

Direct Inhibitory Effect of Rotavirus NSP4(114-135) Peptide on the Na⁺-D-Glucose Symporter of Rabbit Intestinal Brush Border Membrane

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The direct effect of a rotavirus nonstructural glycoprotein, NSP4, and certain related peptides on the sodium-coupled transport of D-glucose and of L-leucine was studied by using intestinal brush border membrane vesicles isolated from young rabbits. Kinetic analyses revealed that the NSP4(114-135) peptide, which causes diarrhea in young rodents, is a specific, fully noncompetitive inhibitor of the Na⁺-D-glucose symporter (SGLT1). This interaction involves three peptide-binding sites per carrier unit. In contrast, the Norwalk virus NV(464-483) and mNSP4(131K) peptides, neither of which causes diarrhea, both behave inertly. The NSP4(114-135) and NV(464-483) peptides inhibited Na⁺-L-leucine symport about equally and partially via a different transport mechanism, in that Na⁺ behaves as a nonobligatory activator. The selective and strong inhibition caused by the NSP4(114-135) peptide on SGLT1 in vitro suggests that during rotavirus infection in vivo, NSP4 can be one effector directly causing SGLT1 inhibition. This effect, implying a concomitant inhibition of water reabsorption, is postulated to play a mechanistic role in the pathogenesis of rotavirus diarrhea.

A rotavirus nonstructural glycoprotein, NSP4, and a synthetic peptide, NSP4(114-135), corresponding to residues 114 to 135 of this protein, both have been shown to induce age- and dose-dependent diarrhea in young rodents (5). Because the induction of diarrhea and alterations in chloride secretion, unaccompanied by any histological lesions, occurred within a period of about 3 h, similar to those induced by the heat-stable toxin β of *Escherichia coli*, NSP4 was proposed to be a viral enterotoxin which, by activating a Ca²⁺-dependent signal transduction pathway, impairs intestinal epithelial transport (5). However, it is known that the ionic concentrations in the stools of rotavirus-infected animals, although high, are considerably less than those occurring in the secretory diarrheas caused by secretagogues such as the enterotoxins of *Vibrio cholerae* and *E. coli* (11, 15). Since the mechanisms by which rotavirus and NSP4 cause diarrhea are not completely understood, investigating possible, alternative mechanisms might prove useful. Recently, others have shown that rotavirus infection alters regulation of the expression of digestive enzymes (13).

Independently, we have demonstrated that both natural and experimental infection by a lapine group A rotavirus, La/RR510 strain, isolated in our laboratory impairs Na⁺-D-glucose (SGLT1) and Na⁺-L-leucine symport activity across intestinal brush border membrane (BBM) vesicles isolated from young rabbits. Because infection reduces D-glucose transport capacity (V_{max}) without affecting the density of phlorizin-binding sites and of SGLT1 protein antigen present in the BBM

vesicles, we concluded that the rotavirus effect on this symporter is direct (10). Transport inhibition preceded viral shedding into the lumen and the onset of diarrhea. No intestinal lesion was seen in the experimentally infected rabbits, confirming that diarrhea is not necessarily a consequence of the intestinal lesion but can precede it, as if cell dysfunction were the cause, not the consequence, of the histological damage (5, 6, 10, 17, 18, 22).

Since SGLT1 is involved in the reabsorption of large volumes of water under physiological conditions, we proposed that the mechanism of rotavirus diarrhea involves a generalized inhibition of symport systems and hence of water reabsorption. The resulting massive water loss through the intestine would eventually contribute to overwhelming the organ's capacity for water reabsorption, thereby facilitating establishment of the main symptom of enteritis, diarrhea.

In this work, we investigated the hypothesis that NSP4 acts, at least in part, by specifically inhibiting sodium-coupled solute transport across the intestinal BBM. In particular, we examined whether NSP4(114-135) and certain related peptides directly affect the kinetics of Na⁺ cotransport with either D-glucose or L-leucine. To avoid possible problems of interpretation, due for instance to indirect metabolic effects, changes in intestinal structure, and/or changes in rotavirus protein synthesis, transport was assayed with a rapid in vitro technique, based on using jejunal BBM vesicles isolated from specific-pathogen-free (SPF) young rabbits.

(A preliminary account of this work has been presented elsewhere [2].)

MATERIALS AND METHODS

BBM vesicle preparation. SPF 4- and 7-week-old New Zealand albino hybrid rabbits were obtained from Charles River. Jejunal segments from 7-week-old rabbits and the entire small intestine from 4-week-old rabbits were removed, rinsed with saline at room temperature, everted, and distributed into plastic bags for storage at -80°C as described elsewhere (21).

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TABLE 1. Comparative effects of NSP4-related peptides on the kinetics of D-glucose and L-leucine uptake by intestinal BBM vesicles from young rabbits^a

Peptide (1 mM)	Kinetic parameter (mean ± SD)			F test	
	V_{\max} (pmol · s ⁻¹ · mg of membrane protein ⁻¹)	K_T (mM)	K_d (nl · s ⁻¹ · mg of membrane protein ⁻¹)	F (df)	F ^b
4-wk-old rabbits (substrate: D-Glucose)					
Control	992 ± 65	0.41 ± 0.06	3.5 ± 1.2	0.536 (8, 54)	a
NV(464-483)	753 ± 54	0.38 ± 0.06	5.2 ± 1.1	1.003 (7, 47)	a
mNSP4(131K)	843 ± 42	0.40 ± 0.04	9.0 ± 1.1	2.026 (8, 49)	a
Overall fit	878 ± 35	0.41 ± 0.04	5.4 ± 0.7	1.442 (8,171)	a
NSP4(114-135)	165 ± 20	0.53 ± 0.12	5.1 ± 0.8	1.422 (8, 50)	b
7-wk-old rabbits					
Substrate: D-Glucose					
Control	389 ± 38	0.42 ± 0.09	4.9 ± 1.1	0.864 (8, 49)	a'
NV(464-483)	339 ± 32	0.39 ± 0.08	3.2 ± 0.8	0.229 (8, 22)	a'
mNSP4(131K)	223 ± 24	0.37 ± 0.08	2.9 ± 0.7	2.148 (7, 17)	a'
Overall fit	337 ± 24	0.41 ± 0.06	3.6 ± 0.6	0.350 (9,108)	a'
NSP4(114-135)	48 ± 11	0.25 ± 0.13	3.4 ± 0.6	0.940 (6, 18)	b'
Substrate: L-Leucine					
Control	322 ± 39	0.36 ± 0.09	7.5 ± 1.7	0.580 (11, 49)	a''
NV(464-483)	270 ± 32	0.31 ± 0.08	5.5 ± 1.5	0.493 (12, 73)	a''
NSP4(114-135)	165 ± 22	0.36 ± 0.10	3.4 ± 1.0	0.432 (10, 56)	a''
Overall fit	248 ± 21	0.36 ± 0.06	5.2 ± 0.9	0.500 (12,205)	a''

^a BBM vesicles from 4- or 7-week-old rabbits were mixed in the appropriate volume of membrane buffer in either the absence or presence of the indicated peptides, calculated to give concentrations of 1 mM in the final (transport) incubation mixtures. After preincubation for 5 min at 35°C, aliquots were used to assay for either D-glucose or L-leucine uptake as a function of [S], under standard conditions. Uncorrected uptake data were used to estimate the relevant kinetic parameters. For each condition, F values were all found to be nonsignificant (see Materials and Methods).

^b Letters identify fits that are statistical other ($P < 0.01$). Relevant data have been pooled and fitted again to yield the indicated overall fit values. For further details, see text.

Intestinal BBM vesicles, prepared according to the magnesium precipitation method of Hauser et al. (12), were suspended at about 40 mg of membrane protein/ml in membrane buffer (20 mM HEPES–10 mM Tris HCl, supplemented to a total osmolality of 600 mosM with the inert sugar D-arabinose and adjusted to pH 7.4 with Tris base [9, 21]) and stored in liquid nitrogen until the day of assay, as described elsewhere (24). Membrane protein concentrations were measured with a Bio-Rad protein assay kit, using serum globulin as the standard.

Viral protein and synthetic peptides. The SA11 NSP4 protein and all peptides used in this work were synthesized and purified as described elsewhere (5). They were dissolved in the appropriate volume of membrane buffer to obtain either 8 μM protein or 10 mM peptide stock solutions, which were stored at –20°C until use. The peptides used were Norwalk virus NV(464-483) (DTGRNLGEFKAYP DGFLTCV), NSP4(114-135) (DKLTTREIEQVELLKRIYDKLT), and mNSP4(131K) (DKLTTREIEQVELLKRIKDKLT).

Transport assay and expression of results. Transport was assayed by using a rapid filtration technique and either D-[¹⁴C]glucose or L-[¹⁴C]leucine as the substrate (7). BBM vesicle aliquots suspended in membrane buffer were mixed with appropriate amounts of the same buffer containing either the intact protein or a given peptide. After preincubation for 5 min at 35°C (unless stated otherwise), 10-μl aliquots were used to carry out transport measurements by mixing with 40 μl of transport buffer formed by membrane buffer supplemented with either constant or variable (see below) concentrations of unlabeled substrate, ¹⁴C-labeled substrate as a tracer, and 100 mM sodium cyanide (all concentrations given as the final ones in the incubation mixtures). Initial uptake rate measurements were then carried out for 2.6 s at 35°C in an automatic, temperature-controllable short-time incubation apparatus constructed in our laboratory. Uptake data were statistically compared by applying a global one-way analysis of variance (19). Results are shown as absolute uptake rates, v , in picomoles per second per milligram of protein ± standard deviation (SD).

Kinetic analyses. Uncorrected uptake rates as a function of either the substrate ($v = f[S]$ at [I] constant) or inhibitor ($v = f[I]$ at [S] constant) concentration were fitted by nonlinear least-squares regression analysis (23) to the appropriate equation as indicated. To perform each fit, the procedure of Fletcher and Powell as modified by van Melle and Robinson (23) was used. To test the fit of data to each relevant equation, we used the commercial program Stata (Integral Software, Paris, France). For statistical evaluation, fits were compared either within each given condition (F test) or between pairs of conditions (F' test) as described elsewhere (23). For each given data set, comparison between a lack-of-fit and a pure-error component, with the degrees of freedom given in that order in parentheses in the tables, yielded F values that provide a quantitative assessment

of the goodness of fit. All individual F values listed in the tables were found to be not significant, meaning that the data points did not differ statistically ($P < 0.01$) from the theoretical fit of the equation used to perform the fit. This was true for each of the curves forming each set, as well as for the overall fit of the set. All calculations were done on Apple Macintosh microcomputers.

RESULTS

Effect of rotavirus NSP4 on intestinal BBM Na⁺-solute symport activities in young rabbits. Jejunal BBM vesicles from 7-week-old rabbits were mixed with appropriate amounts of purified NSP4. After incubation for 5 min at 35°C, aliquots were used to assay uptake under standard conditions that included a constant (0.1 mM) substrate concentration and a zero-trans sodium cyanide gradient. Because of its low solubility, the maximal protein concentration that could be reached in the incubation mixtures was 1 μM. This dose, however, was found to be too low to have any significant effect on the uptake of either D-glucose or L-leucine (results not shown). The question posed having remained unanswered, we turned our attention to more soluble peptides derived from NSP4, some of which have been shown to induce diarrhea in young rodents, with kinetics similar to that of the intact protein (5).

Comparative effects of NSP4-related peptides on the kinetics of D-glucose and L-leucine uptake by intestinal BBM vesicles from young rabbits. In preliminary assays, we established that doses in the order of 1 mM peptide were necessary to cause significant effects on these symporters. This information was then used to investigate the possible mode of action of each of these peptides on intestinal transport, using first the $v = f[S]$ approach, at constant [I].

Saturation curves were performed with either D-glucose or L-leucine as the substrate (Table 1). As previously established

with young rabbits (10), all uptake curves were found to fit an equation involving the sum of one Michaelian, saturable transport component and one nonsaturable, diffusion-like component,

$$v = \{[V_{\max}/(K_T + [S])] + K_d\} \cdot [S] \quad (1)$$

where V_{\max} and K_T are, respectively, the capacity and affinity parameters of classical Michaelis-Menten kinetics and K_d is an apparent diffusion constant. Except for minor fluctuations that fall within normal limits (9, 10), both K_T and K_d remained practically unchanged, regardless of the substrate used, the peptide present in the transport assay, and the age of the animals (average $K_T = 0.38 \pm 0.11$ [SD], $n = 610$; average $K_d = 5.1 \pm 2.2$, $n = 610$).

If the results are considered by groups, a similar conclusion applies to V_{\max} with the exception only of D-glucose. Thus, in contrast with L-leucine, the D-glucose saturation curves obtained in the presence of the NSP4(114-135) peptide are the only ones found to be different by an F' test (Table 1).

Because K_d remained constant under all conditions studied, and because K_d is not merely a measure of the diffusion rate but also can be interpreted as a function of the vesicular volume, that is, of the physical integrity of the vesicles (8), we concluded that the apparent vesicular volume was constant, meaning that there was no evidence of vesicle lysis in any of these experiments. Because the preceding initial velocity experiments involved very short (2.6-s) incubations, we also measured the vesicular volume after incubations for 90 min at 35°C. Again, K_d was found to remain constant, regardless of the presence or absence of any of the NSP4-related peptides used (data not shown). Taken as a whole, these results agree with the observations of Tian et al. (20) indicating that even though NSP4 and the NSP4(114-135) peptide have membrane destabilization activity, this seems to be true only for liposomes and endoplasmic reticulum vesicles, not for plasma membrane vesicles such as the BBM vesicles used in the present work. Because the integrity of the membrane was not affected, we conclude that the observed inhibitions must have taken place directly at the transport level.

Because the strong inhibitions caused by the NSP4(114-135) peptide affected only the D-glucose transport V_{\max} (on the average, 81 and 85% inhibition with 4- and 7-week-old animals, respectively), it can be concluded that this peptide acts as a fully noncompetitive inhibitor of SGLT1. Interestingly, a similar kinetic result, a selective inhibitory effect on the V_{\max} of SGLT1, was obtained in *in vivo* studies of rotavirus infection of young rabbits (10).

L-Leucine uptake was used as a control for a distinct but mechanistically quite similar symport system, that of Na^+ with neutral amino acids. Although an effect of NSP4(114-135) on L-leucine transport is suggested by the data in Table 1, the results are borderline; that is, the control and NSP4(114-135) curves were kinetically indistinguishable according to an F' test at $P < 0.01$. A significant difference was apparent when the limits of probability were increased to $P < 0.05$, but no definitive conclusion on this point could be reached. The existence of a small inhibition of the Na^+ -L-leucine symporter by the NSP4(114-135) peptide, although conceivable, can be considered to be quantitatively negligible compared with the strong inhibition caused by this peptide on SGLT1.

The C-terminal Norwalk virus capsid, NV(464-483) peptide, was tested as a possible negative control since this peptide does not cause diarrhea even though it has physical properties quite similar to those of NSP4(114-135), including a practically identical amphipathic score (5). The NV(464-483) peptide was

found to have a slight inhibitory effect on both D-glucose and L-leucine transport (13 to 24% inhibition of V_{\max}). But again, in both cases, an F' test revealed no significant difference compared to the control group. Interestingly, compared with NSP4(114-135), the NV(464-483) peptide behaved essentially in the same manner toward the Na^+ -L-leucine symporter, agreeing with the results of peptide inhibition on leucine uptake shown in Table 2. Obviously, such results strongly support the conclusion that the effects of NSP4(114-135) on the D-glucose symporter are indeed specific.

The third peptide investigated, mNSP4(131K), differs from NSP4(114-135) only by having an L-lysine residue substituting for the L-tyrosine at position 131. Similar to NV(464-483), the mNSP4(131K) peptide had a slight effect on SGLT1, but once again, this effect was not significant according to the F' test at $P < 0.01$ (Table 1). The fact that mNSP4(131K) neither inhibits SGLT1 nor causes diarrhea (5) is perhaps the best evidence for the specificity of the NSP4(114-135) effect on D-glucose transport mentioned above. We conclude that for SGLT1 inhibition to occur, the presence of an L-tyrosine in position 131 of the peptide is required. However, the question of why this particular L-tyrosine is necessary for inhibition or, alternatively, why the presence of a positively charged L-lysine in its place blocks the inhibitory effect remains open.

Concerning the statistical relevance of the kinetic results presented above, we propose the following considerations. First, the total number of experimental points forming each saturation curve (Table 1, df) is generally sufficient to obtain statistically reliable fits to an equation (23). Second, even if small quantitative differences are apparent, when taken by groups (age of the animal, peptide, and substrate used), all of the data are roughly equivalent. Hence, certain of the $v = f[S]$ results have been pooled into a single curve, such as those listed as overall fits in Table 1 (e.g., upper curve in Fig. 1). As mentioned, the only exceptions to this rule are the D-glucose curves obtained in presence of the NSP4(114-135) peptide (lower curve in Fig. 1). Finally, the fact that the D-glucose transport results obtained for 4- and 7-week-old rabbits were quantitatively equivalent reinforces the statistical validity of this entire set of kinetic results.

Dose-dependent peptide effects on the Na^+ -solute symporters. To confirm that the NSP4(114-135) effect on D-glucose (but not on L-leucine) transport is indeed specific, experiments were performed by using the $v = f[I]$ approach, at a constant (0.1 mM) concentration of each substrate.

(i) Effects on D-glucose uptake. With the NSP4(114-135) peptide we found a progressive effect, approaching 78% inhibition of the D-glucose symporter at 1 mM peptide (Fig. 2). A significant but lesser inhibition was observed when the NV(464-483) peptide was used (29% inhibition at 1 mM peptide [Fig. 2]). However, the two curves have different shapes, which can be taken as evidence that different transport pathways are involved, as discussed below.

Focusing on SGLT1, we considered whether the NSP4(114-135) peptide inhibits preferentially from the outer (*cis*) or from the inner (*trans*) side of the membrane. To answer this question, right-side-out vesicles were treated with the peptide initially present only in the *cis* side, but half of the vesicle-plus-peptide mixtures were subjected in parallel to two cycles of freezing and thawing in liquid nitrogen to permit equilibration of the peptide across the membrane (see reference 24 for the rationale of this procedure). The D-glucose uptake rates were found to be the same (Fig. 2), regardless of whether *cis*- or *cis-plus-trans* conditions were used. This result can be interpreted in several ways. First, the peptide may act either preferentially or exclusively from the external side of the mem-

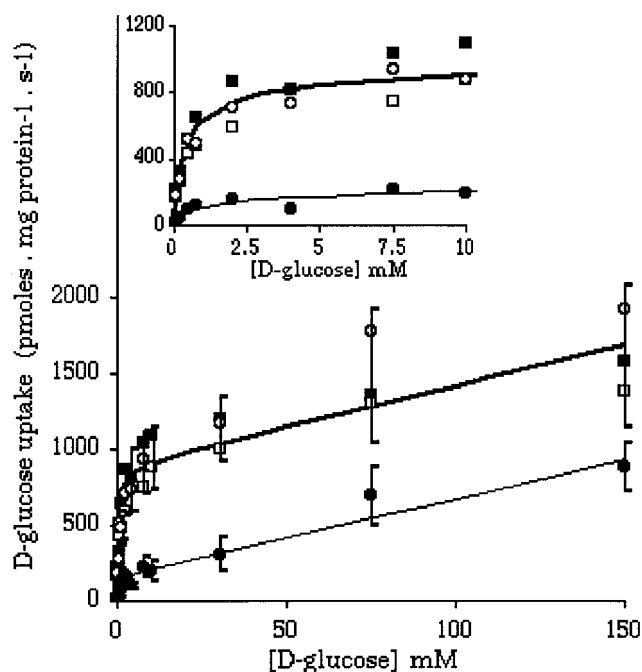


FIG. 1. Kinetic effects of NSP4-related peptides on D-glucose uptake by intestinal BBM vesicles from 4-week-old rabbits. Initial rates of D-glucose uptake, expressed as the uncorrected, absolute uptake rates, are plotted as a function of substrate concentration in either the absence (■) or presence of 1 mM NV(464-483) (□), mNSP4(131K) (○), and NSP4(114-135) (●). Solid lines represent theoretical fits computed by using the kinetic parameters listed in Table 1. However, because the control, NV(464-483), and mNSP4(131K) results were all statistically indistinguishable, they have been pooled and are shown as a single curve corresponding to the overall fit for 4-week-old rabbits in Table 1. The insert shows the results obtained by using only the lowest [D-glucose] range used, 0.1 to 10 mM.

brane. Second, the amphipathic peptide can equilibrate rapidly across the membrane, in which case freezing and thawing would not be necessary to obtain a maximal effect if it acts preferentially from the inner side. The most likely and simplest interpretation is that the peptide is equally effective from either side of the membrane.

To evaluate the stability of the peptide and whether the inhibition was time dependent, we varied the time of contact between peptide and membrane. Before assaying for transport, vesicles were incubated with or without 0.4 mM (final concentration) NSP4(114-135) peptide for time periods of 1, 5, 15, and 30 min at 35°C. The D-glucose uptake rates were found to remain constant, regardless of the time of preincubation. However, they were significantly different in either the absence ($174.5 \pm 28.7 \text{ pmol} \cdot \text{s}^{-1} \cdot \text{mg of protein}^{-1}$, $n = 21$) or presence (127.9 ± 21.5 , $n = 21$) of the peptide, indicating a 27% inhibition. Quantitatively, this result agrees fully with that shown in Fig. 2, indicating that (i) the effect is practically instantaneous (it does not increase with time) and (ii) the peptide is stable. This result proves that the observed inhibition does not involve any NSP4 interference with the targeting of SGLT1 to the BBM, a time-dependent process (incompatible with an instantaneous effect) that furthermore requires an intact cell and would not occur with isolated BBM vesicles.

(ii) **Hill equation analysis.** Because of the shape of the curves obtained, the results in Fig. 2 suggest strongly that an empirical equation of the Hill type might be appropriate to describe the observed peptide effects on D-glucose uptake (although not necessarily those on L-leucine uptake). We there-

fore refitted this set of data by using an equation adapted from Alvarado and Vasseur (3),

$$v = V/(1 + \{([I]/K_i)^{n_i}\}) \quad (2)$$

where V is an apparent maximal velocity; K_i is the apparent affinity constant for the inhibitor, I (the peptide); and n_i is the Hill number. Nonlinear regression analysis of these data gave the numerical results in Table 2. Both NV(464-483) and NSP4(114-135) results could be fit very well by using equation 2, but the two curves were statistically different according to an F' test. The NSP4(114-135) peptide gave a Hill number larger than 1 ($n_i = 2.8$), indicating the participation of at least three peptide-binding sites in the interaction, each with an average K_i of 0.67 mM. In contrast, the NV(464-483) peptide yielded a Hill number of 1, with a lower affinity ($K_i = 1.7 \text{ mM}$), suggesting that although NV(464-483) inhibits D-glucose uptake, the effect is relatively small and probably involves a different mechanism that might be nonspecific.

(iii) **Effects on L-leucine uptake.** The most salient feature of the L-leucine results is that both the NV(464-483) and NSP4(114-135) peptides cause a practically identical, partial inhibition of L-leucine uptake (Fig. 3; Table 2). Consequently, because they are statistically indistinguishable, these data have been pooled and fitted again to obtain the overall fit listed in Table 2. As illustrated in Fig. 3, this result strongly suggests that L-leucine symport inhibition is nonspecific and is not mediated by NSP4(114-135), a specific inhibitor of SGLT1. Analysis of the L-leucine results according to the Hill equation (Table 2) reveals that the two peptides used, NV(464-483) and

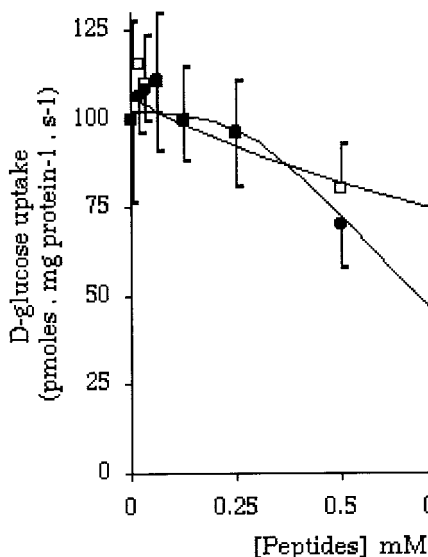


FIG. 2. Dose-dependent effects of the NSP4(114-135) and NV(464-483) peptides on the initial rate of D-glucose uptake by jejunal BBM vesicles from 7-week-old rabbits. Fixed amounts of vesicles were mixed with variable amounts of peptide in the appropriate volume of membrane buffer to give the indicated (final) peptide concentrations. These mixtures were either used directly (*cis* peptide conditions) or subjected first to two cycles of freezing and thawing to permit equilibration of the extra- and intravesicular contents (*cis-plus-trans* conditions), as described elsewhere (24). After incubation for 5 min at 35°C, aliquots were used to measure the initial rate of 0.1 mM D-glucose uptake under standard conditions. Because the results at all given *cis* and *cis-plus-trans* peptide concentrations were statistically indistinguishable, they have been pooled and fitted again to obtain relevant, overall fits. These are illustrated by the same symbol, ● for NSP4(114-135) and □ for NV(464-483). Results are shown as absolute substrate uptake rates \pm SD. The solid lines represent theoretical fits of either set of data to equation 2, computed by using the kinetic parameters listed in Table 2.

TABLE 2. Kinetic parameters used to compute the theoretical curves in Fig. 2 and 3^a

Substrate	Peptide	Kinetic parameter (mean \pm SD)			F test	
		V (pmol·s ⁻¹ · mg of membrane protein ⁻¹)	K_t (mM)	n_i	F (df)	F'
0.1 mM D-glucose	NV(464-483)	106 \pm 4	1.69 \pm 0.42	1.1 \pm 0.4	0.903 (7,71)	a
	NSP4(114-135)	102 \pm 3	0.67 \pm 0.04	2.8 \pm 0.4	0.532 (6,80)	b
0.1 mM L-leucine	NV(464-483)	97 \pm 7	2.00 \pm 1.50	0.4 \pm 0.2	0.763 (7,22)	a'
	NSP4(114-135)	100 \pm 9	0.31 \pm 0.17	0.4 \pm 0.1	1.473 (6,56)	a'
	Overall fit	99 \pm 7	0.49 \pm 0.20	0.4 \pm 0.1	1.622 (7,87)	a'

^a Kinetic parameters for the function $v = f[I]$ (at $[S]$ constant = 0.1 mM) were estimated by applying equation 2. Details for F and F' tests are given in footnotes to Table 1. The L-leucine results being statistically homogeneous, regardless of the peptide present, the relevant data have been pooled and fitted again to yield the indicated overall fit values. Further details are given in the text.

NSP4(114-135), gave the same Hill number, 0.4. The fact that these n_i values are smaller than unity strongly indicates deviation from simple Michaelian kinetics, as further discussed below. Interestingly, such differences in the peptide effects on the sodium-coupled transport of either D-glucose or L-leucine strongly suggest that the two symporters do not use the same mechanism.

Testing whether a three-site symport model can explain the L-leucine and D-glucose transport inhibitions caused by NSP4-related peptides. To test the preceding hypothesis, we reanalyzed the relevant data to see whether they can be explained in terms of the three-site symport model recently proposed by Alvarado and Vasseur to explain the interaction of another BBM symport system, that of the enterocyte H⁺ and Cl⁻ symporter, with the amphipatic inhibitor carbonyl cyanide *m*-chlorophenylhydrazone (4). According to this model, certain Na⁺-solute cotransporters may involve a carrier, C, with three distinct, specific binding sites: one for the substrate, S; one for the allosteric activator, A (the Na⁺ ion); and a third for the inhibitor, I (a peptide in the present paper). In the general

model, it is further assumed that all of the possible substrate-bound carrier complexes, C-S, I=C-S, A=C-S, and IA=C-S, can form and translocate. To use well-established jargon, the model includes slippage; i.e., there is no need to propose that the C-S complex is not mobile. Kinetically, the system is defined by equation A1 in reference 4, that is,

$$v = [(V_1 \cdot [I]) + (V_2 \cdot K_t)] / ([I] + K_t) \quad (3)$$

where v is the total substrate uptake, V_1 and V_2 are two distinguishable maximal velocities resulting when $[I]$ tends to infinity and zero, respectively; and K_t is the apparent affinity constant for I .

Accordingly, the L-leucine data in Fig. 3 were refitted by using equation 3; the kinetic results are presented in Fig. 4 and Table 3. It is clear that both equation 2 (Table 2; Fig. 3) and equation 3 (Table 3; Fig. 4) fit the data satisfactorily; in both cases, the results obtained with the NSP4(114-135) and NV(464-483) peptides are statistically indistinguishable. This result permitted a simpler analysis consisting of pooling the data to obtain the overall fits listed in the tables. The experimental data are of course the same, but the equations used to

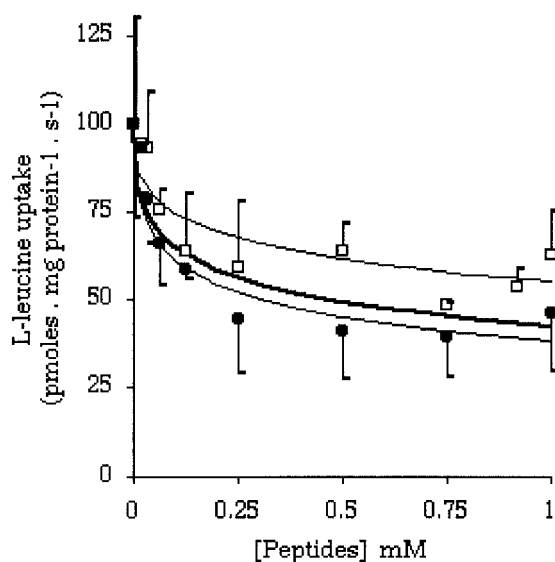


FIG. 3. Dose-dependent effects of the NSP4(114-135) and NV(464-483) peptides on the initial rate of L-leucine uptake by jejunal BBM vesicles from 7-week-old rabbits. Details are as for Fig. 2, except for the use of L-leucine as the substrate and peptides only in the *cis* side. Symbols: ●, NSP4(114-135); □, NV(464-483). Because the two peptides gave statistically indistinguishable results, the two sets of data have been pooled to obtain the overall fit in Table 2 (heavy middle line).

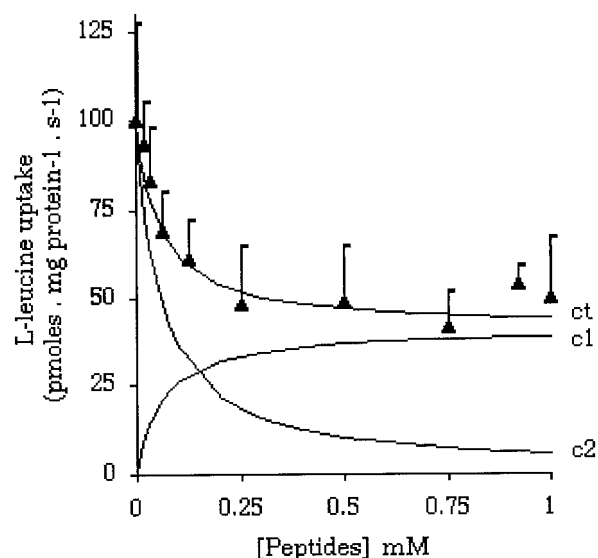


FIG. 4. Effect of the NSP4(114-135) peptide on the kinetics of L-leucine uptake, according to the full, nonobligatory three-site symport model. Experimental L-leucine data (taken from Fig. 3) were fitted by nonlinear regression analysis to equation 3. The theoretical fits thus computed show the overall result to be the sum (ct) of two distinct hyperbolas: one that is Michaelian (concave; c1) and another that is convex (c2).

TABLE 3. Kinetic parameters used to compute the theoretical curves in Fig. 4

Peptide	Kinetic parameter			<i>F</i> test ^a	
	<i>V</i> ₁ (pmol · s ⁻¹ · mg of membrane protein ⁻¹)	<i>V</i> ₂ (pmol · s ⁻¹ · mg of membrane protein ⁻¹)	<i>K</i> _{<i>T</i>} (mM)	<i>F</i> (df)	<i>F</i> '
NV(464-483)	54 ± 5	98 ± 7	0.062 ± 0.05	0.491 (7,22)	a
NSP4(114-135)	37 ± 3	100 ± 8	0.050 ± 0.02	0.540 (6,56)	a
Overall fit	41 ± 3	99 ± 6	0.057 ± 0.02	0.675 (7,87)	a

^a See footnotes to Table 1. Further details are provided in the text.

perform each fit are different, as mentioned. As we shall show, this difference is important, as it concerns interpretation of the mechanisms involved.

The results show, first, that equation 3 yields the best fit, as indicated by its lower *F* value (*F* = 0.68 versus *F* = 1.62 for equation 2). Second and more important, equation 3 furnishes meaningful information about the mechanism because it permits separating the total uptake into two distinct components, as defined by the general three-site model. Here, the two components of equation 3 can be regarded as representing two distinct pathways for leucine uptake, even when each of them involves the same molecular entity, the three-site symporter (4). The first pathway consists of a Michaelian concave hyperbola, [$v_1 = V_1 \cdot [I]/([I] + K_T)$], and involves L-leucine fluxes via the I=C-S and IA=C-S complexes (curve c1 in Fig. 4). The second pathway consists of a convex hyperbola, [$v_2 = V_2 \cdot K/([I] + K_T)$], and involves L-leucine fluxes via the C-S and A=C-S complexes (curve c2 in Fig. 4). Thus, as *[I]* increases, fluxes via these last two complexes will tend toward zero (curve c2), whereas fluxes via the I=C-S and IA=C-S complexes will tend to increase (curve c1). This means that at saturating *[I]* values, *V*_{max} will tend to *V*₁, and *v* cannot possibly be zero. This is the reason why these peptides inhibit L-leucine transport, but only partially (Fig. 3). In conclusion, the mechanism of L-leucine transport inhibition by the amphiphilic peptides can be explained in terms of the full, nonobligatory three-site symport model in which all substrate-bound carrier complexes can form and translocate. It seems conceivable that certain of the submodels previously described as special cases (4) could explain the results even better. Such an analysis has not been attempted here because it is not necessary: regardless of the submodel used, the conclusion stated above would be valid.

When the same analysis was applied to the D-glucose uptake data in Fig. 2, the results were quite different, agreeing with the previous suggestion that this sugar and L-leucine use different symport mechanisms. It is known (1, 16) that SGLT1 follows an obligatory sodium activation mechanism according to which, even though it can form, the C-S complex is not mobile; only fluxes involving the IA=C-S and A=C-S complexes are possible. When these D-glucose data were analyzed with equation 3, the fits all yielded a zero value for *V*₁ (results not illustrated), meaning that only equation 2 can fit these results. Kinetically, such a result agrees fully with the conclusion stated previously in this report that the NSP4(114-135) peptide is a fully noncompetitive inhibitor of SGLT1. Summing up, apart from the fact that the NSP4(114-135) peptide specifically inhibits D-glucose but not L-leucine transport, we conclude that both substrates conform to the three-site symport model but differ in one respect: whereas L-leucine fits the full, general model where Na⁺ is a nonobligatory activator, D-glucose follows a restricted model where Na⁺ is an obligatory activator.

DISCUSSION

Our results show that in vitro, the NSP4(114-135) peptide strongly and specifically inhibits the SGLT1 activity of intestinal BBM vesicles from young rabbits. The Na⁺-L-leucine symporter also is inhibited (Fig. 3), but this effect is much weaker and probably nonspecific. Indeed, it seems conceivable that this effect involves nonspecific, lipophilic interactions of the amphiphilic peptides with the membrane.

Interestingly, in in vivo experiments with young rabbits infected either naturally or experimentally with a lapine rotavirus, La/RR510 strain, the Na⁺-L-leucine symporter was inhibited strongly and to an extent similar to that of SGLT1. In both cases, *V*_{max} was the only kinetic parameter impaired after rotavirus infection (10). Because with D-glucose qualitatively and quantitatively similar effects are obtained, either during rotavirus infection in vivo (10) or in in vitro experiments with isolated NSP4 peptides (this report), the in vitro effects exhibited by certain NSP4-related peptides on SGLT1 might explain the rotavirus transport effects observed in vivo.

The case of L-leucine appears to be special. In contrast with the in vivo experiments just mentioned, the Na⁺-L-leucine symport activity was not significantly inhibited when the isolated NSP4(114-135) peptide was used directly (Table 1). Such a result strongly suggests that if rotavirus infection results in inhibition of the Na⁺-L-leucine symporter, this inhibition, in contrast with that of the SGLT1, is unlikely to be mediated by the NSP4(114-135) peptide. More work will be required to establish whether or not the Na⁺-L-leucine symporter inhibition is caused by some other rotavirus protein or by some NSP4 peptide distinct from NSP4(114-135).

The concentrations of NSP4(114-135) that cause full inhibition of SGLT1, about 1 mM according to the data presented here, are of the same order of magnitude as those used by Ball et al. (5) to induce diarrhea in young rodents. In these experiments, both intraperitoneal and intraduodenal NSP4(114-135) peptide were effective, but only in young (6- to 8-day-old) mice. Peptide administration to slightly older (11- to 13-day-old) animals was much less effective. Our experiments were carried out by using vesicles from either 4- or 7-week-old rabbits. The possibility clearly exists that stronger effects may have been obtained if younger rabbits had been used. But such work was not attempted at this time, mainly because working with SPF rabbits younger than 4 weeks would have posed practical problems of availability.

The very low concentration of the NSP4 protein that can be maintained in the incubation mixtures (1 μM) was found to have no significant effect on the uptake of either D-glucose or L-leucine. Such a result was not surprising because to induce diarrhea in young rodents (5), doses as high as 2 μM were necessary. However, the validity of comparing biologically effective doses derived from either in vivo or in vitro work appears to be limited (25).

The specific and strong inhibition caused *in vitro* by the NSP4(114-135) peptide on SGLT1 strongly suggests that this effect can be one (but not necessarily the only) important factor in the pathogenesis of rotavirus diarrhea. Based on our *in vivo* work with rotavirus-infected rabbits (10), we proposed earlier that a generalized inhibition of Na⁺-solute cotransport systems might be at the basis of the inhibition of intestinal water reabsorption caused by rotavirus in the absence of any histological damage to the intestinal mucosa. The subsequent massive water loss might eventually overwhelm the intestinal capacity for water reabsorption, thereby contributing to establishment of the main symptom of enteritis, diarrhea (10).

The fact that the mNSP4(131K) peptide does not inhibit SGLT1 constitutes perhaps the strongest evidence for our conclusion that the specific inhibition of this cotransporter is a key factor in the pathogenesis of rotavirus diarrhea. In closing, we have established the existence of the following correlation: NSP4(114-135) inhibits SGLT1 and causes diarrhea, whereas mNSP4(131K) neither inhibits SGLT1 nor causes diarrhea.

Concerning the possible role of NSP4 in the pathogenesis of rotavirus diarrhea, the present *in vitro* work strongly suggests that NSP4 is at least one among other effectors directly causing SGLT1 inhibition during rotavirus infection *in vivo*. Whereas *in vitro* NSP4(114-135) inhibits SGLT1 practically instantaneously, the situation *in vivo* is different. Being absent from the mature, infective virion particle, the nonstructural protein NSP4 needs to be synthesized after infection, and time will be required for the newly synthesized protein to be released into the cytoplasm and/or the intestinal lumen and migrate to its target, the symporter molecule.

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