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# Calcium/calmodulin inhibition of coupled NaCl transport in membrane vesicles from rabbit ileal brush border

(trifluoperazine/intestinal secretion)

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**ABSTRACT** **The role of $Ca^{2+}$ and calmodulin in regulating coupled NaCl transport has been investigated in membrane vesicles from rabbit ileal brush border. Uptake of $^{22}Na^+$ and $^{36}Cl^-$ was determined by a rapid filtration technique in vesicles isolated with a sucrose density gradient ultracentrifugation method. $Ca^{2+}$ on the inside of the vesicle inhibited $Na^+$ uptake when $Cl^-$ was the anion and $Cl^-$ uptake when $Na^+$ was the cation by ≈30%. $Ca^{2+}$ on the outside had no effect. When gluconate was the anion or when choline was the cation, $Na^+$ or $Cl^-$ uptake was reduced by only 9–12%. A similar inhibition of D-[$^3$H]mannitol uptake (10–17%) suggests this was due to a nonspecific decrease in the membrane permeability. Other cations such as $Ba^{2+}$ and $Mg^{2+}$ had no effect, but $La^{3+}$ inhibited $Na^+$ and $Cl^-$ uptake to the same degree as $Ca^{2+}$. Calmodulin (2 μM) in combination with $Ca^{2+}$ (1 μM, free concentration) significantly inhibited $Na^+$ uptake when $Cl^-$ was the anion by 21–32% and $Cl^-$ uptake when $Na^+$ was the cation by 20–27%. This effect was completely reversed by 10 μM trifluoperazine. When gluconate was the anion or when choline was the cation, $Na^+$ or $Cl^-$ uptake was unaffected by $Ca^{2+}$/calmodulin and trifluoperazine. The $K_i$ for $Ca^{2+}$ inhibition of $Cl^-$-coupled $Na^+$ uptake was reduced from 200 μM to 0.2 μM by incubation with 20 μM calmodulin. The $K_i$ for exogenously added calmodulin studied at 1 μM $Ca^{2+}$ was 0.2 μM. The $K_i$ for trifluoperazine inhibition of the $Ca^{2+}$/calmodulin response was 3 μM. These results represent compelling evidence for intracellular $Ca^{2+}$/calmodulin regulation of coupled NaCl transport across the intestinal microvillus membrane. The exact mechanism of this regulation remains to be delineated.**

Recent *in vitro* studies of intestine indicate a role for intracellular calcium in the regulation of basal and stimulated active electrolyte transport. Alterations in serosal solution $Ca^{2+}$ concentration change $Na^+$ and $Cl^-$ fluxes in a fashion that cannot be ascribed to modifications of epithelial passive permeability (1–4). Calcium deprivation increases $Na^+$ and $Cl^-$ absorption, whereas high $Ca^{2+}$ concentrations inhibit $Na^+$ and $Cl^-$ absorption, cause $Cl^-$ secretion, or both. The effect of $Ca^{2+}$ deprivation is reproduced by the $Ca^{2+}$-channel blocker verapamil (1). Furthermore, NaCl absorption is inhibited or $Cl^-$ secretion is stimulated by the $Ca^{2+}$ ionophore A23187 (3, 4), by serotonin (5), and by carbamoylcholine (3) only if $Ca^{2+}$ is present in the serosal solution. The serotonin effect is also prevented by verapamil (5).

Calmodulin may be necessary for $Ca^{2+}$-regulated transport. This protein has been identified in the intestinal microvillus, where it is primarily associated with the microfilament core (6, 7). Indirect evidence for calmodulin-mediated transport comes from inhibition of electrolyte secretion by trifluoperazine (TFP) (8, 9). However, the concentration of TFP necessary in whole gut for such an effect (100–500 μM) is far above that reported for other $Ca^{2+}$/calmodulin-dependent processes (10, 11).

The mechanism of calcium's effect is not known, but it could involve inhibition of coupled NaCl influx across the apical cell membrane as postulated by Field for cyclic nucleotides as well (12). Indeed, cAMP inhibition of coupled NaCl influx has been demonstrated in rabbit ileum (13) and gall bladder (14).

We have recently demonstrated a coupled NaCl influx process in membrane vesicles of rabbit ileal villus brush border (15). These vesicles were isolated in $Ca^{2+}$-free solutions and, therefore, represent a system in which to study $Ca^{2+}$/calmodulin regulation of electrolyte transport. In the experiments reported here, we present direct evidence for such a process.

## EXPERIMENTAL PROCEDURES

**Materials.** D-[$^3$H]Mannitol, $^{36}Cl^-$, and $^{125}$I-labeled calmodulin radioimmunoassay kits were obtained from New England Nuclear; $^{22}Na^+$ and $^{45}Ca^{2+}$ were from Amersham and ICN, respectively. Tris, Hepes, and EGTA were obtained from Sigma. Calmodulin (isolated from rat testis) was obtained from CAABCO (Houston, TX). TFP was a generous gift from Smith Kline & French. Purified troponin C was a gift from T. C. Vanaman (Duke University Medical Center). Rabbit antiserum against aminopeptidase from human small intestinal brush border was generously provided by W. D. Heizer (University of North Carolina School of Medicine).

**Preparation of Brush Border Membrane Vesicles.** Isolation and characterization of brush border membrane vesicles from rabbit ileal villus epithelial cells have been described (15). Enterocytes obtained by incubation and then vibration of ileal segments of 3- to 4-kg male New Zealand White rabbits in $Ca^{2+}$-free EGTA solutions are collected by centrifugation in a $Ca^{2+}$-free solution and homogenized in an Omni-mixer (Sorvall), and the brush border fraction was separated by a sucrose density gradient ultracentrifugation of the pellet after suspension with a Dounce homogenizer. The enrichment of maltase, a brush border membrane marker enzyme, for each preparation was between 16- and 23-fold. Usually batches of vesicles were prepared from several animals and then pooled and stored in liquid nitrogen for transport studies the following week.

To ascertain the orientation of our vesicles, electron microscopy of vesicles negatively stained with phosphotungstate revealed that virtually all had exterior particles similar to the knobs observed on the external surface of intestinal microvilli (courtesy of C. R. Hackenbrock, University of North Carolina School of Medicine). To quantitate the percentage of right-side-out vesicles, the immunological accessibility of brush border aminopeptidase to external antibody was determined as described by Haase *et al.* (16). In our vesicles, 85% inhibition of aminopeptidase activity was obtained with rabbit antiserum to human brush border aminopeptidase. Thus, a minimum of 85% of our vesicles are right-side-out.

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Abbreviation: TFP, trifluoperazine.

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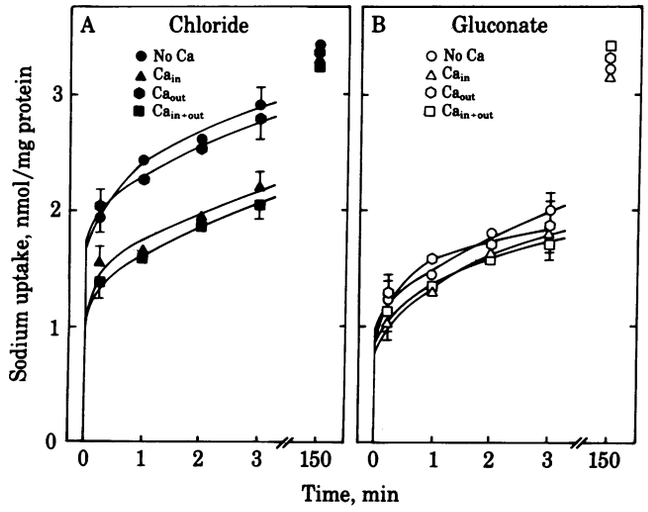


FIG. 1. Effect of 1 mM $Ca^{2+}$ on the inside, outside, or both sides of the vesicle membranes on $Na^+$ uptake in the presence of $Cl^-$ (*A*) or gluconate (*B*). The membrane vesicles were loaded in a solution (inside) containing 1 mM $CaCl_2$ or EGTA and 2 mM NaCl/98 mM KCl (*A*) or 2 mM sodium gluconate/98 mM potassium gluconate (*B*), 100 mM mannitol, 20 mM Tris/Hepes at pH 7.4 and then incubated at 22°C in the transport solution (outside) containing 1 mM $CaCl_2$ or EGTA, 2 mM $^{22}NaCl$/98 mM KCl (*A*) or 2 mM $^{22}Na$ gluconate/98 mM potassium gluconate (*B*), 100 mM mannitol, and 20 mM Tris/Hepes at pH 7.4.

**Measurement of Vesicle Calmodulin.** Calmodulin concentrations in membrane vesicles were determined by radioimmunoassay using preheated calmodulin standards. Assays were performed before and after solubilization of the vesicles in 1% Triton X-100 at room temperature for 30 min. In addition, vesicles were preincubated with 10 μM calmodulin for 4 hr at 4°C and then washed in 100 mM mannitol/20 mM Hepes buffer adjusted to pH 7.4 with Tris, and the assays were repeated. Calmodulin concentrations in our vesicles were compared to those isolated by the $Ca^{2+}$ precipitation method (17).

**Transport Studies.** Uptakes of $^{22}Na^+$, $^{36}Cl^-$, and D-[$^3$H]-mannitol were determined as follows (15, 18): membrane vesicles were preincubated at 4°C for 4 hr in predesignated solutions (as indicated in the figure legends) that also contained 20 μg of valinomycin per mg of vesicle protein. Uptake was initiated by the addition of membrane vesicles (12 mg/ml) to transport medium containing isotopes and other solutes as described in the figure legends. These vesicles were incubated at 22°C in a final volume of 300 μl. Samples (50 μl) were removed at intervals and transferred to 2 ml of ice-cold stopping solution containing 100 mM mannitol, 150 mM KCl, and 10 mM Tris/Hepes, pH 7.4. Vesicles were rapidly filtered and washed on membrane filters (Millipore, 0.45 μm pore diameter) and were dried and dissolved in a Triton X-100/xylene-based scintillation fluid and their radioactivities were measured. Uptake was calculated from the transport medium specific activities and expressed as nmol/mg of protein, based on Lowry protein determinations (19) using bovine serum albumin as standards. All experiments were repeated a minimum of three times and the data are presented as the means ± SEM.

Preparation of solutions with $Ca^{2+}$ concentrations in the micro- and nanomolar range was facilitated by the computer program of Perin and Sayce (20) for calculating free $Ca^{2+}$ concentrations in $Ca^{2+}$–EGTA-buffered solutions. Absolute stability constants for the interactions of EGTA and ATP with $H^+$, $Ca^{2+}$, and $Mg^{2+}$ were chosen from Fabiato (21).

Preliminary experiments indicated that $Na^+$, $Cl^-$, and $Ca^{2+}$ uptake into the vesicles reached equilibrium after incubation for 3–4 hr at 4°C. This assured us that ionic concentrations, particularly $Ca^{2+}$, inside the vesicle were those prepared and calculated from the equilibrium constants.

## RESULTS

Fig. 1 shows the effect of $Ca^{2+}$ location on the time course of $Na^+$ uptake by the brush border membrane vesicles. Sodium uptake in the presence of $Cl^-$ was rapid (>40% at 15 sec), and the uptake values of control and $Ca^{2+}$-treated vesicles at 150 min were similar. The latter result indicates that the different uptake of $Na^+$ under the four conditions tested was not due to a different volume or size of the vesicles. When $Cl^-$ was the counter anion (Fig. 1*A*), 1 mM $Ca^{2+}$ inhibited $Na^+$ uptake by 22–30% when $Ca^{2+}$ was present on either the inside or both sides of the membrane vesicles ($P < 0.01$). No effect was observed when $Ca^{2+}$ was present on the outside of the vesicle.

Fig. 2 demonstrates the effect of $Ca^{2+}$ on $Cl^-$ uptake by the brush border vesicles. When $Na^+$ was the counter cation, $Ca^{2+}$ (1 mM) on the inside or both sides of the vesicle inhibited $Cl^-$ uptake by 29–35%, but no inhibition was observed when $Ca^{2+}$ was present only on the outside (Fig. 2*A*).

The $Ca^{2+}$ inhibition of $Na^+$ and $Cl^-$ uptake could be due to a decrease either in $Na^+$ and $Cl^-$ binding or in transmembrane

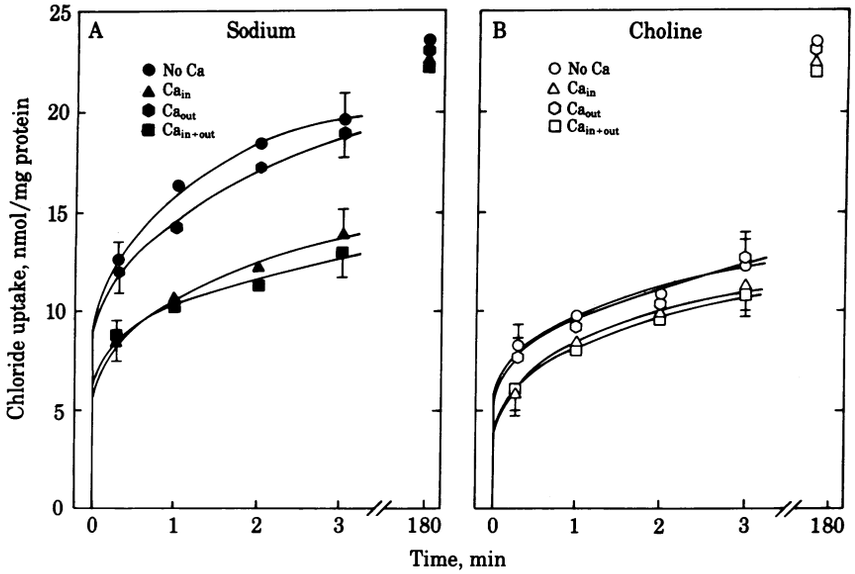


FIG. 2. Effect of 1 mM $Ca^{2+}$ on the inside, outside, or both sides of the vesicle membranes on $Cl^-$ uptake in the presence of $Na^+$ (*A*) or choline (*B*). The membrane vesicles were loaded in a solution containing 1 mM calcium acetate or EGTA, 20 mM NaCl, 100 mM mannitol, and 20 mM Tris/Hepes at pH 7.4 and then incubated in a transport solution containing 1 mM calcium acetate or EGTA and 20 mM $Na^{36}Cl$ (*A*) or choline $^{36}Cl$ (*B*), 100 mM mannitol, and 20 mM Tris/Hepes at pH 7.4.

Table 1. Calmodulin concentrations in rabbit ileal brush border membrane vesicles

| Vesicle preparation (*n*) | Calmodulin, pmol/mg protein: Before Triton | After Triton |
|---|---|---|
| Calcium-precipitated vesicles (5) | 17.1 ± 0.9* | 68.3 ± 11.5† |
| Sucrose density gradient vesicles (7) | 4.9 ± 0.3 | 49.8 ± 2.0† |
| Sucrose density gradient vesicles incubated with 10 μM calmodulin (7) | 13.4 ± 1.4* | 85.1 ± 6.2*† |

*$P < 0.01$ as compared to sucrose density gradient vesicles.
†$P < 0.01$ as compared to before Triton X-100.

transport. To determine which, uptake experiments were performed in transport solutions of various osmolalities. A plot of $Na^+$ and $Cl^-$ uptakes versus the reciprocal of osmolality showed the same intercept on the ordinate, but different slopes, in the presence or absence of inside $Ca^{2+}$. Thus, the decrease in electrolyte uptake by $Ca^{2+}$ was due to inhibition of transport into the intravesicular space. Furthermore, the inhibition was virtually complete within the first 15 sec, indicating a decrease in unidirectional influx rather than an increased efflux.

A small but not statistically significant effect of $Ca^{2+}$ location on $Na^+$ uptake was found when gluconate was the counter anion (Fig. 1*B*), whereas the small (9–12% inhibition) effect of inside $Ca^{2+}$ on $Cl^-$ uptake when choline was the counter cation (Fig. 2*B*) was significant ($P < 0.05$). The slight inhibition by $Ca^{2+}$ of $Na^+$ uptake in the presence of gluconate and of $Cl^-$ uptake in the presence of choline could be due to an effect of $Ca^{2+}$ on the general permeability of the brush border vesicles. To examine this possibility, the effect of $Ca^{2+}$ on D-[$^3$H]mannitol uptake was determined. $Ca^{2+}$ (1 mM) only on the inside or both sides of the vesicle significantly ($P < 0.05$) inhibited mannitol uptake by 10–17%. No effect on mannitol uptake was noted at $Ca^{2+}$ concentrations less than 10 μM (data not shown).

The effects of other polyvalent cations (1 mM $Ba^{2+}$, $Mg^{2+}$, and $La^{3+}$) on $Na^+$ uptake in the presence of $Cl^-$ and on $Cl^-$ uptake in the presence of $Na^+$ were investigated. $Ba^{2+}$ and $Mg^{2+}$ had no significant effect, but $La^{3+}$ inhibited $Na^+$ and $Cl^-$ uptake to the same degree as $Ca^{2+}$. The inhibitory effect of $Ca^{2+}$, but not $La^{3+}$, could be reversed by incubating preloaded vesicles in $Ca^{2+}$- or $La^{3+}$-free solution for 4 hr at 4°C.

Many $Ca^{2+}$-regulated cellular functions are calmodulin dependent. In order to understand the effect of calmodulin on $Ca^{2+}$ inhibition of $Na^+$ and $Cl^-$ uptake, it was necessary to measure the concentration of endogenous calmodulin in our membrane preparation and to determine if exogenously added calmodulin would increase the measured concentration. Table 1 indicates that the calmodulin concentration of calcium-precipitated vesicles (17) was significantly greater than the concentration in our vesicles, which were isolated by a sucrose density gradient technique (15). In addition, two pools of calmodulin are evident: one pool is immunologically accessible to the externally added antibody; the other pool is revealed only after solubilization of the vesicles in Triton X-100 and is 4–10 times larger. Incubation of our vesicles with 10 μM calmodulin for 4 hr at 4°C significantly increased the calmodulin concentration in the Triton-accessible space.

The simple addition of calmodulin to the external transport solution had no effect on $Ca^{2+}$ inhibition of $Cl^-$-coupled $Na^+$ uptake. Four hours of incubation of the vesicles with 1 μM calmodulin did have an effect, as shown in Fig. 3*A*. Compared to the response to $Ca^{2+}$ alone (Fig. 3*B*), calmodulin shifted the dose–response curve for $Ca^{2+}$ inhibition of $Na^+$ uptake to the left by nearly 3 $\log_{10}$ units (Fig. 3*C*). Incubation with higher concentrations had little additional effect. The $K_i$ values for $Ca^{2+}$

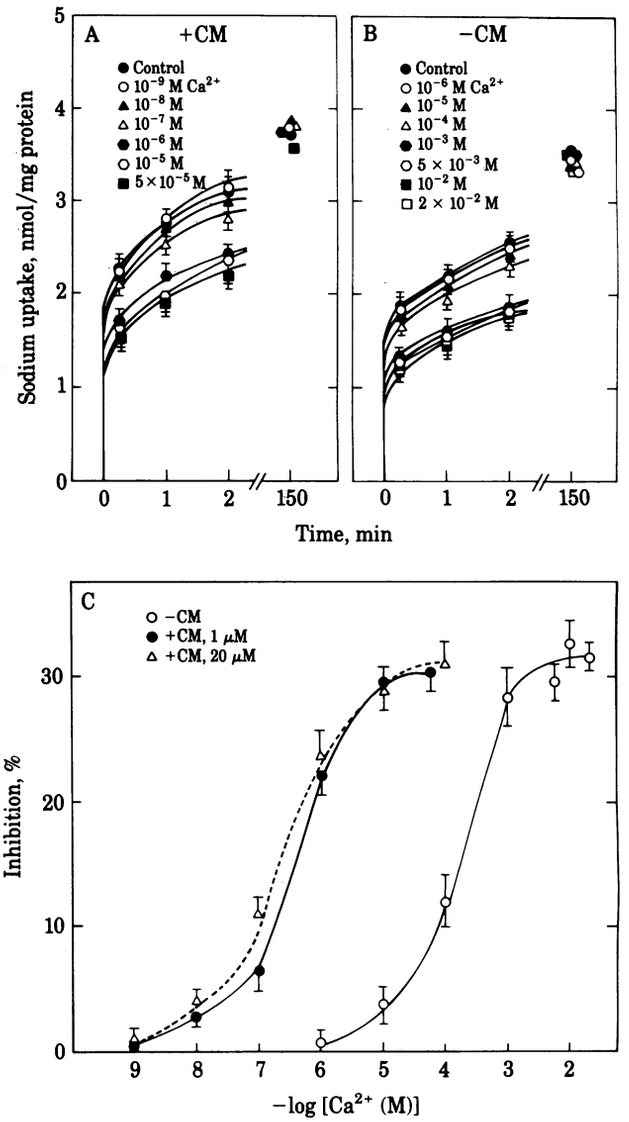


FIG. 3. Effect of various free $Ca^{2+}$ concentrations on $Na^+$ uptake in the presence or absence of calmodulin (CM). (*A*) The membrane vesicles were incubated at 4°C for 4 hr in a solution containing various concentrations of free $Ca^{2+}$ ($10^{-9}$ to $5 \times 10^{-5}$ M), 1 μM calmodulin, 2 mM NaCl/98 mM KCl, 100 mM mannitol, and 20 mM Tris/Hepes at pH 7.4. The transport solution had the same composition as the inside solution except 2 mM $^{22}$NaCl replaced NaCl. (*B*) The membrane vesicles were incubated at 4°C for 4 hr in a solution containing $10^{-6}$ to $2 \times 10^{-2}$ M $Ca^{2+}$, 2 mM NaCl/98 mM KCl, 100 mM mannitol, and 20 mM Tris/Hepes at pH 7.4. The transport solution contained 2 mM $^{22}$NaCl/98 mM KCl, 100 mM mannitol, and 20 mM Tris/Hepes buffer at pH 7.4. (*C*) Results in *A* and *B* are plotted as the percentage inhibition of $Na^+$ uptake at 15 sec (solid lines). In addition, the inhibition of $Na^+$ uptake by 20 μM calmodulin in the same experimental conditions as *A* is shown (broken line). The $K_i$ values for $Ca^{2+}$ inhibition of $Na^+$ uptake by 20, 1, and 0 μM calmodulin were 0.2, 0.5, and 200 μM free $Ca^{2+}$, respectively.

inhibition after incubation with 20, 1, and 0 μM calmodulin were 0.2, 0.5, and 200 μM $Ca^{2+}$, respectively.

In separate dose–response experiments (not shown), the $K_i$ for inhibition by exogenous calmodulin studied at 1 μM $Ca^{2+}$ was found to be 0.2 μM. To determine the specificity of the calmodulin effect, troponin C was studied with 1 μM $Ca^{2+}$. No inhibition of $Cl^-$-coupled $Na^+$ uptake could be determined even after incubation with troponin C at 100 μM.

We next investigated whether the calmodulin-binding neuroleptic TFP would inhibit the calmodulin-dependent effect on electrolyte transport. At high concentration such drugs react

with membrane lipids and have nonspecific effects on cellular and membrane functions. A significant inhibition of $Na^+$ uptake in $Ca^{2+}$/calmodulin-free solutions was noted with TFP concentration $10^{-4}$ M or greater ($P < 0.05$ at $10^{-3}$ M). TFP concentrations $10^{-5}$ M or less had no effect on $Na^+$ uptake; therefore, we studied the ability of $10^{-5}$ M TFP to prevent the inhibition of $Na^+$ and $Cl^-$ uptake by 1 $\mu$M $Ca^{2+}$ in the presence of 2 $\mu$M calmodulin. At these concentrations, $Ca^{2+}$, calmodulin, and TFP alone had no effect on $Na^+$ or $Cl^-$ uptake, and $Ca^{2+}$ plus calmodulin had no effect on mannitol uptake. As illustrated in Fig. 4*A*, $Ca^{2+}$/calmodulin inhibited $Na^+$ uptake in the presence of $Cl^-$ by 21–32%. TFP (10 $\mu$M) reversed this inhibitory effect. On the other hand, when gluconate replaced $Cl^-$ in the transport solution, there was no stimulation of $Na^+$ uptake, and no effect of $Ca^{2+}$, calmodulin, or TFP would be observed (Fig. 4*B*). Formal dose–response studies (not shown) indicated the $K_i$ for TFP to be 3 $\mu$M.

Similar experiments of the effect of $Ca^{2+}$/calmodulin and TFP on $Cl^-$ uptake in the presence of $Na^+$ and choline are shown in Fig. 5. $Ca^{2+}$/calmodulin inhibited $Cl^-$ uptake in the presence of $Na^+$ by 20–27% and TFP (10 $\mu$M) reversed this effect (Fig. 5*A*). No effect of any agent could be seen when choline was the counter cation (Fig. 5*B*).

The studies depicted in Figs. 4 and 5 were performed after incubation of the vesicles in a solution containing ATP and $Mg^{2+}$ in addition to $Ca^{2+}$ and calmodulin. However, the degree of inhibition (20–30%) was no greater than that observed when ATP and $Mg^{2+}$ were omitted (Fig. 3). The lack of effect of ATP preincubation was confirmed in formal comparison experiments in which $Na^+$ uptake was determined after incubation with identical solutions containing $10^{-6}$ or $10^{-7}$ M free $Ca^{2+}$ and 20 $\mu$M calmodulin, with or without 5 mM ATP and 2 mM

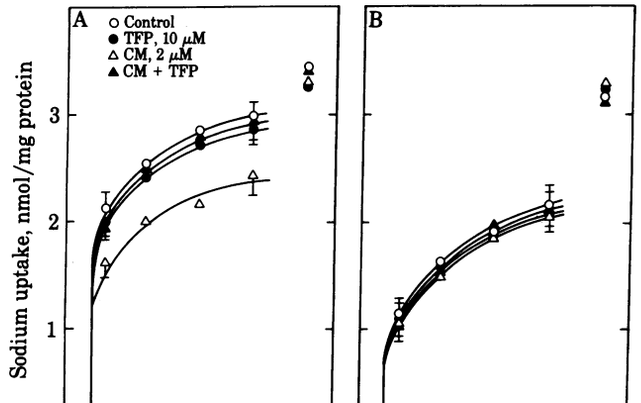


FIG. 4. (*A*) Inhibition of $Cl^-$-coupled $Na^+$ uptake by $Ca^{2+}$/calmodulin (CM) and reversal of this effect by the addition of TFP. (*B*) Lack of a $Ca^{2+}$/calmodulin effect when gluconate is the anion. Vesicle suspensions were split into four aliquots. In *A*, vesicles were incubated for 2–4 hr at 4°C in 2 mM NaCl/98 mM KCl/2 mM $MgSO_4$/5 mM ATP/100 mM mannitol/20 mM Tris/Hepes, pH 7.4, and this alone was the composition of the incubation solution for controls (○). For the second aliquot (△), 1 $\mu$M free $Ca^{2+}$ and 2 $\mu$M calmodulin were also present in the incubation solution. In the third aliquot, TFP (10 $\mu$M final concentration) was added to the control solution after 2 hr of incubation and the vesicles were incubated for an additional 2 hr (●). In the fourth aliquot, 1 $\mu$M $Ca^{2+}$ and 2 $\mu$M calmodulin were present in the incubation solution and TFP (10 $\mu$M final concentration) was added after 2 hr (▲). The transport solution had the same $Na^+$, $Cl^-$, mannitol, and Tris/Hepes concentrations as the inside solution except that $^{22}NaCl$ replaced NaCl. In *B*, sodium gluconate and potassium gluconate replaced the $Cl^-$ salts.

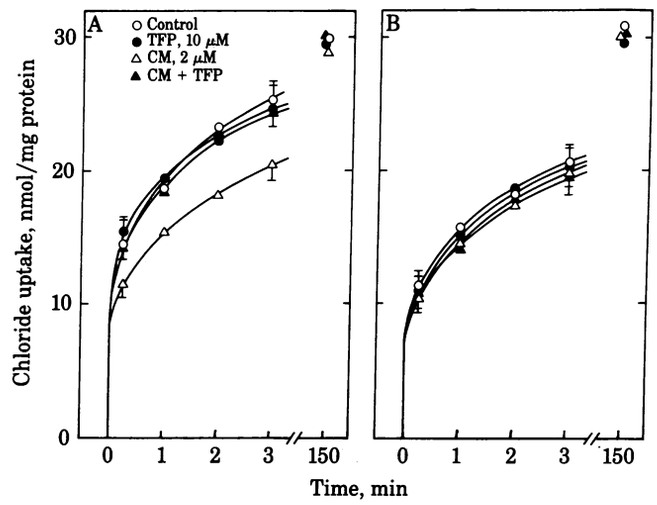


FIG. 5. (*A*) Inhibition of $Na^+$-coupled $Cl^-$ uptake by $Ca^{2+}$/calmodulin (CM) and reversal of this effect with TFP. (*B*) Lack of inhibition of $Cl^-$ uptake by $Ca^{2+}$/calmodulin when choline replaces $Na^+$. Controls (○) were incubated for 4 hr at 4°C in 20 mM NaCl/2 mM $MgSO_4$/5 mM ATP/100 mM mannitol/20 mM Tris/Hepes, pH 7.4. For a second aliquot of vesicles (△), 1 mM free $Ca^{2+}$ and 2 $\mu$M calmodulin were present in the incubation solution. TFP at 10 $\mu$M was added to the incubation solution of a third aliquot (●). For the fourth aliquot, 1 $\mu$M $Ca^{2+}$ and 2 $\mu$M calmodulin were present in the incubation solution, and after 2 hr 10 $\mu$M TFP was added and incubation was continued for an additional 2 hr (▲). The transport solutions had the same ionic composition as the inside solutions except $Na^{36}Cl$ replaced NaCl. In *B*, choline Cl replaced NaCl.

$MgSO_4$. Preliminary studies have demonstrated considerable ATP hydrolysis under these conditions, so it is doubtful that ATP entered the vesicles in significant concentrations.

## DISCUSSION

We have used isolated membrane vesicles for studying the role of $Ca^{2+}$/calmodulin in regulating $Na^+$ and $Cl^-$ transport. We have purified brush border membrane vesicles with a technique not involving calcium precipitation and have previously presented evidence for an electrically neutral, carrier-mediated, coupled $Na^+$–$Cl^-$ transport process (15). However, the nature of the coupling of $Na^+$ to $Cl^-$ transport remains uncertain. We also demonstrated $Na^+$:$H^+$ and $Cl^-$:$OH^-$ (or $HCO_3^-$) antiport and showed inhibition of $Na^+$-dependent $Cl^-$ uptake by $Na^+$ transport inhibitors such as harmaline or amiloride and inhibition of $Cl^-$-dependent $Na^+$ uptake by anion-exchange inhibitors such as 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid (SITS). Therefore, as suggested by Leidtke and Hopfer (22, 23), it may be simultaneous $Na^+$:$H^+$ and $Cl^-$:$OH^-$ exchange rather than directly coupled NaCl influx that accounts for the comovements of $Na^+$ and $Cl^-$ across the villus cell apical membrane.

In the studies reported here, we present direct evidence for $Ca^{2+}$/calmodulin-mediated inhibition of apical membrane NaCl uptake. The effect of $Ca^{2+}$ on NaCl coupled uptake was found to be specific for the inside of the vesicle, was reversible, and was not the result of altered $Na^+$ or $Cl^-$ binding to the vesicle membrane. The inhibition was observed in the first 15 sec and therefore represents a decrease in influx rather than an increase in efflux. The effect was not mimicked by other divalent cations, such as $Ba^{2+}$ and $Mg^{2+}$, although $La^{3+}$ was equally effective. The $La^{3+}$ effect could not be reversed.

These findings contrast with those of Schulz and Heil (24) in cat pancreatic membrane vesicles, where $Ca^{2+}$ outside the vesicle decreased membrane $Na^+$ permeability, whereas $Ca^{2+}$ inside increased permeability. Those effects might well have been

nonspecific because they were reproduced by other divalent cations. Indeed, we found both nonspecific and specific alterations in permeability in our studies. $Ca^{2+}$ concentrations of 1 mM decreased $Na^+$ uptake in the presence of gluconate, $Cl^-$ uptake in the presence of choline, and mannitol uptake by a similar degree (10–17%). It is likely that the general membrane permeability of our vesicles, initially isolated in $Ca^{2+}$-free media, was decreased by addition of millimolar concentrations of $Ca^{2+}$. A similar nonspecific effect on permeability was demonstrated with TFP at greater than $10^{-5}$ M, concentrations at which the drug may interact with the vesicle membrane (25).

When studied at $Ca^{2+}$, calmodulin, and TFP concentrations that by themselves had no effect on electrolyte or mannitol transport, a TFP-inhibitable, $Ca^{2+}$/calmodulin-regulated NaCl uptake process was demonstrated. After incubation with 20 $\mu$M calmodulin, the $K_i$ for $Ca^{2+}$ inhibition of coupled NaCl uptake was reduced three orders of magnitude, from 200 to 0.2 $\mu$M. This inhibition was specific for calmodulin (troponin C had no effect) and was reversed by 10 $\mu$M TFP. The $K_i$ (3 $\mu$M) for TFP reversal of the $Ca^{2+}$/calmodulin inhibition of NaCl coupled uptake is similar (1–10 $\mu$M) to that for inhibition of brain cyclic nucleotide phosphodiesterase (26, 27), muscle protein kinase (28), and pituitary prolactin secretion (29).

The concentration of $Ca^{2+}$ necessary for half-maximal inhibition of NaCl coupled uptake after incubation with 1 $\mu$M calmodulin was 0.5 $\mu$M, and there was a clearly discernible effect at 0.1 $\mu$M $Ca^{2+}$. This concentration of free $Ca^{2+}$ is certainly in the range expected during stimulus–secretion coupling. This value is also similar to that reported in other calmodulin-dependent systems such as $Ca^{2+}$,$Mg^{2+}$-ATPase (30, 31) and intestinal $Ca^{2+}$ transport (32).

The concentration of exogenous calmodulin incubated with our vesicles that achieved half-maximal inhibition of $Na^+$ uptake in the presence of 1 $\mu$M $Ca^{2+}$ (0.2 $\mu$M) was in the range of that reported for other calmodulin-dependent systems such as $Ca^{2+}$,$Mg^{2+}$-ATPase (4–43 nM) (30, 31), $Ca^{2+}$ transport across rat small intestinal basolateral membranes (0.12 $\mu$M) (32), and protein kinases found in sarcoplasmic reticulum (0.07 $\mu$M) (33) and pancreatic islet cell membranes (0.36 $\mu$M) (34). Because it is necessary to incubate calmodulin with the vesicles (which already contain endogenous calmodulin) before an effect is noted, the $K_i$ for exogenously added calmodulin in our system is difficult to interpret. We cannot be certain which calmodulin pool is responsible, but the important pool is probably the calmodulin that is accessible only after vesicle solubilization—i.e., a pool associated with the lipid in the membrane or actually in the intravesicular space.

Calcium-dependent inhibition of apical cell membrane $Na^+$ permeability has been demonstrated previously in tight epithelia such as frog skin (35) and toad urinary bladder (36, 37). A role for $Ca^{2+}$ and calmodulin in the regulation of intestinal NaCl transport is evident from the present studies and might be inferred for gall bladder and renal tubule, leaky epithelia that also have similar coupled NaCl apical membrane entry processes. Therefore, these results suggest that calcium (and calmodulin) plays an important role in regulating salt absorption in a variety of epithelia.

The mechanism of $Ca^{2+}$/calmodulin inhibition of NaCl transport remains unclear. $Ca^{2+}$/calmodulin could act directly on the ion carriers. Alternatively, protein kinase-mediated phosphorylation of membrane proteins might be responsible; Taylor *et al.* (38) have reported a $Ca^{2+}$/calmodulin-sensitive phosphorylation of rabbit ileal membrane proteins with kinetic values similar to those found here. Exogenous ATP was not necessary to demonstrate a $Ca^{2+}$/calmodulin effect in our membranes. However, this issue needs to be investigated in more detail with methods that ensure entrance of exogenous ATP into the vesicle. An understanding of the specific mechanism of $Ca^{2+}$/calmodulin action should help clarify the intracellular control of epithelial electrolyte transport.

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