Regulation of the Rabbit Ileal Brush-Border Na^+/H^+ Exchanger by an ATP-requiring Ca^{++}/Calmodulin-mediated Process

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Abstract

Brush-border vesicles purified from rabbit ileal villus cells were used to evaluate how Ca^{++}/calmodulin (CaM) regulates the neutral linked NaCl absorptive process, part of which is a Na^+/H^+ exchanger. After freezing and thawing to allow incorporation of macromolecules into the vesicles, the effect of Ca^{++}/CaM on brush-border Na^+ uptake with an acid inside pH gradient, and on Na^+/H^+ exchange was determined. Freezing and thawing vesicles with 0.85 μM free Ca^{++} plus 5 μM exogenous CaM failed to alter Na^+/H^+ exchange as did the addition of exogenous ATP plus an ATP regenerating system, which was sufficient to elevate intravesicular ATP to 47 μM from a basal level of 0.4 μM. However, the combination of Ca^{++}/CaM plus ATP inhibited Na^+ uptake in the presence of an acid inside pH gradient and inhibited Na^+/H^+ exchange, while Na^+ uptake in the absence of a pH gradient was not altered. This effect required a hydrolyzable form of ATP, and did not occur when the nonhydrolyzable ATP analogue, AMP-PNP, replaced ATP. Under the identical intravesicular conditions used for the transport studies, Ca^{++} (0.85 μM) plus exogenous CaM (5 μM), in the presence of magnesium plus ATP, increased phosphorylation of five brush-border peptides. These data are consistent with Ca^{++}/CaM acting via phosphorylation to regulate the ileal brush-border Na^+/H^+ exchanger.

Introduction

Na^+ absorption in rabbit ileal villus epithelial cells occurs via both solute-coupled electrogenic transporters and an electrically neutral, Cl^- dependent process. The latter is thought to consist of two linked neutral exchangers, Na^+/H^+ and Cl^-/HCO_3^- (1–4). In intact ileum in the basal state, it is thought that intracellular Ca^{++} together with calmodulin (CaM)[1] inhibits electroneutral NaCl absorption (5–8). Correlative studies in intact ileum suggest that the mechanism of inhibition may involve protein phosphorylation (8), although the evidence presented thus far is indirect.

Brush-border membrane vesicles purified from rabbit ileal Na^+-absorptive cells contain both the Na^+/H^+ and Cl^-/HCO_3^- exchangers and also Ca^{++}/CaM-dependent protein kinase(s) and substrates (8–10). That both the kinases and the transporters are present in the vesicle preparation is consistent with a role for phosphorylation in the regulation of Na^+ absorption; protein phosphorylation is known to regulate at least two major classes of transport proteins, ion pumps and voltage-dependent ion channels (11–13). We, therefore, investigated the hypothesis that Ca^{++}/CaM-mediated phosphorylation could be involved in the regulation of the brush border Na^+/H^+ exchanger. Since the brush-border vesicles form almost exclusively right side out and are tightly sealed (9), the vesicles were permeabilized to allow ATP, an ATP regenerating system, Ca^{++}, and CaM to be placed inside them. This was accomplished by means of a single cycle of freezing and thawing. The combination of Ca^{++}/CaM plus ATP and the ATP regenerating system inhibited Na^+/H^+ exchange. This effect was not seen with either ATP or Ca^{++}/CaM alone or when ATP was replaced by a nonhydrolyzable ATP analogue (14).

Methods

Brush-border membrane vesicles were prepared by magnesium precipitation, as previously described (9), from male New Zealand white rabbit ileal villus cells obtained from the distal one-third of the small intestine by lightly scraping a sheet of mucosa with a glass slide. The only modification from the method reported previously (9), was to incubate the membranes for 15 min at 25°C immediately after initiating the second magnesium precipitation. This procedure reduced the ATP to a uniformly low concentration in the final vesicles. Brush-border membrane vesicles were stored in liquid nitrogen for up to 2 wk before study.

All brush-border membrane vesicles studied were transiently permeabilized, as previously described (9), by a single cycle of freezing and thawing. On the day of the experiment membrane vesicles were homogenized in a solution containing (in millimolar): 200 mannitol, 40 hydroxy-propane-sulfonic acid (Mopso)/Tris, pH 6.5, 2 EGTA, and 5 $Mg(gluconate)_2$. The vesicles were centrifuged at 100,000 *g* for 1 h at 4°C and resuspended in a buffer containing (in millimolar): 180 mannitol, 36 Mopso/Tris, pH 6.5, 1.8 EGTA, 4.5 $Mg(gluconate)_2$ to a protein concentration of 10 mg/ml. 350 μl of the vesicle suspension plus 36 μl of additives were then frozen in liquid nitrogen for 5 min and thawed slowly for 60 min in an ice-water slurry.

$^{22}Na^+$ influx experiments were performed in the presence of an acid inside pH gradient (pH inside 6.5, pH outside 8.0), with and without 1 mM dimethylamiloride, and in the absence of a pH gradient (pH inside and outside 6.5) over times during which the $^{22}Na^+$ uptake was linear. In most experiments the Na^+ influx was determined by linear regression analyses of Na^+ uptake at three times of study (3, 5, and 8 s). The conditions for the individual experiments are shown in the figure legends. All transport was standardized to the amount of membrane

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1. *Abbreviations used in this paper:* CaM, calmodulin; Mes, morpholino-ethane-sulfonic acid; Mopso, hydroxy-propane-sulfonic acid; TMA, tetramethylammonium.

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protein, determined by the Bio-Rad Coomassie Brillant Blue assay (Bio-Rad Laboratories, Rockville Center, NY) (9).

The ATP content inside the brush-border membrane vesicles after the freeze-thawing process was determined as previously described (9) by the luciferin/luciferase assay (Lumit PM; Lumac, Medical Products Div./3M, St. Paul, MN) (15) after washing the vesicles and then releasing the ATP associated with the vesicles by boiling. Since ATP associated with the vesicles could either be bound to or taken up into the vesicles, ATP taken up into the vesicles was defined as that which could be released from vesicles by exposure to 0.1% saponin. To do this, all the vesicle samples after freeze-thawing were centrifuged at 100,000 *g* at 4°C for 10 min, and resuspended using fine needle homogenization. Half of the vesicles were exposed to 0.1% saponin at 4°C for 10 min. The vesicles were then centrifuged at 100,000 *g* at 4°C for 10 min and resuspended in a solution of (in millimolar): 200 mannitol, 40 Tris, pH 9.2, 5 $Mg(gluconate)_2$, and 2 EGTA in a total volume of 400 μl. An aliquot was removed for protein assay and the remaining sample was boiled for 15 min to release ATP and to precipitate the protein. The sample was centrifuged at 100,000 *g* at 4°C for 5 min and the supernatant was analyzed for ATP content using a standard curve derived from known ATP concentrations. The ATP content in the vesicles was calculated as the amount of ATP associated with the vesicles that was saponin-releasable, divided by the vesicle volume, which was determined on simultaneously studied freeze-thawed vesicles from the same brush-border preparation. The latter were treated identically through the freeze-thaw step before glucose equilibrium values were determined. This was done by mixing 15 μl of the membrane with 30 μl of transport buffer containing (in millimolar) 40 Mopso/Tris, pH 6.5, 20 mannitol, 2 EGTA, 5 $Mg(gluconate)_2$, 90 NaCl, 0.15 [^{3}H]D-glucose (0.03 μCi/μl), and by incubating the sample at 25°C for 90 min, which was followed by stopping in 1 ml of (in millimolar) 40 mannitol, 90 K gluconate, 20 Mopso/Tris, pH 6.5, and by rapid filtration.

Na^+-dependent H^+ efflux was determined using the fluorescent dye acridine orange, as modified from Knickelbein, et al. (3). This was done to show that these vesicles possessed Na^+ gradient–dependent H^+ efflux. 240 μl of vesicles (1.2 mg protein), after being frozen and thawed in a solution of (in millimolar): 180 mannitol, 36 Mopso/Tris, pH 6.5, 1.8 EGTA, 4.5 $Mg(gluconate)_2$, 20 tetramethylammonium (TMA) gluconate, were added to 1,760 μl of transport buffer containing (in millimolar) 180 mannitol, 36 Tris/morpholino-ethane-sulfonic acid (Mes) pH 8.0, 1.8 EGTA, 4.5 $Mg(gluconate)_2$, 6 μM acridine orange, and either 20 mM Na gluconate or 20 mM TMA gluconate. The pH gradient was collapsed after 25 s using nigericin at a concentration of 10 μg/mg protein and 50 mM K gluconate. Hydrogen ion efflux was calculated as the rate of change in fluorescence as a percentage of basal acridine orange fluorescence. The Na^+-dependent H^+ efflux was also determined in the presence of 1 mM dimethylamiloride, with this amiloride analogue added to the membrane and transport buffers 5 min before study.

H^+ permeability was estimated similarly. Vesicular acridine orange uptake was monitored, as previously described (16), and studies were performed to determine whether Ca^{++}/CaM/ATP affected H^+ permeability. Brush-border membranes (in a solution containing [in millimolar] 180 mannitol, 36 Mopso/Tris, pH 6.5, 1.8 EGTA, 4.5 $Mg(gluconate)_2$, 30 K gluconate) were frozen and thawed with or without 0.85 μM free Ca^{++}, 5 μM CaM, 10 U creatine kinase, 10 mM creatine phosphate, and 5 mM ATP. 240 μl of vesicle suspensions (1.2 mg of protein), to which 5 μM valinomycin was added, was added to 1,760 μl of incubation solution which had a concentration of (in millimolar) 180 mannitol, 36 Tris/Mes, pH 8.0, 1.8 EGTA, 4.5 $Mg(gluconate)_2$, 30 K gluconate, together with 6 μM acridine orange. Hydrogen ion efflux was calculated as the rate of change in fluorescence as a percentage of basal acridine orange fluorescence.

Fluorescence in all studies was determined with a LS-5 fluorometer (Perkin-Elmer Corp., Norwalk, CT) using a quartz cuvette, stirred with a teflon-coated stirring disc, and fluorescence was determined at 30°C. Excitation was at 493 nm and emission was at 527 nm.

In parallel studies, it was shown that Ca^{++}/CaM increased phosphorylation of brush-border membrane peptides when conditions identical to those studied in the Na^+ uptake experiments were used. Brush-border membranes were homogenized at a protein concentration of 10 mg/ml in a solution containing (in millimolar) 180 mannitol, 36 Mopso/Tris, pH 6.5, 1.8 EGTA and 4.5 $Mg(gluconate)_2$. 350 μl of the vesicle suspension, containing either 1 mM γ-[^{32}P]ATP (60 μCi), 10 U creatine kinase, and 10 mM creatine phosphate, or 0.85 μM free Ca^{++}, 5 μM exogenous CaM, 1 mM γ-[^{32}P]ATP, 10 U creatine kinase, and 10 mM creatine phosphate was frozen in liquid nitrogen for 5 min, gradually thawed on ice for 75 min, and then warmed to 25°C for 2 min. The phosphorylation was stopped by the addition of a solution consisting of 5% SDS, 0.1 mM EDTA, 200 μM DTT, 50 μg/ml pyronin Y, 20% sucrose (wt/vol), and the solution was boiled for 2 min. Approximately 50 μg of membrane protein was then applied to SDS 5–15% continuous gradient polyacrylamide gels for electrophoresis. The gels were stained with Coomassie Brilliant Blue, destained, dried, and then autoradiograms were made with Kodak XAR-5 film. Analysis was done by scanning densitometry of the autoradiogram, which assumed that phosphorylation of the protein was proportional to height on a densitometry scan.

^{22}Na, [^{3}H]glucose, and γ-[^{32}P]ATP were from New England Nuclear, Boston, MA. Dimethylamiloride was a gift from Dr. E. J. Cragoe, Jr. CaM, ATP (Tris salt), creatine phosphate, and creatine phosphokinase were from Sigma Chemical Co., St. Louis, MO. Adenylyl-imidodiphosphate was from Boehringer Mannheim Biochemicals, Indianapolis, IN.

Statistical analyses were by paired *t* tests on simultaneous studies under varied conditions on the same vesicle preparation.

Results

Na^+/H^+ exchange in frozen and thawed vesicles. Na^+/H^+ exchange was present in these frozen and thawed brush-border vesicles made from ileal villus cells as defined by three criteria: (*a*) the difference in Na^+ uptake in the presence of an acid inside pH gradient, in the presence and absence of 1 mM dimethylamiloride, (*b*) the difference in Na^+ uptake in the presence and absence of an acid inside pH gradient, and (*c*) a Na^+-dependent H^+ efflux having been demonstrated by using acridine orange in a fluorometer.

Initially, two definitions of Na^+/H^+ exchange in a single study were compared (Fig. 1). In these experiments, the initial rate of Na^+ uptake, determined in the absence of an acid inside pH gradient (pH_{in} 6.5/pH_{out} 8.0) 3, 5, and 8 s after Na^+ addition (times at which the rate of Na^+ uptake was linear), was 3.9 pmol/mg protein-s.; this uptake rate was reduced to 0.5 pmol/mg protein-s. by 1 mM dimethylamiloride, and to 0.3 pmol/mg protein-s. by determining Na^+ uptake in the absence of a pH gradient (pH_{in} = pH_{out}, 6.5). Shown in Fig. 1 is Na^+/H^+ exchange defined as: (*a*) the difference in Na^+ uptake in the presence of an acid inside pH gradient with and without dimethylamiloride (3.4 pmol/mg protein-s.), and (*b*) the difference in Na^+ uptake in the presence and absence of an acid inside pH gradient (3.6 pmol/mg protein-s.) It is interesting that 1 mM dimethylamiloride did not affect Na^+ uptake when the pH inside and outside the vesicles was 6.5 (Na^+ uptake was 0.7±0.2 pmol/mg protein-s vs. 0.6±0.1 in the absence and presence of 1 mM dimethylamiloride, respectively, $n = 3$). In contrast, the same concentration of dimethylamiloride did significantly decrease Na^+ uptake in the absence of an acid inside pH gradient at pH_{in} = pH_{out} = 7.0 (Na^+ uptake was 1.0±0.3 pmol/mg protein-s. vs. 0.6±0.1, in the absence and presence of 1 mM dimethylamiloride, respectively, $P < 0.05$, $n = 3$).

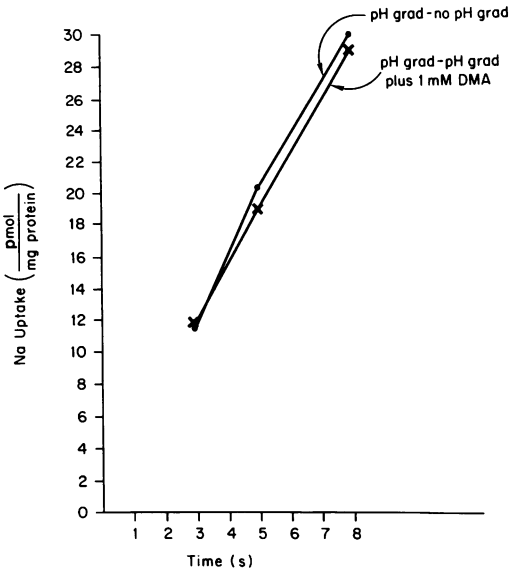

Figure 1. Na^+/H^+ uptake in frozen and thawed ileal brush-border vesicles. Brush-border membrane vesicles were made as described in the Methods. 350 μl of the vesicle suspension plus 36 μl of water were then frozen and thawed as described. $^{22}Na^+$ uptake was begun by mixing 15 μl of the vesicle suspension with 30 μl of a solution containing (in millimolar) either (*A*) 180 mannitol, 36 Tris/Mes, 1.8 EGTA, 4.5 Mg $(gluconate)_2$, 0.1 NaCl and 0.6 μCi $^{22}Na^+$ such that the final combined pH was 8.0 for Na^+ uptake with an acid inside pH gradient, or (*B*) 180 mannitol, 36 Mopso/Tris, 1.8 EGTA, 4.5 $Mg(gluconate)_2$, 0.1 NaCl, and 1.2 μCi $^{22}Na^+$, such that the final pH remained at 6.5, for Na^+ uptake without a pH gradient, (*C*) and (*D*) solutions were identical to A and B, respectively, but also contained dimethylamiloride at a final concentration of 1 mM. After the 60-min thaw, all vesicles were kept for an additional 15 min on ice and at 25°C for 2 min before $^{22}Na^+$ uptake was determined. In the dimethylamiloride studies, 1 mM dimethylamiloride was added to the membrane preparation 5 min before $^{22}Na^+$ uptake was determined. The reaction was stopped 3, 5, or 8 s after the addition of $^{22}Na^+$ by the electronically timed injection of 1 ml of ice-cold stop solution containing (in millimolar) 90 K gluconate, 40 mannitol, and either 20 Tris/Mes, pH 8.0, or 20 Mopso/Tris, pH 6.5. The incubation mixture was vacuum filtered on 45 μm nitrocellulose filters and was rinsed with 6 ml ice-cold stop solution. The filter was then dissolved in 3 ml Liquiscint, counted in a liquid scintillation counter, and $^{22}Na^+$ uptake was expressed per milligram protein, which was determined by Bio-Rad assay. $^{22}Na^+$ uptake was linear over this time and with a zero-time y-intercept not significantly different from zero in all conditions. The data shown that represents Na^+/H^+ exchange are (*a*) the differences in Na^+ uptake rate in the presence of an acid inside pH gradient in the presence and absence of dimethylamiloride (condition A minus C) which equals 3.4 pmol/mg protein-s and (*b*) the difference in Na^+ uptake rate in the presence and absence of an acid inside pH gradient (condition A minus B) which equals 3.6 pmol/mg protein-s. This is a representative experiment and was performed on three separate vesicle preparations.

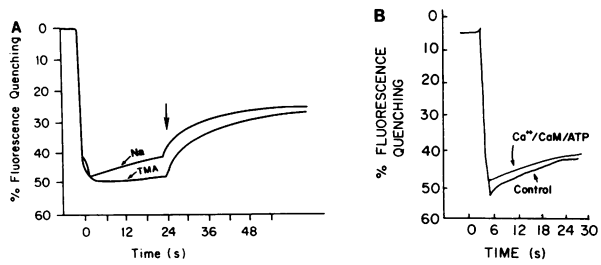

Figure 2. (*A*) Presence of Na^+-dependent H^+ efflux in freeze-thawed brush-border vesicles demonstrated fluorometrically using acridine orange and (*B*) the effect of Ca^{++}/CaM/ATP on ileal brush-border H^+ permeability studied fluorometrically using acridine orange. (*A*) Na^+-dependent H^+ ion efflux was determined using the fluorescent dye acridine orange technique as modified from Knickelbein et al. (3). This was done to show that these vesicles possessed Na^+ gradient-dependent H^+ efflux. Vesicles were loaded using the freeze-thaw technique. 240 μl of vesicles (1.2 mg protein), freeze thawed in (in millimolar) 180 mannitol, 36 Mopso/Tris, pH 6.5, 1.8 EGTA, 4.5 $Mg(gluconate)_2$, 20 TMA gluconate, were added to 1,760 μl of transport buffer containing (in millimolar): 180 mannitol, 36 Tris/Mes, final pH 8.0, 1.8 EGTA, 4.5 $Mg(gluconate)_2$, 6 μM acridine orange, and either Na gluconate (20 mM) or TMA gluconate (20 mM). The pH gradient was collapsed after 25 s using nigericin 10 μg/mg protein and 50 mM K gluconate. Studies were done at 30°C. Hydrogen ion efflux was calculated as the rate of change in fluorescence as a percentage of basal acridine orange fluorescence. Data shown are from a representative experiment, which was repeated on three separate vesicle preparations. In the presence of Na^+, the initial rate of H^+ efflux was changed 20% in fluorescence/initial fluorescence per min per mg protein, while in the absence of Na^+, H^+ efflux was changed 4% in fluorescence/initial fluorescence-min-mg protein. At the arrow, nigericin plus K gluconate was added with the result that the fluorescence returned to the same level both in the presence and absence of Na^+. The difference between the final level of fluorescence and basal fluorescence represents binding of the acridine orange to the brush border (3). (*B*) Ileal brush-border membrane vesicles were prepared and frozen and thawed at pH 6.5 in (in millimolar) 180 mannitol, 36 Mopso/Tris, 1.8 EGTA, 4.5 $Mg(gluconate)_2$, 30 K gluconate, 5 μM valinomycin with either no other additions or with 0.85 μM free Ca^{++}, 5 μM CaM, 10 U creatine kinase, 10 mM creatine phosphate, and 5 mM ATP. At the completion of the freeze-thaw procedure, 240 μl of vesicles (1.2 mg protein) were incubated 15 min at 0°C and then for 2 min at 25°C, and then they were exposed to 1.76 ml of (in millimolar) 180 mannitol, 36 Mes/Tris, pH 8.0, 1.8 EGTA, 4.5 $Mg(gluconate)_2$, 30 K gluconate, 6 μM acridine orange, and fluorescence was determined at 30°C. Data is representative of identical experiments on four separate vesicle preparations.

As further evidence that these frozen and thawed vesicles exhibit Na^+/H^+ exchange, Na^+-dependent H^+ efflux was shown to be present. Shown in Fig. 2 *A*, H^+ efflux from the freeze-thawed vesicles was increased by substituting 20 mM Na gluconate for 20 mM TMA gluconate as measured fluorometrically using acridine orange. In the presence of Na^+, the initial rate of the H^+ efflux was 16±2% change in fluorescence from the initial fluorescence per minute per milligram of protein, while in the absence of Na^+, the initial rate of the H^+ efflux was 7±1%. ($n = 3$, $P < 0.05$). That is, extravesicular Na^+ increased the initial rate of the H^+ efflux 2.3±0.2 times. When identical experiments were performed in the presence of 1 mM dimethylamiloride (preincubated in the transport buffer for 5 min with membranes), Na^+ caused a significantly smaller increase in H^+ efflux (1.3±0.2 times). In subsequent experiments, Na^+/H^+ exchange was defined as the difference in Na^+ uptake in the presence and absence of an acid inside pH gradient (pH_{in} 6.5/pH_{out} 8.0).

Effect of Ca^{++}/CaM and ATP on Na^+/H^+ exchange. Brush-border vesicles purified from villus cells were used to study the effect of Ca^{++}/CaM plus ATP (with an ATP regenerating system) on Na^+ uptake and Na^+/H^+ exchange. In these studies, free Ca^{++} was fixed using an EGTA buffer and Ca^{++}

(gluconate)$_2$ using Ca^{++} binding constants and a computer program (10). Table I shows the results of experiments that demonstrate that vesicles frozen and thawed with ATP and an ATP regenerating system had an increased intravesicular ATP concentration compared with similarly prepared vesicles that were frozen and thawed without the ATP regenerating system (47 μM vs. 0.4 μM). As shown in Fig. 3 *A*, initial rates of $^{22}Na^+$ uptake with an acid inside pH gradient was not altered either by 0.85 μM free Ca^{++} plus exogenous 5 μM CaM or by increasing intravesicular ATP from 0.4 to 47 μM. However, the combination of Ca^{++}, exogenous CaM, and ATP significantly decreased Na^+ uptake with an acid inside pH gradient by 32±7% (Fig. 3 *A*, Table I). In contrast, neither Ca^{++}/CaM, ATP alone, nor together significantly altered $^{22}Na^+$ uptake in the absence of a pH gradient (pH_{in} = pH_{out}, 6.5) (Fig. 3 *B* and Table I). While neither Ca^{++} plus exogenous CaM nor increasing intravesicular ATP significantly altered Na^+/H^+ exchange alone, the combination decreased Na^+/H^+ exchange by 58±8% (Fig. 3 and Table I).

Table I. Effect of Ca^{++}/CaM Plus ATP and ATP Regenerating System on Na Uptake

	(A) $^{22}Na^+$ uptake with acid inside pH gradient	(B) $^{22}Na^+$ uptake without pH gradient	(C) Na^+/H^+ exchange (A − B)	(D) Final ATP concentration in vesicles
		pmol/mg prot-s		*μM*
Control	2.76±0.46	0.73±0.14	2.03±0.37	0.4±0.1
Ca^{++}/CaM/ATP	1.88±0.32	1.01±0.1	0.87±0.20	46.8±0.5
**P*	<0.05	NS	<0.05	<0.001

Effect of Ca^{++}/CaM plus ATP and ATP regenerating system on Na^+ uptake and Na^+/H^+ exchange. Membrane preparation, freeze thaw, and $^{22}Na^+$ uptake were performed as discussed in Fig. 3, with uptake determined at 3, 5, and 8 seconds after Na^+ exposure and rates shown determined as the slope obtained by linear regression analyses for each experiment. Controls were performed by adding 36 μl H_2O to 350 μl of vesicles in membrane buffer. Ca^{++}, CaM, and ATP addition include 0.85 μM free Ca^{++}, 5 μM CaM, 10 U creatine kinase, 10 mM creatine phosphate, and 5 mM ATP. *D* is the final ATP concentration in freeze-thawed vesicles measured using the luciferin/luciferase assay after releasing trapped ATP by boiling as described previously (9). ATP concentrations were calculated by dividing the releasable ATP from the vesicles by the vesicle volumes, which were determined using glucose equilibrium volumes in simultaneously studied vesicles made from the same brush-border preparations. This was done by freeze thawing the vesicles as described above with membrane buffer containing (in millimolar) 180 mannitol, 36 Mopso/Tris, pH 6.5, 4.5 Mg(gluconate)$_2$, 1.8 EGTA with and without Ca^{++}/CaM/ATP, creatine phosphate and creatine kinase. 15 μl of these vesicles were then mixed with 30 μl of transport buffer containing (in millimolar) 20 mannitol, 40 Mopso/Tris, pH 6.5, 5 Mg(gluconate)$_2$, 2 EGTA, 90 NaCl, 0.15 [^{3}H]D-glucose (0.03 μCi/μl), after which they were incubated at 25°C for 90 min, and rapid filtrated with a stop solution of (in millimolar) 40 mannitol, 20 Mopso/Tris, pH 6.5, 90 K gluconate. Glucose equilibrium volumes were not different in freeze-thawed vesicles exposed to control or to Ca^{++}/CaM/ATP conditions. They were 0.87±0.12 and 0.75±0.13 μl/mg protein in control and Ca^{++}/CaM/ATP-exposed vesicles, respectively (n = 5, NS), determined 90 min after exposure to transport buffer. Results are expressed as mean±SEM from five experiments on five separate vesicle preparations.
* *P* refers to the comparison of control and Ca^{++}/CaM/ATP conditions (paired *t* test).

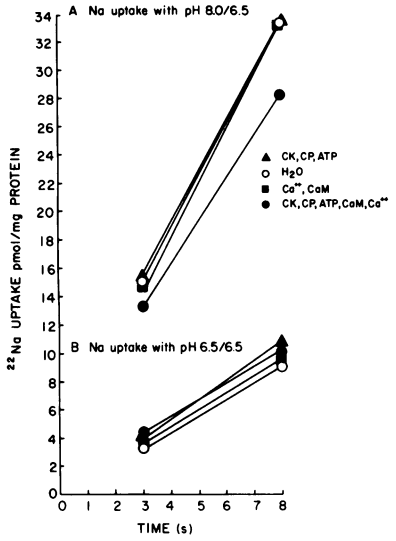

Figure 3. Separate and combined effects of Ca^{++}/CaM and ATP plus an ATP regenerating system on $^{22}Na^+$ uptake (*A*) with an acid inside pH gradient (pH 8.0/6.5) and (*B*) without a pH gradient (pH 6.5/6.5). Brush-border membrane vesicles were prepared as described in the Methods. 350 μl of the vesicle suspension plus 36 μl of one of four conditions were then frozen in liquid nitrogen for 5 min and thawed slowly for 60 min in an ice-water slurry. The four conditions were: control (○), water; 0.85 μM free Ca^{++} plus 5 μM exogenous CaM (■); 10 U creatine kinase, 10 mM creatine phosphate, and 5 mM ATP (Tris salt) (▲); 0.85 μM free Ca^{++}, 5 μM CaM, 10 U creatine kinase, 10 mM creatine phosphate, and 5 mM ATP (●). All reagents except creatine phosphate were added immediately before freezing the vesicles. Creatine phosphate was added immediately after the vesicles thawed. After the 60-min thaw, all vesicles were kept for an additional 15 min on ice and at 25°C for 2 min before $^{22}Na^+$ uptake was determined. $^{22}Na^+$ uptake was begun and conducted as discussed in the legend of Fig. 1. Data shown are from a representative experiment from identical studies using three separate vesicle preparations. $^{22}Na^+$ uptake was linear over this time in all conditions with a zero-time y-intercept not significantly different from zero.

The effect of Ca^{++}/CaM/ATP on Na^+/H^+ exchange was determined in the same experiment using the two definitions of Na^+/H^+ exchange shown in Fig. 1. Simultaneous studies were done on the same membrane preparation with the same conditions as described in Fig. 3 except that 1 mM dimethylamiloride was added 5 min before the uptake study to freeze thawed membranes used for the acid inside pH gradient study and that 1 mM dimethylamiloride was also present in the transport buffers. As shown in Fig. 4, Ca^{++}/CaM/ATP caused statistically significant inhibition of Na^+/H^+ exchange both when it was defined as the difference between acid inside pH gradient–stimulated Na^+ uptake and the absence of pH gradient condition (n = 4, $P < 0.05$), and when it was defined as that Na^+ uptake that was sensitive to 1 mM dimethylamiloride (n = 4, $P < 0.0125$). In addition, the magnitude of the Ca^{++}/CaM/ATP effect on Na^+/H^+ exchange was not statistically different using the two definitions of Na^+/H^+ on the same vesicles (Ca^{++}/CaM/ATP inhibited Na^+/H^+ exchange by 46±13% of control Na^+/H^+ exchange using the former definition of Na^+/H^+ exchange and by 57±17% using the latter definition). Ca^{++}/CaM/ATP did not alter Na^+ uptake in the presence of an acid inside pH gradient when studied in the presence of 1 mM dimethylamiloride (data not shown).

In contrast to these results, when ATP was replaced by a nonhydrolyzable analogue, adenylyl-imidodiphosphate (AMP-PNP), and studied in the presence of the same Ca^{++}/CaM concentrations used above, there was no significant inhibition of Na^+ uptake in the presence of an acid-inside pH gradient (Fig. 5 *A*), of Na^+ uptake in the absence of a pH

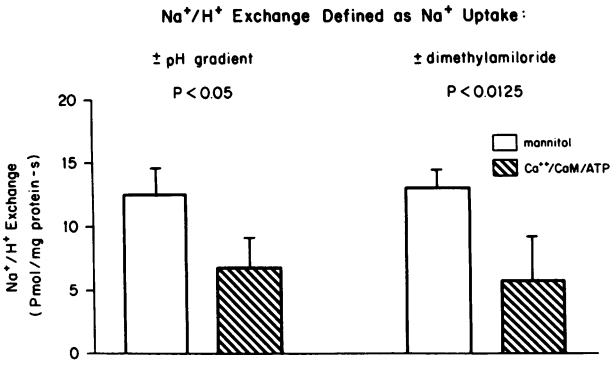

Figure 4. Ca^{++}/CaM/ATP inhibition of Na^+/H^+ exchange using two definitions of Na^+/H^+ exchange. Brush-border membrane vesicles were prepared as described in the methods. 350 μl of vesicles plus 36 μl containing either mannitol or 0.85 μM free Ca^{++}, 5 μM CaM, 10 U creatine kinase, 10 mM creatine phosphate, and 5 mM ATP (all in final concentrations) were frozen and thawed and $^{22}Na^+$ uptake was performed exactly as described in the legend to Fig. 1, except that the final Na^+ concentration was 1 mM. Na^+/H^+ exchange was defined in the bars on the left as the difference between pH gradient (pH_{in} 6.5/pH_{out} 8.0)-stimulated $^{22}Na^+$ uptake, and the Na^+ uptake without a pH gradient (pH_{in} = pH_{out} = 6.5) or, in the bars on the right as that Na^+ uptake that was inhibitable by 1 mM dimethylamiloride. Na^+/H^+ exchange in the control mannitol conditions was not significantly different when comparing the two definitions of Na^+/H^+ exchange. Ca^{++}/CaM/ATP caused significant inhibition of Na^+/H^+ exchange using both definitions of Na^+/H^+ exchange. *P* values refer to the comparison of Na^+/H^+ exchange rates in the presence and absence of Ca^{++}/CaM/ATP separately using both definitions of Na^+/H^+ exchange (paired *t* test). Four separate studies were performed on vesicles from four membrane preparations. Data shown as mean±SEM.

gradient (Fig. 5 *B*) or of Na^+/H^+ exchange (Fig. 5 *C*). Thus a hydrolyzable form of ATP was required for the Ca^{++}/CaM-dependent inhibition of Na^+/H^+ exchange.

It was demonstrated that the Ca^{++}/CaM and ATP-induced change in Na^+/H^+ exchange was not due to a change in H^+ permeability. A change in vesicle H^+ permeability could be responsible for the demonstrated decrease in Na^+ uptake by two possible mechanisms: (*a*) an increase in an H^+ leak which would diminish the buffer capacity inside the vesicles and (*b*) an electrically coupled effect in which decreased electrogenic Na^+ uptake could occur in response to a decrease in the electrogenic H^+ leak. Of these possibilities, the second is less likely, because it has been shown (1–3) that Na^+/H^+ exchange in rabbit ileal brush-border vesicles is not electrically coupled. The brush-border H^+ permeability was determined, as previously described (16), by measuring the rate of uptake and the efflux rate of acridine orange in vesicles without a pH gradient (see Fig. 2 *B*). Ca^{++}/CaM plus ATP and the ATP regenerating system did not alter the rate of uptake or efflux of acridine orange (uptake of acridine orange was 36±6% fluorescence units/initial fluorescence per mg protein, in control, and 41±6% in Ca^{++}/CaM plus ATP-exposed vesicles [n = 4, NS]; H^+ efflux [defined as the rate of efflux of acridine orange] was 12±2% fluorescence units/initial fluorescence-mg protein-min, in control, and 15±1% in Ca^{++}/CaM plus ATP-exposed vesicles [n = 4, NS], and therefore did not affect H^+ permeability.

Ca^{++}/CaM-stimulated phosphorylation of brush-border peptides under the same conditions used for transport. We have previously reported that Ca^{++}/CaM increased the phosphorylation of five ileal villus cell brush-border membrane peptides (8–10). The previous studies were done using membrane sheets, prepared to be freely permeable to macromolecules, including ATP (8, 10). In addition we demonstrated that freeze thawing did not alter Ca^{++}/CaM-dependent phosphorylation of these brush-border peptides (9) and that increasing intravesicular ATP by freeze thawing in the presence of ATP caused a three- to fourfold increase in total brush-border phosphorylation (9). Similar studies were performed in vesicles prepared under identical conditions to those in Fig. 3 so that Ca^{++}/CaM-induced changes in Na^+/H^+ exchange could be correlated with changes in Ca^{++}/CaM-dependent phosphorylation in the same vesicles under the same conditions. Vesicles were prepared as in Fig. 3, freeze thawed, and incubated for 15 min at 0°C and for 2 min at 25°C; conditions were identical to those used for the transport studies except that these vesicles were also freeze thawed with γ-[^{32}P]ATP. As demonstrated by

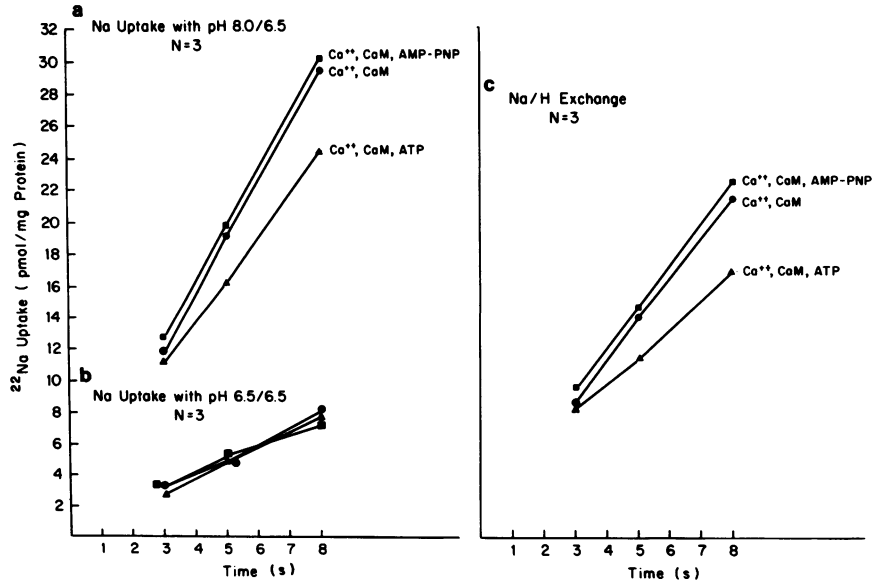

Figure 5. Inhibition of Na^+/H^+ exchange by a process that requires a hydrolyzable form of ATP. Membrane preparation, freeze thaw, and $^{22}Na^+$ uptake were done as in Fig. 1, except that all conditions contained 10 U creatine kinase, 306 μM Ca $(gluconate)_2$ (0.85 μM free Ca^{++}), and 5 μM CaM. In control (●) there was no further addition; (▲) also contained 5 mM ATP (Tris salt) and 10 mM creatine phosphate (Tris salt); (■) also contained 5 mM AMP-PNP (Tris salt). Data shown are the mean of three experiments (n in figure) done with three different vesicle preparations.

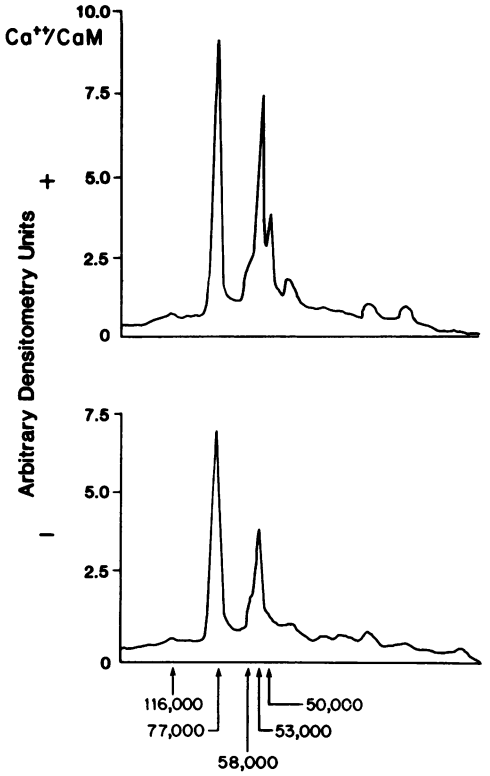

Figure 6. Effect of Ca^{++}/CaM on phosphorylation of brush-border peptides when ATP was freeze thawed into the vesicles under identical conditions used for the transport study. Brush-border vesicles were freeze thawed as in Fig. 3 to contain (in millimolar) 180 mannitol, 36 Mopso/Tris, pH 6.5, 1.8 EGTA, 4.5 $Mg(gluconate)_2$, γ-[^{32}P]-ATP (60 μCi), 10 U creatine kinase, 10 mM creatinine phosphate with or without 0.85 μM free Ca^{++}, plus 5 μM exogenous CaM. After 75 min of thawing on ice and 2 min at 25°C, phosphorylation was stopped by addition of a solution consisting of 5% SDS, 0.1 mM EDTA, 200 μM DTT, 50 μg/ml pyronin Y, 20% sucrose (wt/vol), and the solution was boiled for 2 min. Approximately 50 μg of membrane protein was then applied to 5–15% continuous gradient polyacrylamide SDS gels for electrophoresis. The gels were stained with Coomassie Brilliant Blue, destained, dried, and autoradiograms were made with Kodak XAR-5 film. Analysis was by scanning densitometry of the autoradiogram assuming that the amount of phosphorylation of the protein was proportional to height on a densitometry scan. M_r's of peptides, the phosphorylation values of which were increased by Ca^{++}/CaM, are shown by arrows. Data shown are from a representative experiment. Identical experiments were performed on three separate vesicle preparations.

scanning densitometry, which is shown in Fig. 6, Ca^{++}/CaM increased the phosphorylation of the same brush-border peptides when ATP was incorporated by the freeze thaw process under conditions identical to those used for determining the effect of Ca^{++}/CaM/ATP on the brush-border Na^+/H^+ exchanger, as was previously reported using permeable brush-border vesicles (8–10).

Discussion

This study demonstrates that Ca^{++} plus CaM, acting by a process that requires a hydrolyzable form of ATP, inhibits Na^+/H^+ exchange in rabbit ileal brush-border vesicles. The ATP concentrations were selected to allow the comparison of concentrations above and below the K_m for ATP of the Ca^{++}/CaM-dependent protein kinase that is present in these vesicles (F. Gorelick, M. E. Cohen, G. W. G. Sharp, and M. Donowitz, unpublished observations). The K_m of ATP for this kinase in other tissues has been estimated to be 5–10 μM (17, 18). Evidence has been provided previously that in villus Na^+-absorbing cells, Ca^{++}/CaM regulates neutral, linked NaCl absorption (7) and causes the phosphorylation of specific brush-border peptides that act through a Ca^{++}/CaM-dependent protein kinase that is present in these membranes (10). We now have shown that Ca^{++}/CaM regulates the brush-border Na^+/H^+ exchanger under conditions identical to those at which it regulates the phosphorylation of brush-border membrane peptides.

Na^+/H^+ exchange in these vesicles was equivalently defined as: (*a*) the difference in Na^+ uptake in the presence and absence of an acid-inside pH gradient, while the intravesicular pH was 6.5 and (*b*) the difference in Na^+ uptake in the presence of an acid inside pH gradient with and without 1 mM dimethylamiloride. Na^+ uptake, with the pH both inside and outside the vesicles at 6.5, was not affected by 1 mM dimethylamiloride, but Na^+ uptake was inhibited by dimethylamiloride when intravesicular and extravesicular pH was 7.0. This is different from renal brush-border membrane vesicles in which Na^+ uptake at pH_{out} 6.6/pH_{in} 6.0 was inhibited by amiloride (19). While an explanation for the difference between renal and ileal brush-border Na^+/H^+ exchange is not known, the difference could be explained by a greater affinity for H^+ compared with Na^+ in the ileal exchanger. Further evidence that Na^+/H^+ exchange could be defined in brush-border vesicles by either method is the fact that Ca^{++}/CaM and ATP caused similar inhibition of Na^+/H^+ exchange in experiments using both definitions of Na^+/H^+ exchange. In the latter studies, there was the possibility that 1 mM dimethylamiloride might have affected protein kinase activity (20); under the conditions of these studies, however, 1 mM dimethylamiloride did not alter Ca^{++}/CaM-dependent phosphorylation in these ileal brush-border membrane vesicles (unpublished observations).

The data presented here, particularly that concerning the requirement for exogenous ATP to demonstrate an effect of Ca^{++}/CaM on Na^+/H^+ exchange, are contrary to the findings of Fan and Powell who found that Ca^{++}/CaM could inhibit NaCl absorption in brush-border vesicles without exogenous ATP (21). Ca^{++}/CaM has been demonstrated to act either directly, by regulating an enzyme, or indirectly via protein kinase regulation of phosphorylation. The erythrocyte plasma membrane Ca^{++}-Mg^{++}-ATPase, several adenylate cyclases, and cyclic nucleotide phosphodiesterases are examples of enzymes directly regulated by Ca^{++}/CaM, but most identified Ca^{++}/CaM regulation occurs via the control of phosphorylation. The difference in results between the Fan and Powell study and this one may be due to the fact that our vesicles were deliberately depleted of endogenous ATP before use (see Table I). In some small intestinal brush-border vesicles, the ATP concentrations have been found to be as high as 90 μM (22), which is far above the K_m for ATP of most Ca^{++}/CaM-dependent protein kinases. By reducing the vesicle ATP concentration to below 1 μM, the vesicles used in this study became dependent upon exogenous ATP.

The fact that a hydrolyzable form of ATP is necessary for regulation of the Na^+/H^+ exchanger by Ca^{++}/CaM, most likely indicates that phosphorylation of membrane proteins is involved. The Na^+ uptake process affected in the presence of an acid inside pH gradient, does not depend on metabolic energy and is not affected by or associated with an Na^+ pump since

this pump does not occur in rabbit ileal brush-borders. Furthermore, these membranes contain Ca^{++}/CaM-dependent protein kinase(s) and substrates, which appear not to be altered by the freeze-thaw procedures used (9). Importantly, the Ca^{++}-dependence of Ca^{++}/CaM-dependent phosphorylation of specific brush-border membrane proteins, as previously described in similar rabbit ileal apical membrane vesicles (10), is very similar to the Ca^{++} dependence of this Ca^{++}-, CaM-, and ATP-dependent regulation of Na^+/H^+ exchange (unpublished observations). Taken together, these facts suggest that Ca^{++}/CaM-dependent phosphorylation of brush-border membrane proteins is involved in the regulation of ileal villus cell NaCl absorption and brush-border Na^+/H^+ exchange. Whether the specific phosphorylated substrates of Ca^{++}/CaM kinase(s) demonstrated in these membranes are involved in the regulation of Na^+/H^+ exchange has not been established. These studies represent strong evidence that in addition to established involvement in the regulation of ion pumps and ion channels, protein phosphorylation appears to be involved in the regulation of a third type of transport protein, an epithelial electrolyte exchanger.

The approach described here in intestinal villus cells represents the use of plasma membrane vesicles to study regulation of the transport proteins present in the vesicles. In the past, vesicle studies have been used primarily to identify plasma membrane transport processes and to define their kinetics and stoichiometry. The current study demonstrates that by incorporation of macromolecules and the definition of intravesicular contents (in this case, intravesicular ATP and Ca^{++}) plasma membrane vesicles can be used to probe the regulation of the plasma membrane transport processes by protein kinases and by other regulating enzymes. For instance, the conditions standardized in this work should allow for the investigation of the effects of the protein kinases that are present in the brush-border membrane on brush-border transport processes. These protein kinases include not only the Ca^{++}/CaM protein kinase(s) but also cyclic AMP– and cyclic GMP–dependent protein kinases (10, 21, 23) and under some conditions protein kinase C (24).

Acknowledgments

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