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Improved stability of rabbit and rat intestinal brush border membrane vesicles using phospholipase inhibitors

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The initial rates of Na^+-dependent D-aspartate and D-glucose uptakes were shown to decline from the time of resuspension of brush border membrane vesicles isolated from rabbit and rat jejunum by standard divalent cation precipitation procedures. The former were however more stable than the latter and followed quite closely the decrease in the intravesicular volume, thus suggesting that the loss of transport activity may involve both nonspecific opening of the vesicles and either direct or indirect specific inactivation of the transporters. Uptake rates for both substrates did tend to stabilize at 6–24 h from resuspension, however this final 'next day' uptake activity was too low to be of practical use in kinetic studies. Freezing aliquots of rabbit jejunal vesicles in liquid N_2 until the time of assay resulted in complete stabilization of D-glucose uptake. A modified homogenate buffer designed to inhibit a broad spectrum of phospholipase activities resulted in a partial stabilization of glucose transport by rabbit jejunal vesicles with, on average, an over 6-fold enrichment in the 'next day' stable specific activity of uptake as compared to unfrozen vesicles. The modified homogenate buffer also improved the stability and the 'next day' specific activities of D-glucose uptake in rat jejunal brush border vesicles and D-aspartic acid uptake in rabbit jejunal vesicles. It also completely stabilized the intravesicular volume in the latter preparation. An evaluation of the kinetic parameters of Na^+-dependent D-glucose transport in rabbit vesicles prepared from either the standard homogenate media and frozen in liquid N_2 or the modified media and allowed to stabilize overnight, revealed a single transport system with a K_m of 0.31–0.32 mM as the best model to fit the data. As such the modifications to the homogenate media do not appear to effect the functional properties of D-glucose transport in the membrane. While being less efficient in stabilizing the vesicles than the rapid freezing protocol, it is shown that the modified homogenate should however be preferred when dealing with slowly permeant ions like choline since it provides in this case the only alternative to reliable measurement of uptake rates across a stable and equilibrated vesicle preparation.

Introduction

Characterization of transport phenomena using preparations of membrane vesicles is dependent upon the following criteria [1,2]: (1) purity of the preparation; (2) accurate measure of uptake rates under all conditions tested; (3) stable specific activity of uptake from the first to the last assay on the day of the experiment; and (4) complete preequilibration of solutes across the vesicles prior to the assay.

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The purity of intestinal and renal brush border membrane vesicles can be considered as adequate for the purpose of uptake experiments since they generally display an over 10-fold enrichment in the specific activity of brush border marker enzymes with little apparent contamination by other cellular organelles [1,2]. As such, uptake measured in an experimental setting is likely to represent interaction of the substrate with the brush border membrane.

A common procedure used in the kinetic analysis of transporter function is the single time point measure of substrate uptake. This procedure has the advantage of being relatively easy to perform and it is possible to accomodate a large number of such assays in a single experiment. However, the assumption underlying this approach is that the single time point is an accurate

estimate of the steady state rate of transport under all conditions tested [1,2]. Recently, our laboratory has developed a fast sampling rapid filtration apparatus (FSRFA) that permits us to collect up to 18 aliquots from the same incubation mixture at a maximal rate of 4 samples/s [3]. The FSRFA thus allows to use a dynamic approach in the evaluation of transport kinetics, i.e. true initial rates can be directly determined from uptake time-courses recorded under all sets of experimental conditions [1,3,4].

Using the FSRFA, we began to characterize the kinetics of sugar and acidic amino acid transport by rabbit jejunal brush border membrane vesicles. However, it soon became apparent that the specific activities of substrate uptake declined over the course of an experiment. In addition, we had indirect evidence that charged solutes in the resuspension media may not have fully equilibrated across the membrane at the time of performing the uptakes. Clearly, these experimental difficulties could lead to an erroneous characterization of transporter kinetics and prompted the studies to be reported in this paper. We demonstrate herein the instability of rabbit and rat intestinal brush border membrane vesicles to Na^+-dependent substrate uptake and we present a protocol aimed at solving both the instability and the solute pre-equilibration problems.

Materials and Methods

Preparation of brush border membrane vesicles

The proximal small intestine of rat (male Sprague-Dawley, 250–300 g) or rabbit (male, New Zealand White, 2.0–2.5 kg) were removed and flushed with ice-cold 0.9% NaCl. Mucosal scrapings were taken with a spatula on a cold glass plate and placed in mucosal homogenate media at a 20:1 (v/w) of scraping ratio. The standard homogenate media contained 50 mM mannitol in 2 mM Tris-HCl buffer (pH 7.0) for the Ca^{2+}-precipitation procedure [5]; or 50 mM mannitol and 5 mM EGTA-Tris (pH 7.0) in 2 mM Tris-HCl buffer (pH 7.0) for the Mg^{2+}-precipitation procedure [6]. Alternatively, the modified homogenate media described in Table I was used for rabbit and rat intestinal scrapings. The tissues were homogenized with a Waring blender for 1 min at full speed and either 10 mM $CaCl_2$ or 10 mM $MgCl_2$ was added to the homogenate and the vesicles were prepared as far as the second centrifugation (P_2) as described previously [7]. Routinely, the P_2 was resuspended in 50 mM Hepes-Tris buffer (pH 7.0) containing 300 mM mannitol and was frozen in liquid N_2 until the day of use when the P_2 was thawed, diluted 10:1 (v/w of original scraping) in the same buffer, and prepared to the final (P4) vesicle pellet as described previously [7]. All procedures were performed at 0°C and the vesicles were kept on ice for the duration of the uptake assays. Alternatively, the vesicle suspension was divided into 30 μl aliquots and kept frozen in liquid N_2 until assay. Any modification of this basic protocol will be indicated where appropriate in the Results section.

TABLE I

Composition of the modified homogenate media used for the preparation of jejunal brush border vesicles

The modified homogenate media without EGTA can also be used for the $CaCl_2$ precipitation protocol.

Mannitol	50 mM
Tris-HCl (pH 7.0)	2 mM
Mepacrine-Tris (pH 7.0)	1 mM
EGTA-Tris (pH 7.0)	30 mM
BSA	1 mg/ml
LiF	5 mM
TPEN	0.01 mM
PMSF	0.10 mM

In one experiment, rabbit jejunal brush border vesicles were gel-filtered on a Sepharose 4B column in an attempt to remove contaminants that may destabilize the preparation [8]. The final vesicle pellet was resuspended in 150 mM KCl, 50 mM Hepes-Tris buffer (pH 7.4) and then chromatographed at 4°C according to published procedures [1,2,8]. The vesicles collected in the column void volume were then diluted in the same buffer, centrifuged, resuspended and washed twice by centrifugation-resuspension before final resuspension of the vesicle pellet.

Rabbit renal brush border vesicles were prepared following the same procedures as used for jejunal vesicles except that the kidney cortex was dissected free and used to prepare the vesicles [9]. Dog jejunal brush border vesicles were prepared following the same Ca^{2+}-precipitation procedure as used for rabbit jejunum and confirmed by Laganière et al. [10].

Routinely, rabbits were obtained from La Ferme Lapro (Stuckley-sud, Québec). Alternate sources were Maple Lane Farms (Clifford, Ontario) and specific pathogen free rabbits from Cunipur (St-Valérien, Québec). Dog jejunum was kindly supplied by Dr. F. Vinay from the Department of Physiology, Université de Montréal.

Transport assays

All transport assays were performed using the FSRFA recently developed in our laboratory [3,4] as follows. Routinely, 20 μl of brush border membrane vesicles (0.4–1.0 mg protein) were loaded into the apparatus and uptake was initiated by injecting the vesicles into 480 μl of uptake media containing 50 mM Hepes-Tris buffer (pH 7.0), 150 mM NaI, 0.5 mM amiloride, 50 μM D-glucose or D-aspartic acid, and 10 μCi ^{3}H-D-glucose or 20 μCi ^{3}H-D-aspartic acid. D-Glucose uptake was measured at 20°C by a 9-point sam-

pling of the uptake mixture at 0.5 s intervals. The route of D-aspartic acid transport has been defined in our laboratory as occurring through the specific high affinity acidic amino acid transporter [11] and uptake of this substrate was determined at 35°C by a 9-point sequential sampling at 1.5 s intervals from injection of the vesicles. At each sampling time, the apparatus automatically injected 50 μl of the uptake mixture (40–100 μg protein) into 1 ml of ice-cold stop solution (50 mM Hepes-Tris buffer (pH 7.0) containing 150 mM NaCl, and either 1 mM phlorizin or 0.05 mM $HgCl_2$ for D-glucose and D-aspartic acid, respectively), then filtered the stopped sample through 0.65 μm cellulose nitrate filters (MFS) and washed the filters three times with 1 ml ice-cold stop solution. Substrate uptake was then determined by liquid scintillation counting as described previously [12].

Under these conditions, the uptake time courses of both D-glucose and D-aspartic acid were linear (results not shown). Accordingly, the slopes of these straight lines represent the true initial rates of transport and these were determined by linear regression analysis using the Enzfitter program (R.J. Leatherbarrow, 1987) and an IBM compatible microcomputer.

The kinetic parameters of D-glucose transport were determined as described in the legend to Fig. 5. under conditions of a Na^+-gradient with the membrane potential clamped at 0 mV using iodide as a highly permeant anion [12]. Since we have established that the diffusion coefficients for D-glucose and D-mannitol are identical in our rabbit vesicle preparation, as also shown recently for human jejunal brush border membranes [3,4], the ^{3}H-D-glucose uptake values were corrected for the mannitol space in order to extract the carrier-mediated component of transport. The initial rates of tracer D-glucose transport were then determined par linear regression as described above and the initial rate data was analyzed as described recently [1,4] using non-linear regression analysis and model equations for displacement curves describing transport through either one or two carrier-mediated processes working in parallel with diffusion. Only the Eadie-Hofstee plot corresponding to the best model is reported here for simplicity and visual appraisal of the data.

Intravesicular volume determinations

Intravesicular volumes were estimated from the D-glucose equilibrium space by adding 20 μl of brush border vesicles to 480 μl of the same media used to measure initial rates of D-glucose uptake and incubating the mixture at room temperature for 1 h. The mixture was then loaded into the FSRFA and 9×50 μl samples were taken at 0.5 s interval and processed as described above in order to determine the mean equilibrium uptake of D-glucose.

Enzyme and protein assays

γ-Glutamyltranspeptidase was assayed according to Szasz [13] and Na^+/K^+-ATPase activity was determined by the method of Schwartz et al. [14]. Protein was measured with the BCA assay kit using bovine serum albumin as the standard.

Chemicals

^{3}H-D-Aspartic acid (25 Ci/mmol) and ^{14}C-D-mannitol (59 mCi/mmol) were obtained from Amersham while ^{3}H-D-glucose (15 Ci/mmol) was purchased from New England Nuclear. Unlabelled glucose, EGTA, bovine serum albumin, phenylmethylsulfonyl fluoride (PMSF) and *N,N,N',N'*-tetrakis(2-pyridyl-methyl)ethylenediamine (TPEN) were purchased from Sigma. Unlabelled D-aspartic acid and phlorizin were obtained from Aldrich, mepacrine was supplied by ICN and the BCA protein assay kit was purchased from Pierce.

Results

Instability of rabbit and rat brush border vesicles

Rabbit jejunal brush border vesicles prepared by either Ca^{2+} or Mg^{2+}-precipitation techniques were characterized by a declining specific activity of Na^+-dependent D-glucose and D-aspartic acid uptakes from the time of resuspension of the final vesicle pellet until at least 6 h post resuspension (Fig. 1A). A decline in the specific activity of uptake occurred in all preparations, however, the overall magnitude and the rate of the decline varied between preparations. D-Glucose uptake rates showed a tendency to decline at a faster rate in vesicles prepared by Ca^{2+}-precipitation although this difference was not statistically significant at any one time point and, at 6 h from resuspension, both Ca^{2+} and Mg^{2+}-prepared vesicles had lost a similar % of the uptake rates obtained immediately after resuspension (Fig. 1A). In comparison to glucose, the initial rates of D-aspartic acid uptake in vesicles prepared by Mg^{2+}-precipitation were found to be more stable with time (Fig. 1A).

The instability of rabbit jejunal vesicles was not confined to a local source of rabbits and was also seen in pathogen free animals (data not shown). In addition, biopsies performed on the jejunum of rabbits obtained from the local source showed no evidence of abnormality or infection.

Na^+-dependent D-glucose uptake in rat small intestinal brush border vesicles prepared by Mg^{2+} or Ca^{2+}-precipitation was also found to be unstable from the time of resuspension of the final vesicle pellet (Fig. 1B). The decline in uptake rates also showed considerable variation between preparations; however, vesicles prepared by Mg^{2+}-precipitation appeared to be more stable to D-glucose uptake than those prepared by Ca^{2+}-precipitation.

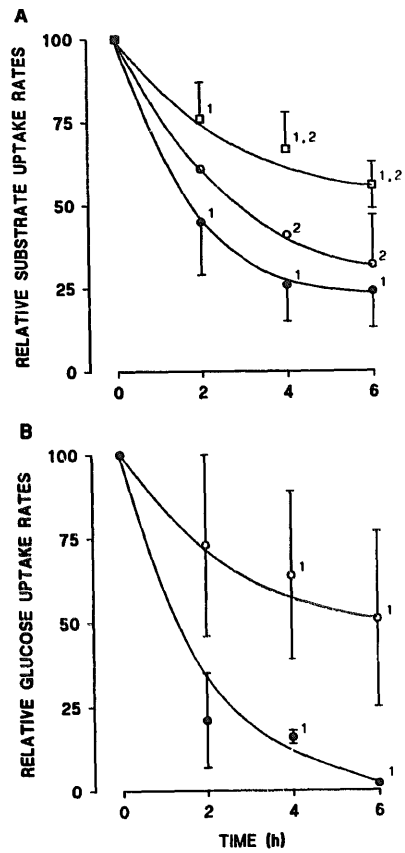

Fig. 1. Instability of rabbit (A) and rat (B) jejunal brush border vesicles to Na^+-dependent D-glucose (○, ●) and D-aspartic acid (□) uptakes. Vesicles were prepared by the standard Mg^{2+} (○, □) or Ca^{2+} (●) precipitation methods described under the Materials and Methods section. Points shown are the mean ± standard deviation for three to six different vesicle preparations and represent the initial rates of substrate uptake expressed as a % of the 0 time uptake rate. Common numbers at the same time from resuspension indicate a significant difference in relative substrate uptake rates ($P \leq 0.05$).

In contrast with the above results, brush border membrane vesicles from rabbit kidney and dog jejunum both maintained a constant specific activity of D-glucose uptake between 0 and 6 h from resuspension of the final pellet (data not shown).

Initial attempts to stabilize rabbit jejunal brush border vesicles

A number of variations were performed in the standard protocol for vesicle preparation, but failed to increase the stability of the preparations (data not shown): (i) omitting the P_2 freezing step (daily fresh vesicles); (ii) replacement of mannitol in the P_2 and P_4 resuspension media with choline Cl, choline I, KCl or KI, or replacement of NaI with NaCl in the uptake media; (iii) addition of proteinase inhibitors (12.5 μg/ml pepstatin A, 12.5 μg/ml chymostatin, 1.0 μg/ml aprotinin, 80 μg/ml bacitracin) to either or both of the P_4 and homogenate media; (iv) Changes in the physical conditions of vesicle preparation like resuspension of P_4 at 37°C, sonication, or multiple freeze-thaw cycles in liquid N_2 of the resuspended P_4 (interestingly, maintaining the vesicles at 0°C offered little protection in comparison to a room temperature incubation); (v) Passing the vesicles through a Sepharose 4B column; and (vi) addition to the homogenate and/or P_4 fractions of antioxidants and free radical scavengers including ascorbic acid, dithiothreitol, indomethacin and vitamin E, alone or in combination.

The first evidence of an improvement in the stability of a jejunal brush border vesicle preparation was seen when a combination of 1 mM mepacrin, 1 mg/ml bovine serum albumin (BSA), and 0.1 mM PMSF was included in the initial homogenization buffer as a protocol to inhibit phospholipid breakdown [15–18]. Following this initial observation, several experiments were performed in order to determine the optimum concentrations of these ingredients as well as those of EGTA using a Mg^{2+}-precipitation protocol (data not shown). Both TPEN and LiF were added latter at concentrations used by others (C. Le Grimellec, personal communication) and the final composition of the modified homogenization buffer is given in Table I.

Improved stability of rabbit and rat jejunal brush border vesicles using a modified homogenization buffer

Fig. 2A demonstrates the effect of the modified homogenization media on the stability of D-glucose uptake in rabbit jejunal vesicles prepared by Mg^{2+}-precipitation. The media does not completely stabilize the vesicles; however, in all preparations the rate of decline in uptake activity is less than controls. At 24 h from resuspension, uptake rates appeared to have been stabilized. In the case of control vesicles, 90 ± 3% of the zero time uptake activity is lost and the remaining D-glucose uptake capacity (6 ± 3 pmol/mg protein per s) is too low to be of practical use. In contrast, vesicles prepared from the modified homogenate buffer retained an average of 40 ± 16% of the zero time uptake activity which represents an over 6-fold enrichment (39 ± 22 pmol/mg protein per s) in the specific activity as compared to controls.

The modified homogenization buffer caused some improvement in the stability of D-glucose uptake across rabbit jejunal vesicles prepared by the Ca^{2+}-precipitation technique. However the magnitude and the consistancy of the improved specific activity of uptake was less than obtained with Mg^{2+}-precipitated vesicles. As such all subsequent experiments were performed using the Mg^{2+}-precipitation protocol.

The improvements in stability and final specific activity of D-glucose uptake by rabbit jejunal vesicles obtained with the modified homogenate media were also observed with Na^+-dependent D-aspartic acid uptake (Fig. 2B). At 24 h from resuspension, the specific activity in control vesicles (7 ± 3 pmol/mg protein per

s) represented 40 ± 5% of the zero time uptake activity as compared to a 73 ± 10% (14 ± 6 pmol/mg protein per s) in vesicles prepared from the modified media. This difference represents a 1.9-fold enrichment in the stable 24 h specific activity of D-aspartic acid uptake.

In these series of experiments, the integrity of the vesicle preparations was evaluated by estimating the intravesicular volume from the D-glucose equilibrium space. At 1 h from resuspension, the mean ± standard error for equilibrated D-glucose spaces were 1.48 ± 0.12 and 1.55 ± 0.17 μl/mg protein for vesicles prepared from control and modified homogenate media, respectively. As can be seen from Fig. 2C, vesicles prepared from the control media displayed a steady decline in

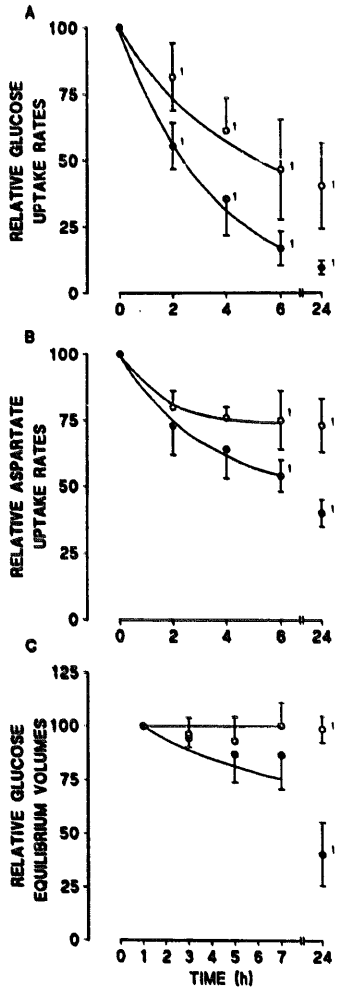

Fig. 2. Increased stability of D-glucose (A) and D-aspartic acid (B) uptakes and of D-glucose equilibrium volume (C) in rabbit jejunal brush border membrane vesicles prepared by Mg^{2+}-precipitation from the modified (○) or the standard (●) homogenate media as described under the Materials and Methods section. For graphs A and B, the initial rate data are expressed as a % of the 0 time uptake rate while for graph C the D-glucose equilibrium volumes are given as a% of the volume obtained at 1 h from resuspension. Values shown are the mean ± standard deviation for three to four different vesicle preparations. A common number at the same time from resuspension indicates significant differences ($P \leq 0.05$).

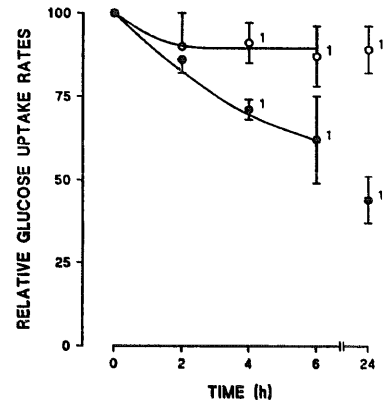

Fig. 3. Increased stability of D-glucose uptake in rat intestinal brush border membrane vesicles prepared by Mg^{2+}-precipitation from the modified (○) or the standard (●) homogenate media as described under the Materials and Methods section. Values shown are the mean ± standard deviation for three different vesicle preparations. A common number at the same time from resuspension indicates significant differences ($P \leq 0.05$).

intravesicular volume and, at 24 h, the average trapped volume/mg protein was 41 ± 15% of the value obtained at 1 h from resuspension. In contrast, vesicles prepared from the modified homogenate media maintained a constant D-glucose equilibrium volume between 1 and 24 h post resuspension.

Homogenization of the mucosal scrapings in the modified buffer was also shown to improve the stability and the final specific activity of D-glucose uptake in rat small intestinal vesicles (Fig. 3). When prepared from the modified media, stability was reached by 2 h from resuspension and 89 ± 7% (41 ± 5 pmol/mg protein per s) of the zero time uptake activity was retained at 24 h. In comparison, vesicles prepared from the standard homogenate media had only retained an average of 44 ± 7% (22 ± 2 pmol/mg protein per s) of the zero time uptake rates. The difference in specific activities represents a 1.9-fold enrichment in the 24 h D-glucose uptake activity in rat vesicles.

The modified homogenate media had no effect on the protein content and the purity of the vesicle preparation. Using γ-glutamyltranspeptidase as a brush border marker enzyme, 4 preparations of control vesicles had a mean ± S.D. (nmol/mg protein per min) of 427 ± 75 as compared to 402 ± 46 obtained when vesicles were prepared from the modified homogenate media. The enrichment factor relative to the specific activity in the initial homogenate was 20.1 ± 5.8 for control vesicles and 18.9 ± 2.9 for vesicles prepared from the modified media. In addition, the modified homogenate media did not affect the degree of contamination by basolateral membrane as determined by oubain-sensitive Na^+/K^+-ATPase (88 ± 30 and 114 ± 33 pmol/mg protein per min in vesicles from standard and modified media, respectively).

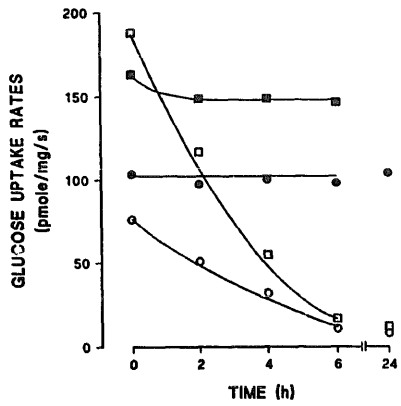

Fig. 4. Increased stability of D-glucose uptake in frozen aliquots of vesicles (■, ●) versus 0 °C-maintained (□, ○) rabbit brush border membrane vesicles prepared by the standard Ca^{2+} (■, □) or Mg^{2+} (●, ○) precipitation methods described in the Materials and Methods section. The points shown are initial rates of transport ± standard error of regression (actually smaller than the symbol sizes) obtained in a representative experiment.

Improved stability of rabbit jejunal brush border vesicles by freezing in liquid N_2

Dividing the P_4 resuspended vesicles into multiple aliquots suitable for uptake assays and then freezing the aliquots in liquid N_2 until the time of assay should reduce or eliminate any specific process that may destabilize the vesicles. Indeed, freezing either Ca^{2+} or Mg^{2+}-precipitated vesicle preparations resulted in virtually identical specific activities of D-glucose uptake at all time points that were assayed from the time of resuspension of the P_4 (Fig. 4).

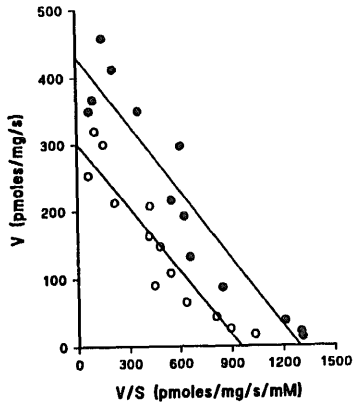

Fig. 5. Hofstee plot of D-glucose transport in rabbit jejunal brush border membrane vesicles prepared by Mg^{2+}-precipitation from control (●) or modified (○) homogenate media. The P_2 fractions were resuspended in the presence (●) or absence (○) of valinomycin in 50 mM Hepes-Tris buffer (pH 7.0) containing 150 mM KI and 125 mM mannitol. The P_4 fractions were resuspended in the valinomycin-free buffer and either frozen in liquid N_2 as 30 μl aliquots (●) or incubated overnight at 4 °C (○) until assay. Uptake media contained 50 mM Hepes-Tris buffer (pH 7.0), 150 mM NaI, 0.5 mM amiloride, 31 μCi ^{3}H-D-glucose, 5 μCi ^{14}C-mannitol, and 125 mM D-glucose + mannitol with the concentrations of D-glucose varied from 0.016 to 100 mM. Transport assays were performed as described in Materials and Methods.

Kinetics of D-glucose transport in vesicles prepared from control and modified homogenate media

The kinetic parameters of Na^+-dependent D-glucose transport were determined in rabbit jejunal vesicles prepared by Mg^{2+}-precipitation from the control and modified homogenate media and the results of this experiment are shown in Fig. 5. In both cases, the best model fit to the data was that of a single transport system with K_m's of 0.316 ± 0.034 and 0.312 ± 0.034 mM for the control and modified media, respectively. As expected from the results of Fig. 2A, however, the vesicles from the modified media had a lower V_{max} of 300 ± 20 pmol/mg protein per s as compared to control vesicles (424 ± 26 pmol/mg protein per s). This experiment also permitted to determine the passive diffusional coefficient (k_D) for mannitol transport in the two preparations of vesicles and values of 10.2 ± 1.8 and 10.8 ± 2.2 pmol/mg protein per s per mM (corresponding to $t_{1/2}$ for vesicle equilibration of 102 ± 18 and 96 ± 20 s) were obtained in control and modified media, respectively.

Equilibration of charged solutes across vesicles prior to transport assay

In a last series of experiments, we compared the effect of intravesicular replacement of 300 mM mannitol with 150 mM of either choline I (ChI) or KI on the apparent stability of glucose uptake in rabbit jejunal vesicles prepared from the modified homogenate media by a Mg^{2+}-precipitation protocol. As shown in Fig. 6, the glucose uptake rates are higher in the mannitol resuspended vesicles (open circles), a result which was expected and corresponds, as demonstrated previously (12), to a membrane potential effect on D-glucose uptake due to the presence of a I^- diffusion potential during the transport assay under these conditions. However, Fig. 6 also shows that KI resuspended vesicles (open triangles) appear more stable than their ChI counterpart (open squares) and that their relative stability closely matches that seen in mannitol (53 and 58% loss of glucose transport activity at 24 h from resuspension, respectively). Since identical uptake rates are recorded from 4 to 24 h following resuspension in either KI or ChI media, the early difference in the apparent stability between ChI and KI resuspended vesicles is best explained by a lower permeability of the vesicles to choline as compared to K^+ since failure of ChI to have fully preequilibrated across the vesicles at the time of assay would generate an interior negative membrane potential (I^- out > I^- in). Accordingly, it can be inferred that a minimum of 4 h is required for complete preequilibration of ChI at 0°C. It should also be noted that resuspending the P_2 in a ChI media prior to freezing in liquid N_2 (Fig. 6, closed squares) did not improve the process of ChI pre-equilibration.

Discussion

A clear-cut interpretation of uptake experiments using membrane vesicles requires a constant specific activity of transport during the course of an experiment. Rabbit and rat jejunal brush border membrane vesicles prepared in our laboratory using standard Ca^{2+} or Mg^{2+}-precipitation protocols do not conform to this expectation since they demonstrate a steady decline in the initial rates of Na^+-dependent D-glucose transport from 0 to 6 h of vesicle resuspension (Fig. 1). Moreover, the low specific activities of transport recorded once the vesicles have stabilized (Figs. 2 and 3) are unsuitable for detailed kinetic characterization of transporter function.

The nature of the mechanism(s) responsible for vesicle instability has been addressed in our studies by systematically testing for different hypotheses. It would first appear that the problem of vesicle instability is both tissue and species specific and not the result of any obvious pathogenicity. Next, our initial attempts to stabilize rabbit jejunal vesicles have ruled out the presence of external contaminants in our solutions and have shown that passing the vesicles through a Sepharose 4B column failed to stabilize the rabbit vesicles. This last result clearly establishes that the destabilizing agent(s) is (are) intimately associated with the membrane preparation. Finally, our results demonstrate a continuous reduction in the equilibrated D-glucose space from 1 to 24 h of vesicle resuspension (Fig. 2C). However, D-glucose (and to a lesser extent D-aspartic acid) uptake rates declined more than the intravesicular volume (Fig. 2). It can thus be concluded that a nonspecific opening of the vesicles can only account for part of the drop in uptake rates. Moreover, D-glucose uptake rates were shown to decline to a greater extent than those of D-aspartic acid (Figs. 1 and 2), a result that also rules out a nonspecific increase in vesicle permeability to solutes and ions as the sole complementary cause for transport instability. In agreement with this conclusion, the passive diffusion of mannitol was not affected under conditions where the V_{max} for glucose transport dropped by some 30% (Fig. 5). One must then refer to some kind of specificity in the mechanism(s) leading to transport and vesicle instabilities like direct effects on the transport proteins themselves and/or indirect effects on the transport activities through modifications in the environment of the transport proteins. This conclusion is in fact supported by the demonstration that glucose transport activity is fully stabilized when the vesicles are frozen in liquid N_2 until the time of assay (Fig. 4). Such (a) specific mechanism(s) do(es) not seem to involve proteolysis and oxidation phenomena. This conclusion does depend, however, on our choice of proteinase inhibitors, antioxidants, and free radical scavengers and on the permeability of the membrane to these molecules and their ability to reach the agent(s) responsible for vesicle instability. Notwithstanding these restrictions and considering that our only success at (partly) stabilizing both rabbit and rat intestinal brush border membrane vesicles (Figs. 2 and 3) made use of a wide spectrum of agents (Table I) selected for their potential as phospholipase inhibitors, we can tentatively conclude that (a) phospholipase activity(ies) is (are) involved in the mechanism leading to vesicle instability and decline in transport activities. Further work would however be required to clarify this point since our studies have not directly addressed the (lyso)phospholipid content of the membrane vesicles. Nevertheless, the intestinal mucosa of both rat [19,20] and rabbit [6,21] have been reported to contain high levels of phospholipase activities and there has been much debate as to the lysophospholipid content of Ca^{2+}- as compared to Mg^{2+}-prepared membranes [6,21–23]. Other investigators have also shown that rat [22,24] and rabbit [23] brush border phospholipase activities may not be Ca^{2+}-dependent. In this context, it is interesting to note that the instability of rabbit vesicles was marginally sensitive to Ca^{2+} replacement by Mg^{2+}. Rat vesicles however showed less of a decline in glucose transport by Mg^{2+}-prepared membranes (Fig. 1).

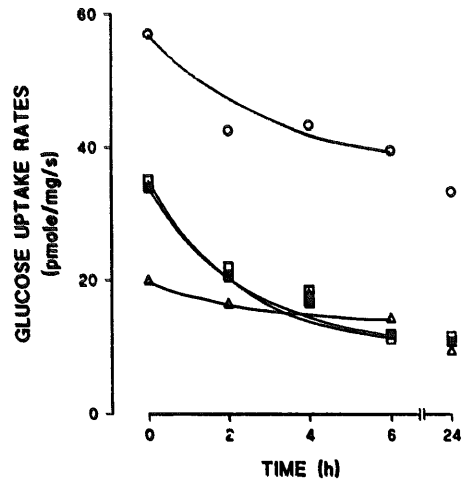

Fig. 6. Effects of vesicle resuspension media on the apparent stability of Na^+-dependent D-glucose transport. Rabbit jejunal mucosal scrapings were homogenized in the modified media of Table I and all but one of the P_2's were resuspended in 500 μl of 300 mM mannitol, 50 mM Hepes-Tris (pH 7.0), 0.1 mM $MgSO_4$ and frozen in liquid N_2. The remaining P_2 (■) was resuspended in a media containing 150 mM ChI prior to freezing. On the day of use the P_2's were thawed and processed to the final vesicle pellet using resuspension media containing 300 mM mannitol (○) or 150 mM ChI [□, ■] or 150 mM KI (△) plus 50 mM Tris-Hepes (pH 7.0) and 0.1 mM $MgSO_4$. Vesicles were maintained at 0°C and the data points represent the initial rates of D-glucose uptake at each of the indicated times from resuspension of the final pellet.

Whatever the exact mechanism(s) leading to transport and vesicle instabilities, our studies demonstrate that stable brush border membrane preparations can be obtained by either using a modified homogenate media and allowing for overnight stabilization of vesicles (Figs. 2 and 3) or, at least with rabbit vesicles, by dividing the resuspended membranes into aliquots suitable for uptake assays and freezing the aliquots in liquid N_2 until the time of the assay (Fig. 4). Comparing the kinetic parameters of D-glucose transport under zero-trans and voltage-clamped (0 mV) conditions in vesicles prepared following these two protocols revealed similar K_m values (0.31–0.32 mM) in the two preparations but lower V_{max} in vesicles prepared using the modified homogenate media (Fig. 5), in accordance with the fact that vesicle stability under these conditions is obtained to the detriment of the overall specific activity of transport (compare Figs. 2 and 4). As such, the modifications to the homogenate media do not appear to effect the functional properties of D-glucose transport in the brush border membrane. A corollary observation of these experiments is that glucose transport in rabbit jejunum vesicles would seem to be accounted for by the presence of a single transport system only since we did not find any evidence of a second, lower affinity system as has been described for intestinal brush border vesicles prepared from human fetus [25], human adult [26], rabbit [27], bovine [28], guinea pig [29] and rat [30]. This result does agree however with that obtained with human adult jejunal brush border vesicles during our recent re-evaluation of uptake data using the FSRFA [4]. A detailed kinetic analysis of D-glucose transport in rabbit jejunal brush border vesicles using our FSRFA and a protocol for measuring uptake across stable, equilibrated, vesicle preparations is currently underway in our laboratory.

Comparing the two methods found to stabilize rabbit brush border membrane vesicles, it would appear that the freezing protocol has a few advantages over the other one since: (i) it is not necessary to modify any of the solutions used in the preparation of the vesicles; (ii) it is possible to use vesicles prepared by either Ca^{2+} or Mg^{2+}-precipitation protocols; (iii) vesicles can be used immediately after preparation; and (iv) the specific activity of transport are maximized. Using this protocol however, it is essential to insure that the components of the resuspension media have preequilibrated across the vesicles prior to freezing since, as demonstrated in Fig. 6, incomplete preequilibration of ionic species may effect uptake rates and preclude a meaningful interpretation of the data. In the case of impermeant solutes like choline, it appears then that the only choice is a protocol whereby the vesicles are prepared from the modified homogenate media by Mg^{2+}-precipitation and the resuspended P_4 is preincubated overnight at 0°C. Moreover, in the case of lengthy experiments, it may be advisable, and indeed it is our practice, to preincubate the vesicles overnight at 0°C, to divide the preparation into aliquots, and to freeze them in liquid N_2 until the time of assay. This procedure does allow for a stabilization of uptake activities and does insure complete pre-equilibration of the components of the resuspension media.

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