibition of Na^+/H^+ exchange and stimulated basal linked NaCl absorption [10]. At this concentration, W_{13} did not alter phosphorylation of cytosolic protein kinase C in ileal villus absorbing cells [8]. That this was not a nonspecific hydrophobic effect of W_{13} was established since the naphthalenesulfonamide W_{12}, which is a hydrophobic control for W_{13}, failed to reverse the Ca^{2+}/CaM/ATP inhibition of Na^+/H^+ exchange. Lastly, 60 μM H-7, which inhibits ileal villus cell protein kinase C activity but does not alter Ca^{2+}/CaM-dependent phosphorylation in brush borders from ileal villus cells, did not alter the inhibition of Na^+/H^+ exchange by Ca^{2+}/CaM. These results are consistent with an action of Ca^{2+} through calmodulin to regulate brush border Na^+/H^+ exchange. It is important to establish which Ca^{2+}-dependent protein kinase regulates Na^+/H^+ exchange since protein kinase C systems and Ca^{2+}/CaM systems can regulate the same substrate separately and have different interactions in multiple cell systems; and ileal brush-border membranes obtained from ileal absorbing cells in the basal state, contain both protein kinase C and Ca^{2+}/CaM kinase(s) [6, 8]. The absence of an

Table 2. Initial rate of Na^+ uptake with an acid inside pH gradient (pH_{in} 6.5/pH_{out} 8.0) (pmol/mg protein-sec)[a]

Drug addition	Mannitol control	Ca^{2+}/CaM/ATP	*P#*
None (n = 4)	21.1 ± 3.6	13.8 ± 2.4	<0.02
H-7 (60 μM) (n = 4)	16.7 ± 3.0	13.0 ± 2.9	<0.02
P+	NS	NS	
W_{13} (45 μM) (n = 4)	15.1 ± 1.7	15.1 ± 2.8	NS
P+	NS	NS	
W_{12} (45 μM)	15.5 ± 2.8	12.9 ± 3.9	<0.05
P+	NS	NS	

[a] Effect of the isoquinolenesulfonamide H-7 and the naphthalenesulfonamides W_{13} and W_{12} on the initial rates of pH gradient-driven Na^+ uptake both in the mannitol control and in the presence of Ca^{2+}/CaM/ATP. Brush-border membrane vesicles were prepared as described in the legend to Fig. 1*A* and loaded, using the freeze-thaw technique, with either 45 mM mannitol or 0.85 μM free Ca^{2+}, 5 μM calmodulin, 5 mM ATP, 26 U/ml creatine kinase, and 10 mM creatine phosphate added just subsequent to the thawing period. W_{13}, W_{12} and H-7 were added just before freezing and thawing and also were present in equal concentrations in the respective transport buffers. The effect on initial rates of Na^+ uptake was determined by adding 15 μl of the membrane preparation to 30 μl of transport buffer containing (in mM): 180 mannitol, 4.5 $Mg(gluconate)_2$, 1.8 EGTA and 36 Tris/Mes such that the combined pH was 8.0. Na^+ uptake is expressed as pmol/mg protein-sec and values are mean ± SEM. *P+* refers to the comparison of Na^+ uptake in the presence or absence of H-7, W_{13}, or W_{12}, respectively (paired t test), analyzing separately the mannitol and Ca^{2+}/CaM/ATP conditions. *P#* refers to the comparison of the rate of Na^+ uptake in mannitol control *vs.* Ca^{2+}/CaM/ATP under identical conditions of addition of H-7, W_{13}, or W_{12}, (paired t test). n refers to the number of separate vesicle preparations studied on separate days.

effect of H-7 on Ca^{2+}/ATP inhibition of ileal brush-border Na^+/H^+ exchange, under conditions in which the drug alters secretagogue-induced changes in ileal villus cell NaCl absorption and alters ileal protein kinase C activity [15], indicates that the regulation of ileal brush border Na^+/H^+ exchange by Ca^{2+} demonstrated here does not involve protein kinase C. As shown in Table 2, all three agents caused nonsignificant inhibition of Na^+ uptake with acid inside pH gradient. The mechanism of the effect is not known, but given the similarity of the effect caused by W_{12} and W_{13} probably is related to the hydrophobic nature of these drugs.

The brush-border membranes used in these studies contain endogenous calmodulin, and we have not been able to deplete them of this calmodulin while leaving the Ca^{2+}/CaM protein kinase(s) fully functional [6]. This failure to remove endogenous calmodulin may explain why exogenous calmodulin gives only a slight and not significant additional effect on Na^+/H^+ exchange over that of Ca^{2+} plus ATP alone.

The Ca^{2+}-concentration dependence of the Ca^{2+}/CaM/ATP effect on Na^+/H^+ exchange also is consistent with a role for the Ca^{2+}/calmodulin-dependent protein kinase. As determined by Woolf-Hanes analysis, the maximal Ca^{2+}-dependent inhibitory effect occurred at free-Ca^{2+} concentrations between 300 and 850 nM, 50% of the maximum effect was at 120 nM, and a decrease in the effect occurred at concentrations above 850 nM. This Ca^{2+} dependence is remarkably similar to the Ca^{2+}-concentration dependence of Ca^{2+}/CaM phosphorylation of specific peptides in the apical membrane made from ileal villus cells [4]. In previous studies, Ca^{2+}/CaM increased phosphorylation of six apical membrane peptides, with a 50% effect at a mean free-Ca^{2+} concentration of 105 nM (range 60–110 nM), maximum effects at 300–800 nM and Ca^{2+} concentrations above 300–800 nM caused a decrease in the effect [6]. The similarity between the Ca^{2+}-concentration dependence of Ca^{2+}/CaM-induced phosphorylation of specific peptides and of Na^+/H^+ exchange in the apical membranes of the same villus cells is consistent with involvement of Ca^{2+}/CaM-dependent phosphorylation in the regulation of Na^+/H^+ exchange.

Regulation of Na^+/H^+ exchange by protein kinases has been suggested before [3, 12, 13] and there is a single additional convincing example in an epithelial tissue, that of a partially reconstituted Na^+/H^+ exchanger from renal brush border, which was inhibited by a cAMP-dependent protein kinase [26, 27]. This current study, be demonstrating the dependence of Na^+/H^+ exchange on Ca^{2+}, CaM and ATP under conditions which are associated with Ca^{2+}-dependent phosphorylation of membrane proteins in the same vesicles, represents further evidence that Na^+/H^+ exchangers can be regulated by protein kinases.

Speculation is justified concerning the similarity between the Ca^{2+} concentration at which Ca^{2+}/CaM/ATP causes both a 50% of maximum inhibition of Na^+/H^+ exchange and a 50% maximum increase in phosphorylation of specific ileal membrane proteins and the cytosol free-Ca^{2+} concentration of ileal villus epithelial cells. The level of cytosol free Ca^{2+} in ileal villus epithelial cells has been estimated to be approximately 130–140 nM, based on preliminary measurements with the fluorescent Ca^{2+}-sensitive dye Fura-2 using a fluorescence microscope/imaging system interfaced with a low intensity TV camera and computer [L. Reinlib, D. Zahniser, R. Mikkelsen and M. Donowitz, *unpublished observations*]. In intact ileal mucosa, protein kinase C, Ca^{2+}/calmodulin, cAMP and cGMP all decrease neutral linked NaCl absorption [9]. If this estimate of ileal cytosol free Ca^{2+} proves to be correct, it would indicate that the cytosol free Ca^{2+} under basal conditions is near the K_m of the Ca^{2+}/CaM regulation of the ileal brush-border Na^+/H^+ exchanger. This would allow both small increases and small decreases in cytosol free Ca^{2+} to regulate the Na^+/H^+ exchanger. Since, after a meal, the intestine must switch quickly from absorption to secretion to absorption again, it can be speculated that small changes in cytosol free Ca^{2+} or other changes in the state of activation of the Ca^{2+}/CaM-dependent protein kinase could be involved in a physiologic rapid change in Na^+/H^+ exchange.

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