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Calcium-induced inhibition of taurine transport in brush-border membrane vesicles from rabbit small intestine

Yusei Miyamoto, Palaniappan Kulanthaivel, Vadivel Ganapathy, Gary M. Whitford and Frederick H. Leibach

Department of Cell and Molecular Biology, Medical College of Georgia, Augusta, GA (U.S.A.)

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The influence of Ca^{2+} on the activity of the taurine transport system was investigated in rabbit small intestinal brush-border membrane vesicles. Preincubation of the brush-border membrane vesicles with Ca^{2+} prepared by the Mg^{2+}-aggregation method markedly decreased the NaCl gradient-dependent uptake of taurine in these vesicles. Uptake of glucose and alanine, both dependent on a Na^+ gradient, were also decreased by Ca^{2+}-treatment, but their reduction was very small compared with that of taurine uptake. The inhibitory effect of Ca^{2+} was dose- and time-dependent. The inhibition was reduced by the presence of ethylene glycol-bis(β-amino ethyl ether)-*N,N,N'-N'*-tetraacetic acid during treatment of the membrane vesicles with Ca^{2+}. Neomycin partially protected the taurine transporter activity from the Ca^{2+}-induced inhibition, but indomethacin did not. 5-Nitro-2-(3-phenylpropylamino)benzoate, a Cl^--channel blocker, did not increase taurine uptake in the Ca^{2+}-treated membrane vesicles. It is concluded that the Ca^{2+}-induced inhibition of taurine uptake in rabbit intestinal brush-border membrane vesicles is not due to accelerated dissipation of the ion gradient driving forces across the membrane but rather to a direct effect on the transporter, most likely mediated by the activation of the membrane-bound phospholipase C.

Introduction

Many recent reviews have focused on the biological role of taurine, β-aminoethanesulfonic acid, which is an essential compound in animal nutrition [1–4]. Efficient intestinal absorption is crucial to meet the nutritional requirements for this amino acid, particularly in developing animals. This has prompted a number of studies on the mechanisms involved in the transfer of taurine and other β-amino acids across the intestinal epithelium. The purpose of all these investigations was to seek an answer to a basic question: does the intestinal mucosal cell possess a specific transport system for β-amino acids? Unfortunately, these studies have produced contradictory answers to this important question. In fact, there are as many studies which answered the question with a 'yes' [5–12] as those which answered with a 'no' [13–18]. This is intriguing, because contradictory results have been obtained even in the same animal species. For example, we have recently demonstrated the presence of a β-amino acid transport system in rabbit small intestine using isolated brush-border membrane vesicles [11], whereas Stevens et al. [16] and Schell et al. [17] were unable to detect mediated transport of β-alanine in the same animal species using a similar preparation. It seems likely that problems associated with the experimental techniques employed in these studies might have been at least partly responsible for these apparently contradictory results. One obvious difference between the vesicle studies was that those [8,11] which were able to demonstrate the presence of the transport system in the small intestine utilized brush-border membrane vesicles prepared by the Mg^{2+}-EGTA-aggregation method whereas those [16,17] which failed utilized membrane vesicles prepared by the Ca^{2+}-aggregation method. This observation suggested that the presence of Ca^{2+} during isolation of the membrane vesicles might have a deleterious effect on this transport system. Since the vesicles prepared in the presence of Ca^{2+} have been successfully used to investigate the transport systems which serve for many

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid; Tris, tris(hydroxymethyl)aminomethane; EGTA, ethylene glycol-bis (β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; NPPB, 5-nitro-2-(3-phenylpropylamino)benzoate.

Correspondence: F.H. Leibach, Department of Cell and Molecular Biology, Medical College of Georgia, Augusta, GA 30912–2100, U.S.A.

non-β-amino acids, it appears that the effect of Ca^{2+} is specific for the β-amino acid transporter. This prompted us to compare in a recent paper [11] the uptake of taurine in brush-border membrane vesicles prepared by the Ca^{2+}-aggregation method and the Mg^{2+}-EGTA-aggregation method. In this study we found that the activity of the intestinal β-amino acid transporter in brush-border membrane vesicles isolated in the presence of Ca^{2+} was only 10% of the activity in the vesicles isolated in the presence of Mg^{2+}-EGTA. The purpose of the current investigation was to study the mechanisms responsible for the Ca^{2+}-induced reduction in the activity of the β-amino acid transporter.

Materials and Methods

Materials. [2(n)-^{3}H]Taurine (specific radioactivity, 20.1 or 30.0 Ci/mmol), D-[1(n)-^{3}H]glucose (specific radioactivity, 15.5 Ci/mmol) and L-α-[3-^{3}H]alanine (specific radioactivity, 84.0 Ci/mmol) were purchased from DuPont-New England Nuclear, Boston, MA, U.S.A. EGTA, neomycin sulfate, and indomethacin were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. 5-Nitro-2-(3-phenylpropylamino)benzoate was a gift from Dr. Peter S. Reinach, Department of Physiology and Endocrinology, Medical College of Georgia. All other chemicals were of analytical grade.

Preparation of brush-border membrane vesicles. New Zealand white adult rabbits were used to prepare intestinal brush-border membrane vesicles. Rabbits were killed by intravenous administration of a lethal dose of Nembutal. The proximal intestine consisting of the duodenum and the jejunum was taken out and used for the membrane preparation. Brush-border membrane vesicles were isolated from scraped mucosa by a Mg^{2+}-aggregation method in the presence of EGTA as described previously [19]. The membrane vesicles were suspended in 10 mM Hepes-Tris buffer (pH 7.5), containing 300 mM mannitol. The protein concentration of the final membrane suspension was adjusted to 10 mg/ml. The membrane suspension was divided into small aliquots and stored in liquid nitrogen until use.

Treatment of the membrane vesicles. Brush-border membrane vesicles were treated with Ca^{2+} prior to measurement of taurine uptake. Membrane vesicles treated with Mg^{2+} under similar conditions served as control. Chloride salts of Ca^{2+} and Mg^{2+} were used in these experiments. Membrane vesicles were mixed with $MgCl_2$ or $CaCl_2$ solution and incubated at 37°C for a desired time. Protein concentration during this treatment was 7.5 mg/ml. Loading of the vesicles with neomycin or indomethacin was done by a freeze-thaw procedure described previously [20,21]. Stock solutions of neomycin and indomethacin were prepared in the membrane suspension buffer and in ethanol, respectively. Control membrane vesicles were mixed with buffer or ethanol and taken through the freeze-thaw procedure under similar conditions.

Uptake studies. Uptake measurements were made at room temperature (22–23°C) by a rapid filtration technique using Millipore filters (DAWP type, 0.65 μm pore size) as described previously [22]. Uptake was initiated by mixing 40 μl of membrane suspension (300 μg protein) with 160 μl of uptake buffer containing radiolabeled substrates. The uptake buffer was 10 mM Hepes-Tris (pH 7.5) containing 150 mM NaCl. Uptake was terminated by adding 3 ml of the ice-cold stop buffer (5 mM Hepes-Tris, 155 mM KCl (pH 7.5)) and the mixture was filtered. The filter was washed with 3 × 5 ml of the stop buffer and then transferred to a counting vial. The radioactivity associated with the filter was measured by liquid scintillation spectrometry.

Determination of the calcium content in membrane preparations. The total calcium concentrations in intestinal brush-border membrane vesicle preparations were determined by atomic absorption spectrometry (Varian, Model Spectra A-20) using a nitrous oxide/acetylene flame. The membrane suspensions were diluted in 2000 ppm KCl solution prior to the experiment. The calcium standards contained 0.5, 1.0 and 2.0 μg calcium/ml in 2000 ppm KCl solution.

Each experiment was done with two or three different membrane preparations. The results are expressed as mean ± S.E. Statistical difference was determined by Student's *t*-test. A P value less than 0.05 was considered significant.

Results

Uptake of taurine in brush-border membrane vesicles treated with Mg^{2+} or Ca^{2+}

The time course of taurine uptake was studied in rabbit intestinal brush-border membrane vesicles which were preincubated with either Mg^{2+} or Ca^{2+}. The concentration of the cations during this treatment was 1 mM and the incubation was carried out at 37°C for 1 h. Following the incubation, uptake of taurine (0.2 μM) was measured in these vesicles in the presence of an inwardly directed NaCl gradient. At the initiation of the uptake measurement, the concentrations of Na^+ and Cl^- in the intravesicular and extravesicular media were as follows: $[Na^+]_o = 120$ mM; $[Na^+]_i = 0$; $[Cl^-]_o = 120$ mM; $[Cl^-]_i = 2$ mM. The intravesicular Cl^- was due to the use of chloride salt of Mg^{2+} or Ca^{2+} during the preincubation. Fig. 1 compares the uptake in Mg^{2+}-treated vesicles with that in Ca^{2+}-treated vesicles. Active transport of taurine in Mg^{2+}-treated vesicles was evident from the pronounced 'overshoot', the intravesicular concentration of taurine at the peak of the overshoot (5–10 min) being 8-fold greater than the intravesicular concentration at 180 min. On the other hand, taurine uptake was drastically reduced in Ca^{2+}-

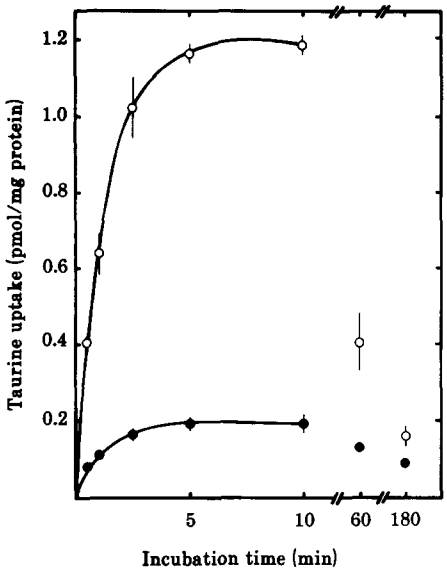

Fig. 1. Effects of Mg^{2+}-treatment and Ca^{2+}-treatment on the time course of taurine uptake in intestinal brush-border membrane vesicles. Brush-border membrane vesicles were incubated with either 1 mM $MgCl_2$ (○) or 1 mM $CaCl_2$ (●) in 10 mM Hepes-Tris buffer (pH 7.5) containing 300 mM mannitol. The incubation was carried out at 37°C for 1 h. Following the incubation, uptake of taurine (final concentration, 0.2 μM) was measured in these vesicles in the presence of an inwardly directed NaCl gradient. The results are given as mean ± S.E. When not shown, the standard error lies within the symbol.

treated vesicles and the active transport of taurine was barely noticeable. The initial rates of uptake measured with short incubations were reduced by about 80% in Ca^{2+}-treated vesicles compared to Mg^{2+}-treated vesicles. These results indicate that the treatment of the intestinal brush-border membrane vesicles with Ca^{2+} leads to severe impairment of taurine uptake.

In order to see whether this Ca^{2+}-induced inhibition of taurine uptake is due to a nonspecific effect of the cation on the properties of the membrane vesicles, we compared the uptake of two other organic solutes, glucose and alanine, in Ca^{2+}- and Mg^{2+}-treated vesicles. Uptakes of taurine (0.3 μM), glucose (1 μM) and alanine (1 μM) were measured with a 30 s incubation in the presence of an inwardly directed NaCl gradient after the treatment of the membrane vesicles with either Mg^{2+} or Ca^{2+}. Table I shows that the uptakes of glucose and of alanine were also reduced by treatment of the vesicles with Ca^{2+}, but the reduction in uptake was much less compared to the reduction in taurine uptake. These data suggest that taurine uptake in intestinal brush-border membrane vesicles was much more severely affected by preincubation with Ca^{2+} than the other uptake systems.

Dose- and time-dependency of the Ca^{2+}-induced inhibition of taurine uptake

The effect of increasing concentrations of Ca^{2+} during the treatment on the uptake of taurine was then

TABLE I

Effect of Ca^{2+}-treatment on the uptakes of taurine, glucose and alanine

Brush border membrane vesicles were incubated with 0.25 mM Ca^{2+} (as $CaCl_2$) for 1 h at 37°C. Membrane vesicles incubated under similar conditions with 0.25 mM Mg^{2+} (as $MgCl_2$) served as controls. After incubation, the uptakes of taurine, glucose and alanine were measured in these vesicles in the presence of an inwardly directed NaCl gradient. Final concentrations of taurine, glucose and alanine during uptake measurement were 0.3, 1 and 1 μM, respectively. The results are given as mean ± S.E. ($n = 6$; two membrane preparations).

Substrate	Taurine uptake			
	Mg^{2+}-treatment		Ca^{2+}-treatment	
	pmol/mg/30 s	%	pmol/mg/30 s	%
Taurine	0.74 ± 0.07	100	0.38 ± 0.02	51
Glucose	18.72 ± 0.95	100	15.52 ± 0.09	83
Alanine	1.79 ± 0.01	100	1.45 ± 0.07	81

studied. We have shown earlier that Cl^- ions play an obligatory role in taurine uptake in rabbit intestinal brush-border membrane vesicles [11]. Because the chloride salt of Ca^{2+} was used in the current study, increasing the concentration of Ca^{2+} during preincubation also results in an increase in the concentration of Cl^- within the vesicles. Therefore, membrane vesicles which were treated with corresponding concentrations of $MgCl_2$ were used as controls in order to minimize the influence of the Cl^- effect, if any, on the interpretation of the experimental data. The results given in Fig. 2 show that the uptake of taurine slightly increased (30–40%) in membrane vesicles which were treated with $MgCl_2$. On the contrary, the presence of $CaCl_2$ during

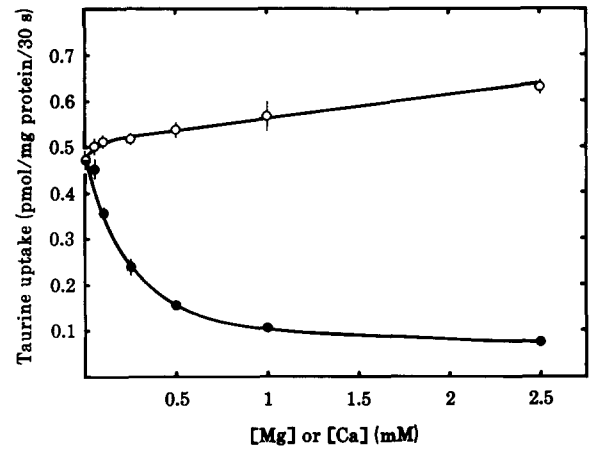

Fig. 2. Dose-response of the Ca^{2+}-induced inhibition of taurine uptake. Brush-border membrane vesicles were incubated with increasing concentrations of either $MgCl_2$ (○) or $CaCl_2$ (●) in 10 mM Hepes-Tris buffer (pH 7.5) containing 300 mM mannitol. The incubation was carried out at 37°C for 1 h. Following the incubation, NaCl gradient-dependent uptake of taurine (final concentration, 0.3 μM) was measured in these vesicles using a 30 s incubation. The data represent mean ± S.E. When not shown, the standard error lies within the symbol.

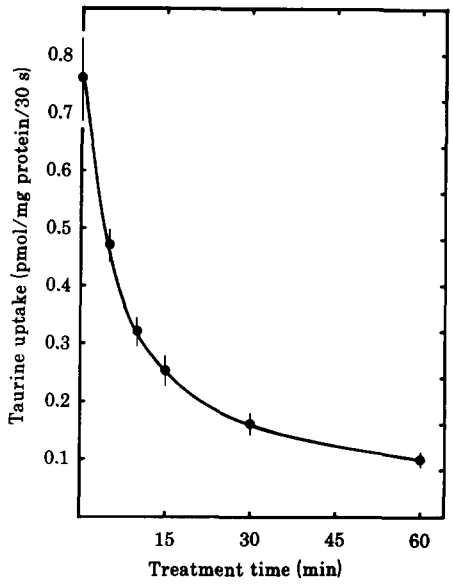

Fig. 3. Dependence of the Ca^{2+}-induced inhibition of taurine uptake on the time of incubation with Ca^{2+}. Brush-border membrane vesicles were incubated with 1 mM $CaCl_2$ in 10 mM Hepes-Tris buffer (pH 7.5) containing 300 mM mannitol. Incubation was carried out at 37°C for varying time periods. Following the incubation, NaCl gradient-dependent uptake of taurine (final concentration, 0.3 μM) was measured in these vesicles using a 30 s incubation. The data represent mean ± S.E.

preincubation decreased the uptake of taurine. This decrease was due to Ca^{2+} rather than Cl^- because a similar effect was not observed in vesicles which were treated with $MgCl_2$. The inhibitory effect of Ca^{2+} on taurine uptake increased as the concentration of Ca^{2+} during preincubation increased. At a concentration of 0.25 mM, Ca^{2+} caused approx. 50% inhibition which increased to about 80% at 2.5 mM Ca^{2+}.

Fig. 3 shows that the Ca^{2+}-induced inhibition of taurine uptake is also a time-dependent phenomenon. In this experiment, the membrane vesicles were treated with 1 mM Ca^{2+} for different time periods and subsequently taurine uptake in these vesicles was measured. The Ca^{2+}-induced inhibition of taurine uptake increased as the time of incubation of the vesicles with Ca^{2+} increased.

Influence of EGTA on the Ca^{2+}-induced inhibition of taurine uptake

The experiments described thus far demonstrate that incubation of intestinal brush-border membrane vesicles with Ca^{2+} results in a time-dependent inhibition of taurine uptake. In order to get an insight into the underlying mechanism, the effect of EGTA, a Ca^{2+} chelator, on the Ca^{2+}-induced inhibition of taurine uptake was studied (Table II). The membrane vesicles were treated with either 0.5 mM Mg^{2+} or 0.5 mM Ca^{2+} in the presence and absence of 1 mM EGTA. The initial rates of taurine uptake (0.3 μM) were measured in these vesicles using a 30 s incubation. Taurine uptake increased by about 60% in membrane vesicles treated with Mg^{2+} plus EGTA compared to the uptake in vesicles treated with Mg^{2+} alone. The presence of Ca^{2+} during treatment caused about 65% inhibition in taurine uptake. But this Ca^{2+}-induced inhibition was totally eliminated if treatment of the vesicles with Ca^{2+} was done in the presence of EGTA. It is clear from this experiment that free Ca^{2+} is necessary to cause the inhibition of taurine uptake.

The observation that the presence of Mg^{2+} plus EGTA during treatment resulted in an increase in taurine uptake compared to the control value measured in vesicles treated with Mg^{2+} alone was interesting. These results suggested that the membrane vesicles prepared by the Mg^{2+}-EGTA-aggregation method might still contain an appreciable amount of endogenous Ca^{2+}. When we measured the calcium content in these membrane preparations, it became evident that this indeed was the case. With two different preparations, the values for the calcium content were 29 and 25 nmol/mg of protein. Since we used 7.5 mg protein/ml during treatment, the concentration of the endogenous calcium under these conditions was approx. 0.2 mM.

Effects of phospholipase inhibitors on the Ca^{2+}-induced inhibition of taurine uptake

Because Ca^{2+} is required for the activity of phospholipase C and of phospholipase A_2, it is possible that the activation of either one of these enzymes may mediate the Ca^{2+} effect on taurine uptake. To test whether the Ca^{2+}-induced inhibition of taurine uptake is the result of Ca^{2+}-dependent activation of phospholipase C, we studied the influence of neomycin on the Ca^{2+} effect. Neomycin, an aminoglycoside antibiotic, is a known inhibitor of phospholipase C and it influences the enzyme activity by binding to the inositol phospholipids which are substrates for the enzyme [23–27]. The membrane vesicles were preloaded with 10 mM neomycin after which they were incubated with either 0.25 mM

TABLE II

Effect of EGTA on the Ca^{2+}-induced inhibition of taurine uptake

Brush-border membrane vesicles were incubated with either 0.5 mM $MgCl_2$ or 0.5 mM $CaCl_2$ in the presence and absence of 1 mM EGTA. Incubation was carried out for 1 h at 37°C. After incubation, uptake of taurine (0.3 μM) was measured in these vesicles with a 30 s incubation. The results are given as mean ± S.E. ($n = 6$; two membrane preparations).

Treatment	Taurine uptake	
	pmol/mg/30 s	%
Mg^{2+}	0.50 ± 0.01	100
Ca^{2+}	0.16 ± 0.01	32
Mg^{2+} + EGTA	0.80 ± 0.01	100
Ca^{2+} + EGTA	0.54 ± 0.05	68

TABLE III

Effect of neomycin on the Ca^{2+}-induced inhibition of taurine uptake

Brush-border membrane vesicles were preloaded with 10 mM neomycin by freezing and thawing. Membrane vesicles treated in a similar way but in the absence of neomycin served as controls. Neomycin-loaded and control vesicles were subsequently incubated with either 0.25 mM Ca^{2+} or 0.25 mM Mg^{2+} for 1 h at 37°C. After incubation, uptake of taurine (0.3 μM) was measured in these vesicles with a 30 s incubation. The results are given as mean ± S.E. ($n = 6$; two membrane preparations).

Treatment	Taurine uptake	
	pmol/mg/30 s	%
Mg^{2+}	0.60 ± 0.04	100
Ca^{2+}	0.27 ± 0.03	44
Mg^{2+} + neomycin	0.47 ± 0.02	100
Ca^{2+} + neomycin	0.33 ± 0.03	70

Mg^{2+} or 0.25 mM Ca^{2+}. Without neomycin, Ca^{2+} treatment caused 55% inhibition in taurine uptake (Table III). This inhibition was however reduced to 30% ($P < 0.01$) if neomycin was present. These data suggest that the inhibitory effect of Ca^{2+} on taurine uptake can be significantly reduced by neomycin, thus implicating phospholipase C in the observed Ca^{2+} effect. We also studied the effects of indomethacin, an inhibitor of phospholipase A_2 on the Ca^{2+} effect. In contrast to neomycin, indomethacin was not able to attenuate the Ca^{2+} effect (data not shown).

Effect of NPPB, a chloride channel blocker, on the Ca^{2+}-induced inhibition of taurine uptake

A transmembrane Cl^- gradient can serve as a driving force for concentrative uptake of taurine in intestinal brush-border membrane vesicles in the presence of Na^+ [11]. Because these membranes possess a Cl^- channel [28] which can be activated by Ca^{2+}, it is possible that the Ca^{2+} effect on taurine uptake is due to a more rapid dissipation of the Cl^- gradient in the presence of Ca^{2+} than Mg^{2+} via the Ca^{2+}-activatable Cl^- channel. To test this possibility, we studied the influence of NPPB, a Cl^- channel blocker [29] on the Ca^{2+} effect. As can be seen in Table IV, NPPB was unable to reverse the Ca^{2+}-induced inhibition of taurine uptake, thus ruling out the involvement of a Cl^- channel in the observed Ca^{2+} effect.

TABLE IV

Effect of NPPB, a Cl^--channel blocker, on the Ca^{2+}-induced inhibition of taurine uptake

Brush-border membrane vesicles were incubated with either 0.25 mM Mg^{2+} or 0.25 mM Ca^{2+} for 1 h at 37°C. After incubation, uptake of taurine (0.3 μM) was measured in these vesicles with a 30 s incubation in the presence and absence of 20 μM NPPB. The results are given as mean ± S.E. ($n = 6$; two membrane preparations).

Treatment	Taurine uptake			
	Control		NPPB	
	pmol/mg/30 s	%	pmol/mg/30 s	%
Mg^{2+}	0.48 ± 0.01	100	0.46 ± 0.01	100
Ca^{2+}	0.21 ± 0.01	43	0.20 ± 0.01	44

Discussion

We have demonstrated in the present study that when intestinal brush-border membrane vesicles prepared in the presence of Mg^{2+}-EGTA were incubated with Ca^{2+}, taurine transport in these vesicles was drastically reduced. This Ca^{2+}-induced inhibition was not instantaneous, but required preincubation. The inhibition also increased with increased concentration of Ca^{2+} during preincubation. The presence of EGTA, a Ca^{2+}-chelator, during the incubation of the vesicles with Ca^{2+} completely abolished the Ca^{2+} effect, indicating that free Ca^{2+} was necessary to elicit this effect. Even though Ca^{2+} treatment also inhibited Na^+-dependent glucose and alanine transport activities, the Ca^{2+}-induced inhibition in these cases was considerably smaller compared to the inhibition of taurine transport. It thus seems highly probable that this Ca^{2+} effect was the reason why membrane vesicles prepared in the presence of Ca^{2+} exhibited little or no transport activity toward taurine or β-alanine [9,16,17].

Brush-border membrane vesicles from polarized cells such as intestinal and renal epithelial cells are generally prepared by either the Ca^{2+}-aggregation method or by the Mg^{2+}-aggregation method. However, there are indications that the presence of Ca^{2+} during the isolation procedure may induce changes in the ion permeabilities of these membrane vesicles [30]. Experimentally imposed transmembrane ion gradients collapse at a faster rate in membrane vesicles prepared by the Ca^{2+}-aggregation method than in membrane vesicles prepared by the Mg^{2+}-aggregation method. This effect of Ca^{2+} on the ion permeabilities leads to a reduction in the transport of organic solutes which are driven by transmembrane ion gradients [21,31,32]. It has been suggested that the Ca^{2+} effect is mediated via Ca^{2+}-activated phospholipases, in particular, phospholipase C [21,30]. Because of this well-documented effect of Ca^{2+} on membrane permeabilities, there is a growing tendency in recent years to avoid Ca^{2+} during the isolation of brush-border membrane vesicles and instead use Mg^{2+} in place of Ca^{2+} as the aggregating divalent cation. The inclusion of EGTA to chelate endogenous Ca^{2+} has also been found to be a desirable modification of the membrane isolation procedure.

The present study suggests that phospholipase C may mediate the Ca^{2+}-induced inhibition of taurine transport in rabbit intestinal brush-border membrane vesicles. The Ca^{2+}-activated phospholipase A_2 and Ca^{2+}-

activated Cl^- channel do not appear to play any role in this Ca^{2+} effect. Furthermore, since the β-amino acid transport system is much more drastically inhibited by incubation of the vesicles with Ca^{2+} than are the glucose- and α-amino acid transport systems, it appears that the accelerated collapse of transmembrane ion gradients, the driving force for taurine transport, may not be the sole mechanism for the Ca^{2+}-induced inhibition of taurine uptake in intestinal brush-border membrane vesicles. Activation of the membrane-bound phospholipase C by Ca^{2+} may have additional effects which have a more direct bearing on the activity of the β-amino acid transport system than the collapse of the ion gradients. For example, the transport system may have specific requirements for certain phospholipids to be optimally active and these lipids may be hydrolyzed by the Ca^{2+}-activated phospholipase C, thus leading to the inactivation of the transport system.

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