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Modulation of Na$^+$/alanine cotransport in liver sinusoidal membrane vesicles by internal divalent cations

Thomas W. Simmons [1], Richard H. Moseley [2], James L. Boyer [3] and Nazzareno Ballatori [1]

[1] *Department of Biophysics, Environmental Health Sciences Center, University of Rochester School of Medicine, Rochester, NY,* [2] *Department of Medicine, University of Michigan School of Medicine and Veterans Administration Medical Center, Ann Arbor, MI and* [3] *Department of Medicine and Liver Center, Yale University School of Medicine, New Haven, CT (U.S.A.)*

Rat liver basolateral plasma membrane (blLPM) vesicles resuspended in 5 mM Mg^{2+}-, Ca^{2+}-, Mn^{2+}- or Co^{2+}-containing media exhibited a markedly lower rate of Na$^+$-stimulated L-alanine transport. Divalent cation inhibition of L-alanine uptake was dose dependent, and was observed only when the vesicles were pre-loaded with the divalent cations. The presence or absence of the metal ions in the extravesicular incubation media had no effect on L-alanine transport. Conversely, pretreatment of the vesicles with 0.2 mM of either EGTA or EDTA resulted in higher initial rates of L-alanine transport. This stimulation was overcome by addition of excess divalent cation to the vesicle suspension solution. Since these blLPM vesicles are primarily oriented right-side-out, the divalent cation inhibition of L-alanine transport appears to be a result of their interaction with cytosolic components of the cell membrane. Total Na$^+$ flux as measured with ^{22}Na$^+$ was not affected by intravesicular 5 mM Mg^{2+} or Ca^{2+}, indicating that the inhibition was not due to dissipation of the Na$^+$ gradient. These observations suggest that intracellular divalent cations may serve to modulate L-alanine transport across the liver cell plasma membrane.

Introduction

Divalent metal cations such as Mg^{2+}, Ca^{2+}, Mn^{2+}, and Co^{2+} serve as co-factors in approximately one-third of all enzyme systems, including membrane-associated enzymes; they serve to chelate metabolic intermediates (e.g. ATP and ADP, α-amino acids, and biogenic amines); and they may function as second or third messengers in cellular signal transduction [1]. Divalent cations also bind to anionic sites on the polar head groups of neighboring membrane phospholipids, thereby decreasing the mobility of phospholipids and increasing membrane rigidity [2,3]. Recent evidence indicates that divalent cations may also interact directly with integral membrane proteins to alter their structural or catalytic functions. The K$^+$/Cl$^-$ co-transporter of the sheep red blood cell plasma membrane has been shown to possess a binding site for divalent metal ions, which when occupied inactivates the K$^+$/Cl$^-$ co-transporter [4]. Similarly, the activity of the electroneutral K$^+$/H$^+$ exchanger in mitochondria appears to be controlled by the concentration of Mg^{2+} in the mitochondrial matrix [5]. In intestinal brush-border membrane vesicles, the Na$^+$-coupled transport of bile acids, glucose, and valine was significantly reduced by the presence of intravesicular Ca^{2+} [6,7]. Our previous studies on the effects of Ca^{2+} on liver plasma membrane fluidity and Na$^+$-coupled taurocholate transport showed that intravesicular concentrations of calcium as low as 0.2 mM were able to significantly inhibit Na$^+$-coupled taurocholate transport [8].

Despite the wide range of divalent cation-induced alterations of membrane structure and function, the effects of divalent cations on transport processes in membrane vesicles are not well characterized, even though chloride salts of metals are routinely added to solutions used in studies with vesicles. The present study is an outgrowth of attempts to define optimal conditions for studying amino acid transport in rat liver basolateral plasma membrane (blLPM) vesicles. The results reveal that inclusion of Mg^{2+} or Ca^{2+} in the

Correspondence: N. Ballatori, Department of Biophysics, Box EHSC, University of Rochester School of Medicine, Rochester, NY 14642, U.S.A.

membrane suspension solution inhibits Na^+-coupled alanine transport. Although the nature of the inhibition is not known, this process has implications for our understanding of hepatic amino acid transport. In addition, our findings should discourage routine addition of divalent cations to vesicle suspension solutions.

Materials and Methods

Preparation of rat liver plasma membrane vesicles. The methods for isolating canalicular (cLPM) and blLPM subfractions, as well as their morphologic and biochemical characterization are described in detail elsewhere [9,10]. In brief, a 'mixed LPM' subfraction was first separated out of a 'crude nuclear pellet' by rate zonal floatation (44/36.5%, w/w, sucrose density interface) in the TZ-28 (Sorvall) zonal rotor. After tight homogenization (Type B Dounce homogenizer, 75 up and down strokes) the vesiculated cLPM and blLPM were separated by high speed centrifugation (195 000 × g_{ave} for 3 h) of the mixed LPM through a 3-step sucrose gradient (31,34, and 38%, w/w), and the membranes were collected at 105 000 × g_{ave} for 60 min. Freshly isolated membranes were divided into aliquots which were resuspended in 300 mM sucrose and 20 mM Hepes-Tris (pH 7.5) containing either no additions (controls), divalent cations (i.e., 5 mM $CaCl_2$, $MgCl_2$, $MnCl_2$, $CoCl_2$), chelating agents (i.e., 0.2 mM EDTA, EGTA), or both. The exact composition of the suspension media is given in the figure and table legends of the individual experiments. The membranes were stored at $-80°C$ until used.

Freeze-fracture analysis revealed that approx. 73% of blLPM vesicles exhibited 'right-side-out' configuration, in which the extravesicular membrane face corresponds to the sinusoidal surface in vivo [11].

The purity of each membrane preparation was routinely analyzed by measurement of Na^+/K^+-ATPase and Mg^{2+}-ATPase activities [12], and γ-glutamyltransferase activity [13]. Protein was determined according to Lowry et al. [14], using bovine serum albumin as a standard.

Transport measurements. Frozen membrane suspensions were quickly thawed by immersion in a $40°C$ water bath, diluted to the desired protein concentration, and passed repeatedly (10 times) through a 25 gauge needle. Uptake of L-[^{3}H]alanine was measured by a rapid Millipore filtration technique. Membrane suspensions (50–100 μg of protein in 20 μl) were preincubated at $25°C$ for 5–10 min. Uptake studies were initiated by the addition of 80 μl of incubation medium also prewarmed to $25°C$, containing L-[^{3}H]alanine. The exact composition of the incubation media is given in the figure and table legends of the individual experiments. Transport was terminated by the addition of 3 ml of ice-cold stop solution; 100 mM NaCl, 100 mM sucrose,

0.2 mM $CaCl_2$, 5 mM $MgCl_2$, and 20 mM Hepes-Tris (pH 7.5). Membrane vesicle-associated ligand was separated from free ligand by immediate rapid filtration (1 ml·s^{-1}) through a 0.45 μm Millipore filter (HAWP). The filter was washed twice with 3 ml of stop solution, dissolved in 4.5 ml of Safety-Count (Research Products International, Mount Prospect, IL) and counted in a Packard TRI-CARB 4350 liquid scintillation counter. Uptake of ^{22}Na was measured by a similar method except that filters were dissolved in Redisolv HP (Beckman Instruments, Palo Alto, CA) and counted in a Beckman LS 1801 liquid scintillation counter. Nonspecific binding of isotope to the membranes was determined in each experiment by addition of cold stop solution to 20 μl of membrane suspension prior to addition of incubation solution. This blank was subtracted from all determinations. Unless otherwise indicated, all incubations were performed in triplicate and all observations confirmed with three or more separate membrane preparations.

All values are expressed as mean ± S.D. The data were compared by the paired t-test and differences were considered to be statistically significant at $P < 0.05$.

Metal analysis. Total calcium levels in buffers and membrane suspensions were determined by atomic absorption spectroscopy according to Christensen [15].

Chemicals. L-[3-^{3}H]alanine (84 Ci·mmol^{-1}) was obtained from New England Nuclear, Boston, MA (U.S.A.); and ^{22}Na from Amersham Corp., Arlington Heights, IL (U.S.A.). All other chemicals and reagents were purchased from either Sigma Chemical Co., St. Louis, MO (U.S.A.), Baker Chemical Co., Phillipsburg, NJ (U.S.A.), or Schwarz/Mann Inc., Spring Valley, NY (U.S.A.).

Results

The time course of L-[^{3}-H]alanine uptake into blLPM vesicles was examined in the absence (Fig. 1A and B) and presence (Fig. 1C and D) of 5 mM intravesicular $MgCl_2$. The concentration of $MgCl_2$ in the incubation media (extravesicular space) was also varied from 0 to 5 mM (Fig. 1). In the absence of Mg^{2+} from both the intravesicular and extravesicular media, transport of L-alanine was stimulated by a Na^+ gradient and an overshoot was observed (Fig. 1A). Comparable results have been reported in similar membrane preparations [10,16]. The presence of 5 mM Mg^{2+} in the incubation media (Fig. 1B), did not influence L-alanine uptake by the blLPM vesicles. However, when the vesicles were preloaded with Mg^{2+} (Fig. 1C and D), the Na^+-gradient dependent transport of L-alanine was significantly decreased, regardless of the extravesicular concentration of Mg^{2+}. The 60 min uptakes (equilibrium values) of L-alanine were similar under all conditions suggesting that vesicle size was unaffected by Mg^{2+} (Fig. 1).
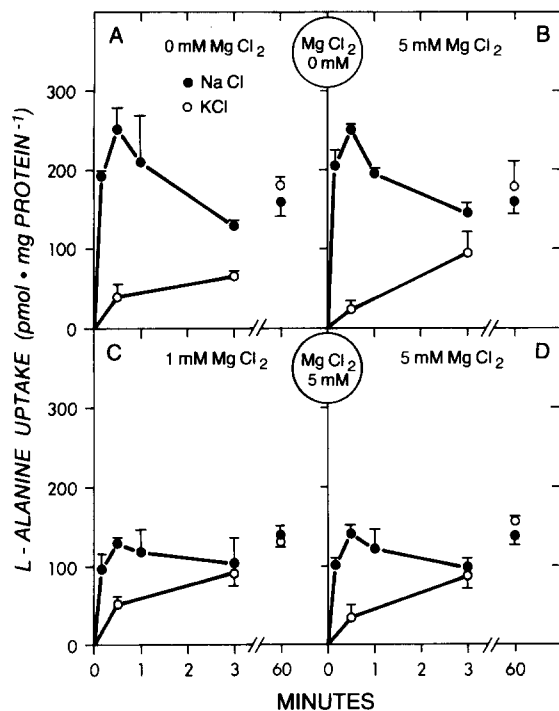
Fig. 1. Effects of intra- and extravesicular Mg^{2+} on L-alanine uptake by blLPM vesicles. Freshly isolated membrane vesicles were divided into two aliquots: one aliquot (panels A and B) was resuspended in a solution containing 300 mM sucrose and 20 mM Hepes-Tris (pH 7.5) without added $MgCl_2$; while the other aliquot (panels C and D) was resuspended in 300 mM sucrose, 20 mM Hepes-Tris (pH 7.5) and 5 mM $MgCl_2$. Uptake of 0.2 mM L-[^{3}H]alanine was measured at 25°C in an incubation solution containing 100 mM of either NaCl (●) or KCl (○), 20 mM Hepes-Tris (pH 7.5) and with (panels B and D) or without (panels A and C) $MgCl_2$, to give the extravesicular $MgCl_2$ concentrations indicated in the figure. Isoosmolarity of the incubation solutions was maintained with sucrose. Results are shown for one representative experiment of three with each data point (mean ± S.D.) performed in triplicate.

Furthermore, the effects of Mg^{2+} were exclusively on the Na^+-coupled uptake mechanism, since uptake in the presence of a K^+ gradient was unaffected.

The inhibitory effects of Mg^{2+} on L-alanine uptake by the blLPM vesicles were mimicked by similar concentrations of Ca^{2+} (Fig. 2). Thus, in $CaCl_2$-preloaded blLPM vesicles (Fig. 2C and D) Na^+-stimulated uptake of L-alanine was significantly lower than in control vesicles (Fig. 2A and B), while extravesicular Ca^{2+} did not appear to influence alanine transport.

To determine if the inhibitory effect of Mg^{2+} was dose-dependent, the time course of L-alanine uptake into vesicles pre-loaded with concentrations of $MgCl_2$ ranging from 0 to 5 mM was measured. The Na^+-stimulated uptake of L-alanine was inversely related to intravesicular Mg^{2+} concentrations (Fig. 3A). A similar inverse relationship was observed between intravesicular Ca^{2+} and L-alanine uptake in vesicles pre-loaded with the same concentrations of $CaCl_2$ (Fig. 3B). As previously shown in Figs. 1 and 2, Na^+-independent

L-alanine uptake and equilibrium values were unaffected by intravesicular Mg^{2+} (Fig. 3A) and Ca^{2+} (Fig. 3B).

Although exogenous $CaCl_2$ was not added to vesicle suspensions used as controls, these suspensions may contain micromolar levels of contaminating Ca^{2+}, as reported by Vaandragen et al. [7]. To examine this issue, total calcium content of the suspension buffer and of the vesicle suspension solution was measured by atomic absorption. Unsupplemented suspension buffer and vesicle suspension solution were found to contain 1.0 μM calcium and 72.9 ± 6.2 nmol calcium per mg protein ($n = 4$), respectively, suggesting substantial binding of calcium to blLPM vesicles. Therefore, the chelator EGTA was used to lower intravesicular ionized calcium, and uptake of L-alanine was measured under these conditions (Table I). Intravesicular EGTA (0.2 mM) enhanced the initial rate of Na^+-stimulated uptake of L-alanine by 17% (Table I), suggesting that micromolar levels of Ca^{2+} can influence L-alanine uptake. Addition of excess Ca^{2+} (5 mM) overcame this enhancement and reduced uptake by 37% compared to control values. In the absence of EGTA, Ca^{2+} inhibited the initial rate of uptake by 33%. The Na^+-independent uptake of L-alanine and the equilibrium values were unaffected by intravesicular Ca^{2+} or EGTA (Table I).

Initial rates of L-alanine uptake were similarly affected by intravesicular Mg^{2+} and/or EDTA. A 29% increase in uptake was seen in vesicles pre-loaded with
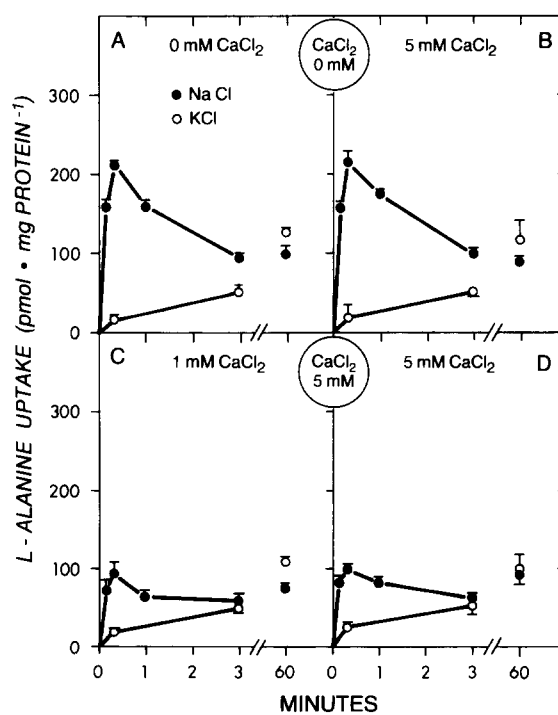

Fig. 2. Effects of intra- and extravesicular Ca^{2+} on L-alanine uptake by blLPM vesicles. The experiment illustrated in Fig. 1 was repeated with $CaCl_2$ instead of $MgCl_2$. See the legend to Fig. 1 for explanatory information.
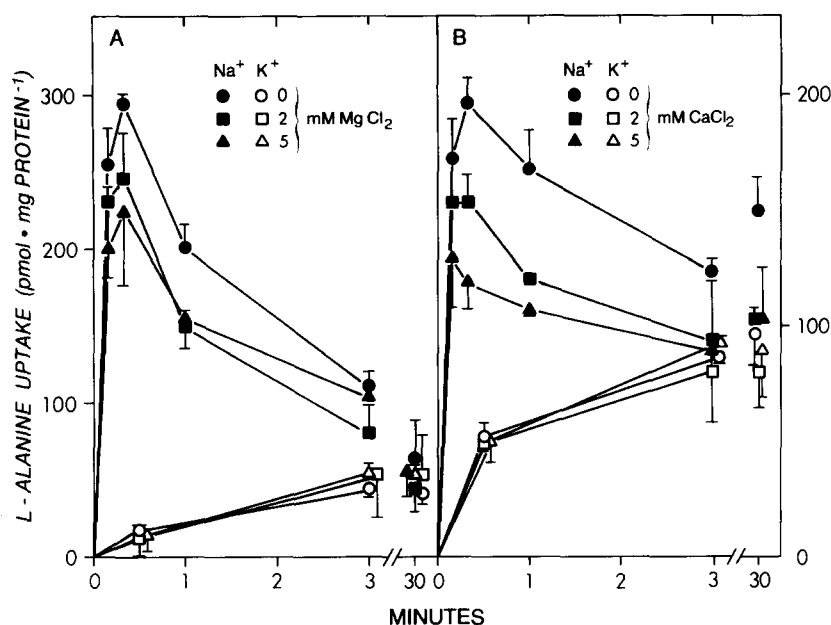
Fig. 3. Effects of increasing concentrations of intravesicular Mg^{2+} and Ca^{2+} on L-alanine uptake by blLPM vesicles. Vesicles were pre-loaded as described in Fig. 1, with 300 mM sucrose, 20 mM Hepes-Tris (pH 7.5) and either no (●, ○), 2 mM (■, □) and 5 mM (▲, △) $MgCl_2$ (A) or $CaCl_2$ (B). Uptake of 0.2 mM L-[³H]alanine was measured at 25°C in an incubation solution containing 20 mM Hepes-Tris (pH 7.5) and 100 mM of either NaCl (●, ■, ▲) or KCl (○, □, △). Isoosmolarity of the incubation solution was maintained with sucrose. Results are shown for one representative experiment of three with each time point (mean ± S.D.) performed in triplicate.

EDTA, while addition of excess (5 mM) Mg^{2+} overcame this stimulation (Table II). Intravesicular Mg^{2+} alone inhibited uptake by 52%. Equilibrium values and Na^+-independent uptake were not significantly affected by intravesicular Mg^{2+} or EDTA (Table II).

To assess whether inhibition of Na^+-stimulated L-alanine uptake was limited to Mg^{2+} and Ca^{2+} or was a property of divalent cations in general, the effects of

intravesicular Co^{2+} and Mn^{2+} were studied. Initial rates of L-alanine uptake were reduced by 54% and 49% in the presence of 5 mM intravesicular $CoCl_2$ and $MnCl_2$, respectively (Table III). These cations did not affect Na^+-independent L-alanine uptake.

It is possible that divalent cations may have indirectly inhibited Na^+-coupled alanine transport by affecting Na^+ permeability, and therefore the concentration gradient which energizes Na^+/alanine cotransport. Separate laboratories using intestinal brush-border membrane vesicles have associated inhibition of Na^+-coupled solute uptake by intravesicular divalent cations

TABLE I

Effects of intravesicular EGTA on intial rates of L-alanine uptake by blLPM vesicles

Vesicles were pre-loaded with 300 mM sucrose, 20 mM Hepes-Tris (pH 7.5), and either no additions (control), 0.2 mM EGTA, 5 mM $CaCl_2$, or both 0.2 mM EGTA and 5 mM $CaCl_2$. Uptake of 0.2 mM L-[³H]alanine by blLPM vesicles at 10 s and 60 min was measured at 25°C in an incubation solution containing 20 mM Hepes-Tris (pH 7.5), and either 100 mM NaCl or KCl. Data are presented as mean ± S.D. of three experiments, each performed in triplicate. * Significantly different from control values at $P < 0.05$.

Extravesicular	Intravesicular	pmol alanine per mg protein	
		10 s	60 min
100 mM NaCl	control	153 ± 29	92 ± 15
	EGTA	179 ± 27 *	98 ± 23
	$CaCl_2$	97 ± 14	95 ± 22
	$EGTA + CaCl_2$	103 ± 19	91 ± 25
100 mM KCl	control	28 ± 15	84 ± 10
	EGTA	33 ± 14	89 ± 24
	$CaCl_2$	20 ± 5	79 ± 16
	$EGTA + CaCl_2$	26 ± 11	90 ± 15

TABLE II

Effects of intravesicular EDTA on initial rates of L-alanine uptake by blLPM vesicles

The experiment presented in Table I was repeated with 0.2 mM EDTA and 5 mM $MgCl_2$ instead of EGTA and $CaCl_2$, respectively. See legend to Table I for details. * Significantly different from control values at $P < 0.05$.

Extravesicular	Intravesicular	pmol alanine per mg protein	
		10 s	60 min
100 mM NaCl	control	153 ± 22	104 ± 27
	EDTA	197 ± 37 *	107 ± 26
	$MgCl_2$	73 ± 10 *	99 ± 30
	$EDTA + MgCl_2$	88 ± 5 *	119 ± 8
100 mM KCl	control	25 ± 6	129 ± 20
	EDTA	32 ± 3	140 ± 20 *
	$MgCl_2$	33 ± 15	117 ± 24
	$EDTA + MgCl_2$	35 ± 10	127 ± 35

TABLE III

Effects of intravesicular Co^{2+} and Mn^{2+} on L-alanine uptake by blLPM vesicles

Vesicles were pre-loaded with 300 mM sucrose, 20 mM Hepes-Tris (pH 7.5), and either no additions (control), 5 mM $CoCl_2$, or 5 mM $MnCl_2$. Initial rates of uptake of 0.2 mM L-[³H]alanine at 10 s were measured at 25°C in an incubation solution containing 20 mM Hepes-Tris (pH 7.5), and either 100 mM NaCl or KCl. Isoosmolarity was maintained in the incubation solution using sucrose. Values are means ± S.D. of three experiments, each performed in triplicate. * Significantly different from control values at $P < 0.05$.

Salt gradients	pmol alanine/mg protein per 10 s		
	Control	$CoCl_2$	$MnCl_2$
100 mM NaCl	232 ± 53	106 ± 12 *	119 ± 45
100 mM KCl	41 ± 24	34 ± 18	44 ± 46

with altered membrane permeability to Na^+ [6,7]. This possibility was tested by measuring uptake of Na^+ into vesicles preloaded with Mg^{2+} and Ca^{2+}. It is important to note that Na^+ permeability was measured at two concentrations of $^{22}Na^+$, a roughly physiological concentration (100 mM) and a low concentration (1 mM) where more subtle effects on Na^+ entry should be detected. Uptake of $^{22}Na^+$ was not altered in vesicles pre-loaded with 5 mM $MgCl_2$ or $CaCl_2$ at either Na^+ concentration (Table IV).

The mechanism by which Na^+-coupled L-alanine uptake was inhibited by divalent cations was further explored by comparing the kinetic parameters of L-alanine uptake with and without intravesicular 5 mM $CaCl_2$. In the presence of a NaCl gradient, initial rates of L-alanine uptake at all concentrations tested (1 to 20 mM) were appreciably reduced by Ca^{2+}, whereas uptake in the presence of a KCl gradient was unaffected (Fig. 4). A double-reciprocal plot of the net Na^+-dependent component of L-alanine uptake revealed that the K_m for L-alanine was increased from 2.6 ± 2.3 to 3.8 ± 0.6 mM, while the V_{max} was decreased from 2.2 ± 1.0 to 1.4 ± 0.7 nmol/mg protein per 10 s by Ca^{2+} (Fig. 4, inset).
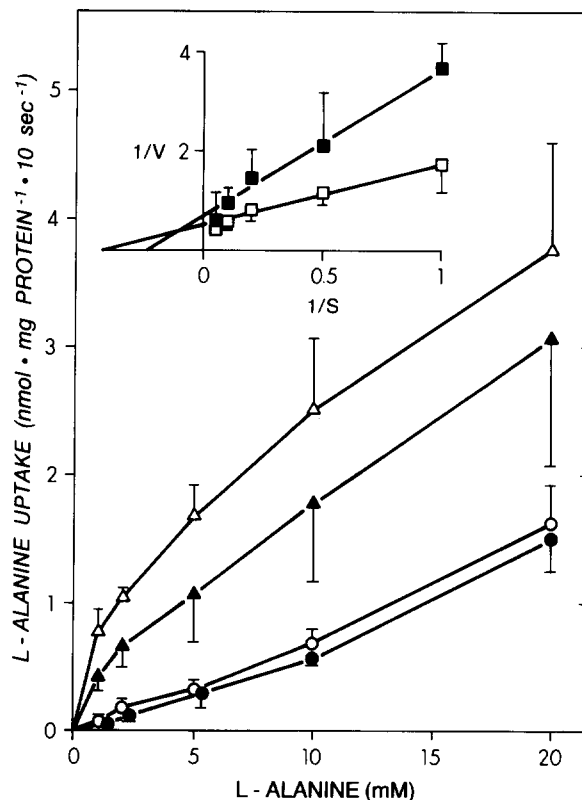

Fig. 4. Effect of intravesicular Ca^{2+} on concentration dependence of L-alanine uptake by blLPM vesicles. Vesicles were pre-loaded with 300 mM sucrose, 20 mM Hepes-Tris (pH 7.5) and either no (○, △) or 5 mM $CaCl_2$ (●, ▲). Initial rates (10 s) of uptake of 1 to 20 mM L-alanine were measured at 25°C in an incubation solution containing 20 mM Hepes-Tris (pH 7.5) and 100 mM of either NaCl (△, ▲) or KCl (○, ●). The inset shows a double-reciprocal plot of the Na^+ gradient-dependent transport system in the presence (■) or absence (□) of Ca^{2+}. Results are expressed as mean ± S.D. of four experiments each performed in triplicate.

However, the variability observed limits interpretation of how Ca^{2+} inhibited alanine uptake. This variability may in part be due to the difficulty of defining kinetic

TABLE IV

Effects of Mg^{2+} and Ca^{2+} on ^{22}Na uptake by blLPM vesicles

Vesicles were pre-loaded with 300 mM sucrose, 50 mM Hepes-Tris (pH 7.5), and either no additions (control), 5 mM $MgCl_2$, or 5 mM $CaCl_2$. Uptake of either 1 mM or 100 mM ^{22}Na was measured at 25°C in an incubation solution containing 300 mM sucrose, 50 mM Hepes-Tris (pH 7.5). Values are means ± S.D. of three experiments, each performed in triplicate.

Extravesicular	Intravesicular	nmol Na^+ per mg protein					
		5 s	10 s	15 s	60 s	2 min	90 min
1 mM Na^+	control	0.19 ± 0.20	0.20 ± 0.13	0.33 ± 0.20	0.45 ± 0.27	0.60 ± 0.27	0.82 ± 0.34
	5 mM $MgCl_2$	0.19 ± 0.12	0.26 ± 0.20	0.41 ± 0.29	0.42 ± 0.08	0.51 ± 0.17	0.60 ± 0.15
	5 mM $CaCl_2$	0.22 ± 0.24	0.28 ± 0.21	0.32 ± 0.20	0.42 ± 0.14	0.51 ± 0.13	0.71 ± 0.38
100 mM Na^+	control	19.9 ± 9.6	26.4 ± 9.2	29.7 ± 11.1	52.2 ± 17.7	57.2 ± 23.9	73.2 ± 18.0
	5 mM $MgCl_2$	22.4 ± 6.5	26.3 ± 8.4	28.0 ± 5.9	56.9 ± 19.4	67.1 ± 18.0	65.0 ± 13.4
	5 mM $CaCl_2$	23.2 ± 16.7	25.5 ± 12.1	41.0 ± 12.8	56.9 ± 13.7	52.8 ± 14.1	77.8 ± 21.4

parameters for a solute such as alanine which is transported via several membrane carriers [17].

Discussion

Alanine, an important oxidative and gluconeogenic substrate of the liver [18–20], is transported across liver sinusoidal membrane mainly by a Na^+-coupled transport process [10,17,21–24]. In rat liver blLPM vesicles uptake of alanine in the presence of Na^+ is characterized by a distinctive overshoot above equilibrium values, and has served as a measure of the functional integrity of these membrane vesicles [10,16]. In the present study, intravesicular divalent cation concentrations of 2 to 5 mM were found to inhibit alanine uptake in blLPM vesicles. Corkey et al. [25] estimated the cytosolic free Mg^{2+} level in hepatocytes to be 0.37 mM, and cytosolic free Ca^{2+} levels are generally in the nanomolar range. Although the concentrations of exogenous divalent cations added to the suspension solutions were greater than would be expected in vivo, stimulation of alanine uptake by the metal chelators EGTA and EDTA suggests that trace amounts of these cations in the suspension solutions may also influence uptake of alanine. This postulate is supported by the micromolar levels of total calcium detected in the unsupplemented suspension solutions and membrane suspensions. Likewise, Vaandrager et al. [7] observed greater glucose uptake in intestinal brush-border membrane vesicles in the presence of a 0.1 μM Ca/EGTA buffer compared to buffer with no additions. This was attributed to endogenous Ca^{2+} concentrations of 5 to 10 μM in the absence of EGTA.

The mechanism by which divalent cations inhibit alanine transport is unclear. Intravesicular Ca^{2+} has been shown to inhibit Na^+-coupled uptake of taurocholic acid, taurochenodeoxycholic acid, valine and glucose in intestinal brush border membrane vesicles [6,7]. Inhibition of uptake of these solutes was associated with changes in membrane permeabilities to ions. However, Vaandrager et al. [7] observed a general enhancement of anion and cation permeabilities, while Fondacaro and Madden [6] noted a decrease in permeability to Na^+. These conflicting results argue against a direct relationship between divalent-cation induced changes in Na^+ permeability and uptake of these solutes. In intact human erythrocytes, the divalent cations Ca^{2+}, Mg^{2+}, Co^{2+} and Mn^{2+} have been shown to inhibit Na^+/K^+ co-transport, and Mg^{2+} also inhibited Na^+-dependent uptake of alanine and serine [26,27]. However, it was not established whether the decrease in flux of Na^+ was related to inhibition of uptake of these two amino acids. In the present study, intravesicular Mg^{2+} and Ca^{2+} did not alter the permeability of blLPM vesicles to Na^+. Thus, the marked inhibition by

divalent cations of alanine uptake was not a result of a change in Na^+ permeability.

It should be emphasized that only intravesicular divalent cations inhibited alanine uptake, irrespective of extravesicular concentrations. Because the blLPM vesicles are primarily oriented 'right-side-out', the inhibitory effect appears to be limited to the cytosolic surface of the cell membrane. Evidence for an intracellular effect of divalent cations on plasma membrane transport processes has also been reported for human erythrocytes. Garay [26] observed a decrease in Na^+/K^+ co-transport when intracellular, but not extracellular Ca^{2+} levels were elevated. Similarly, Vaandrager et al. [7] concluded that the site for inhibition of glucose uptake by Ca^{2+} was located at the inner surface of intestinal brush-border membrane vesicles.

There is a considerable body of evidence showing that Ca^{2+} [3,8,28], and even Mg^{2+} [3], can interact directly and indirectly with lipids to cause a decrease in membrane fluidity. Since changes in the lipid environment of transport proteins can affect their catalytic function [30], it is plausible that divalent cation inhibition of alanine uptake in blLPM vesicles was due to changes in membrane fluidity. Indeed, we have previously demonstrated that Ca^{2+} inhibition of taurocholate uptake in rat liver blLPM vesicles prepared as in this study was associated with a decrease in the fluidity of the membrane [8]. Direct interaction of divalent cations with membrane lipids probably occurs by binding to anionic sites on the polar head groups of phospholipids [2,31]. Since negatively charged phospholipids (e.g. phosphatidyl serine) are more abundant at the inner leaflet of sinusoidal membranes [32], binding to acidic phospholipids may explain why intravesicular, but not extravesicular Ca^{2+} and Mg^{2+} inhibited uptake of alanine by blLPM vesicles.

Divalent cations are also believed to cause a decrease in fluidity indirectly by activating membrane-bound enzymes (e.g. phospholipase C) which biochemically modify the lipid composition of the membrane bilayer [3]. Alternatively, binding of divalent cations at the interior of blLPM vesicles may have altered the surface charge. Since Na^+-coupled uptake of alanine by blLPM vesicles is electrogenic, changes in the surface charge could have affected alanine transport. It is also possible that divalent cations inhibited uptake of alanine by interacting directly with the cytosolic domain of the carrier molecule for Na^+/alanine co-transport. A similar mechanism has been proposed by Lauf [4] to explain inhibition by Ca^{2+} of K^+/Cl^- co-transport by sheep erythrocytes.

Acknowledgements

468

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