Adsorptive-mediated endocytosis of a basic peptide in enterocyte-like Caco-2 cells

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Sai, Yoshimichi, Masahiro Kajita, Ikumi Tamai, J un Wakama, Tateaki Wakamiya, and Akira Tsuji. Adsorptive-mediated endocytosis of a basic peptide in enterocyte-like Caco-2 cells. Am. J. Physiol. 275 (Gastrointest. Liver Physiol. 38): G514–G520, 1998.—The internalization of a basic peptide, 001-C8 [H-MeTyr-Arg-MeArg-o-Leu-NH(CH2)8NH2], into enterocyte-like Caco-2 cells was evaluated. Internalization of 125I-labeled 001-C8 increased time dependently and reached steady state at 60 min. The steady-state internalization of 125I-001-C8 (7.24 ± 0.41 µl/mg protein) was temperature and concentration dependent and was significantly decreased by dansylcadaverine (500 µM), protamine (1 mM), poly-L-lysine (1 mM), E-2078 (1 mM), and ebiratide (1 mM), whereas poly-L-glutamic acid (1 mM), tyrosine (1 mM), and glycyglycine (25 mM) were not inhibitory. Predigestion of acid mucopolysaccharides by heparinase I, heparitinase, and chondroitinase ABC also decreased the internalization. The maximal internalization, the half-saturation constant, and the maximal internalization of 125I-001-C8 were 11.3 ± 0.02 pmdl/mg protein, 0.47 ± 0.43 µM, and 3.13 ± 0.19 µM protein, respectively. Confocal microscopy also indicated the internalization of fluorescence-derived 001-C8 (001-C8-4-nitrobenz-2-oxa-1,3-diazole (001-C8-NBD)). Granular staining seen within the cell, excluding nuclei, indicated the sequestration of 001-C8-NBD within endocytotic vesicles. Dansylcadaverine and protamine strongly decreased the granular distribution of 001-C8-NBD within the cell. These results demonstrate that 001-C8 is taken up by Caco-2 cells via adsorptive-mediated endocytosis.

in intestine; transport; macromolecular uptake; absorptive-mediated drug delivery

SPECIFIC TRANSPORT mechanisms have been evolved in the intestine to facilitate the uptake of various nutrients, including small peptides and macromolecules. For proteins and peptides, four types of uptake mechanisms have been recognized. They include 1) highly specific, receptor-mediated endocytosis (RME), 2) adsorptive-mediated endocytosis (AME) after binding of molecules to cell surface anionic sites, 3) nonspecific fluid-phase endocytosis (AME) of substances dissolved in the extracellular fluid, and 4) carrier-mediated transport for small peptides (3–6, 12, 14, 16–18, 23, 25, 27). RME mechanisms in the intestine have been shown in the transport of various immunoglobulins and growth factors (see Ref. 18 for review). Fluid-phase endocytosis has also been reported with horseradish peroxidase as a model protein in Caco-2 cells (4). As for AME in the intestine, however, very little information is available. Although a dynorphin-like analgesic peptide, E-2078 (23), an ACTH analog, ebiratide (19), and various polycationic proteins, such as β-endorphin-cationized albumin complex (6), histone (14), and avidin (12), are transported via AME into the brain capillaries, it remains unknown whether such a process also occurs in intestinal epithelial cells.

In our previous study, we prepared a novel peptide, 001-C8[H-MeTyr-Arg-MeArg-o-Leu-NH(CH2)8NH2], and its derivatives with different numbers of basic and neutral amino acids and with various carboxy-terminal structures to clarify the structural specificity of AME of brain capillary endothelial cells (21). 001-C8 consists of a partial amino acid sequence of E-2078 and the carboxy-terminal structure of ebiratide, with two arginine residues and an octanediamine residue, and was confirmed to be efficiently taken up by brain capillary endothelial cells via AME (21).

Caco-2, a human colon carcinoma cell line, forms a highly polarized membrane when grown to confluence on plastic dishes and exhibits structural and functional differentiation patterns characteristic of mature enterocytes (15). Caco-2 cells have been used as an in vitro model in various studies, including characterization of the intracellular sorting or cytosis of proteins (1, 4, 9), because of their close morphological and functional similarity to intestinal epithelium. We have also used this system in studies of the carrier-mediated transport mechanism (11, 22, 26).

In this report, we demonstrate the AME of a basic peptide, 001-C8, in enterocyte-like Caco-2 cells by means of inhibition, saturation kinetic, and confocal-microscopic observation studies using radio- or fluorescence-labeled ligands.

MATERIALS AND METHODS

Materials. [3H]polyethylene glycol 900 ([3H]PEG 900) (74–370 MBq/g) and Na125I (629 GBq/mg) were purchased from New England Nuclear (Boston, MA). Tetramethylrhodamine-dextran was obtained from Molecular Probes (Eugene, OR). FCS and rat tail collagen (type I) were purchased from GIBCO (Grand Island, NY) and Collaborative Research (Bedford, MA), respectively. Salmon roe protamine sulfate was purchased from Wako Pure Chemical Industries (Osaka, Japan). Dansylcadaverine, heparin, heparitinase I, and neuraminidase were purchased from Sigma Chemical (St. Louis, MO). Heparitinase, chondroitinase ABC, and hyaluronidase were purchased from Seikagaku (Tokyo, Japan). E-2078, a dynorphin-like analgesic peptide [H-MeTyr-Gly-Gly-Phe-Leu-Arg-MeArg-o-Leu-NH(CH2)8NH2], was kindly supplied by Eisai (Tokyo, Japan). The Caco-2 cells were obtained from American Type Culture Collection (Manassas, VA). All cells used in this study were between passages 33 and 59.

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All other chemicals used were commercial products of reagent grade.

Peptide synthesis and labeling. 001-C8 used in the present study was synthesized in this laboratory, as described previously (21), and was coupled to 4-nitrobenz-2-oxa-1,3-diazole (NBD) as a fluorescence label for direct visualization. Radioiodination of 001-C8 was performed with Na125I and chloramine-T as described previously (21). 125I-001-C8 obtained had a specific activity of ~11.1 TBq/g and a chemical purity of >95%.

Assay for internalization. The cultivation of Caco-2 cells was performed as described previously (26). A typical uptake experiment was as follows. Monolayers of Caco-2 cells grown for 7 days after confluence on four-well multidishes (NUNC, Raskilde, Denmark) coated with collagen were washed three times with Hanks’ balanced salt solution (HBSS) (pH 6.0) (in mM: 0.952 CaCl2, 5.36 KCl, 0.441 KH2PO4, 0.812 MgSO4, 136.7 NaCl, 0.385 K2HPO4, 25 D-glucose, and 10 MES; the osmolarity was 315 mosmol/kg). The uptake experiment was initiated by adding 250 µl of incubation solution (HBSS, pH 6.0) containing 125I-001-C8 (37 kBq) or [3H]PEG 900 (37 kBq) to each well. At designated times after incubation, cultures were washed three times with 1 ml of ice-cold incubation solution to terminate uptake. An acid-wash technique (24) was then used to distinguish surface-bound and internalized 125I-001-C8. The acid treatment removes 001-C8 bound to the cell surface. After the uptake procedure, cells were incubated for 10 min with 1 ml of ice-cold acetate-barbital buffer (28 mM CH3COONa, 120 mM NaCl, 20 mM barbitral-HCl, pH 3.0) at 4°C. The buffer was then removed, and cells were washed four more times with 1 ml of acetate-barbital buffer. The radioactivity in the cells was measured after solubilization with 250 µl of 1 M NaOH for 1 h at room temperature and represents internalized 125I-001-C8. Radioactivity was measured with a gamma counter (ARC-600, Aloka, Tokyo, Japan) and a liquid scintillation counter (LSC-700; Aloka, Japan) for 125I and 3H, respectively. Protein contents of cultured cells were determined as described by Lowry et al. (7) with BSA as a standard. The number of experiments given in Tables 1 and 2 and Figs. 1–6 represents the number of wells of cultured cells used in each measurement.

Confocal microscopy. Caco-2 cells were grown on a glass coverslip equipped with a Flexiperm chamber (Heraeus Instruments, Hanau, Germany) and cultured for 14 days at 37°C with 95% air-5% CO2. The coverslips used were precoated with rat tail collagen under ultraviolet light. Cultured monolayers of Caco-2 cells were rinsed three times with HBSS (pH 6.0), mounted on the microscope stage, and incubated with 10 µM 001-C8-NBD dissolved in 200 µl of HBSS (pH 6.0). Tetramethylrhodamine-dextran (10 mg/ml, labeled overnight and chased for 30 min) was used as an organelle marker. The temperature at 37°C throughout the incubation period. The stage was equipped with a thermostabilizing device to keep the temperature at 37°C throughout the incubation period.

The confocal microscopy used in this study consisted of an MRC-1000 Confocal Imaging System (Bio-Rad, Tokyo, Japan) and an Axiovert 135 inverted microscope equipped with a Neofluar oil objective (magnification ×63, numerical aperture 1.25) and epifluorescence optics (Carl Zeiss, Oberkochen, Germany). This instrument uses a pair of microcomputer-controlled galvanometer mirrors to scan a 488- or 514-nm beam of light from a pinhole illuminated by an argon ion laser in a raster pattern across the specimen being viewed. Images of each optical section were taken, digitized, and stored in a frame buffer in the microcomputer.

HPLC analysis. Unchanged 125I-001-C8 and its metabolites in the incubation solution and in the acid-resistant fraction were analyzed by HPLC. Acid-washed cells were solubilized with 1 M NaOH. Each sample was evaporated to dryness under reduced pressure at room temperature and reconstituted in the mobile phase used for HPLC assay. The HPLC analysis conditions were as follows. The column was a VYDAC 214TP54 (Separations Group, Hasperia, CA); the mobile phase was a mixture of water, acetonitrile, and trifluoroacetic acid (15:85:0.1); and the flow rate was 1.0 ml/min. The eluates were collected with a fraction collector (FRAC-100; Pharmacia, Tokyo, Japan), and the radioactivity in each fraction (0.5 ml) was measured.

Data analysis. Total and acid-resistant bindings were expressed as the cell-to-medium ratio as follows:

\[
\text{acid-resistant binding (µl/mg protein)} = \frac{125I-R}{\text{mg of Caco-2 cell protein}} / \frac{125I-S}{\mu l \text{ medium}} \]  

\[
\text{total binding (µl/mg protein)} = \frac{125I-R + 125I-S}{\mu l \text{ medium}} 
\]

where 125I-R and 125I-S are the radioactivities of 125I in the acid-resistant and acid-soluble fractions, respectively, and 125I-M is the radioactivity of 125I in the incubation medium.

The apparent acid-resistant binding of [3H]PEG 900 was estimated in the same manner.

RESULTS

Time course of internalization of 125I-001-C8 into cultured monolayers of Caco-2 cells. The surface binding and internalization of a cationic peptide, 125I-001-C8 [H-125I-MeTyr-Arg-MeArg-D-Leu-NH(CH2)8NH2], at the apical surface of cultured monolayers of Caco-2 cells were assessed by its total binding and acid-resistant binding and were compared with the surface binding and internalization of the extracellular space and a fluid-phase endocytosis marker, [3H]PEG 900 (Fig. 1). The values were normalized using the initial concentration of each substrate and expressed as the cell-to-medium ratio.

Fig. 1. Time course of total and acid-resistant bindings of 125I-labeled 001-C8 (125I-001-C8 and [3H]polyethylene glycol 900 ([3H]PEG 900) to cultured monolayers of Caco-2 cells. Caco-2 cells were incubated with 125I-001-C8 (circles) or [3H]PEG 900 (squares) for 5 s to 120 min at 37°C. Total and acid-resistant bindings of each substrate were determined as described in MATERIALS AND METHODS. Data are means ± SE of 3 or 4 experiments.
cell-to-medium ratio, calculated as described in MATERIALS AND METHODS. Total and acid-resistant bindings of $^{125}$I-001-C8 increased time dependently, reaching steady-state values of over 13 and 7 µl/mg protein, respectively, at 60 min. Both values were significantly higher than that of acid-resistant binding of [3H]PEG 900. In the following experiments, the acid-resistant binding at 60 min was used to evaluate the steady-state internalization of $^{125}$I-001-C8.

Stability of the peptide in Caco-2 cells. The stability of 001-C8 during the internalization assay period was assessed (Fig. 2). The radio-HPLC chromatograms of $^{125}$I-001-C8 internalized into Caco-2 cells and remaining in the assay medium at 60 min revealed that the intact peptide accounted for 76% and 89% of the radioactivity, respectively.

Temperature dependency and effects of an endocytosis inhibitor and various cationic peptides on internalization of $^{125}$I-001-C8 into Caco-2 cells. Table 1 shows the temperature dependency and effects of an endocytosis inhibitor and several cationic peptides on the internalization of $^{125}$I-001-C8 into Caco-2 cells. At 4°C, the internalization was reduced to 60% of the control value obtained at 37°C. An endocytosis inhibitor, dansylcadaverine (500 µM), significantly decreased the internalization. The polycationic peptides poly-L-lysine (1 mM) and protamine (1 mM) inhibited the internalization. It was also reduced by the structurally analogous compounds E-2078 (1 mM) and ebiratide (1 mM). Unlabeled 001-C8 also decreased the internalization of $^{125}$I-001-C8 at 1 mM, suggesting the existence of a saturable mechanism. No inhibition was seen with anionic poly-L-glutamic acid (1 mM) or the constituent amino acid, tyrosine (1 mM), which is the iodo-labeling site of 001-C8. The dipeptide glycylglycine (25 mM) was not inhibitory, either.

Saturation of the internalization process for $^{125}$I-001-C8. The internalization of $^{125}$I-001-C8 into Caco-2 cells was saturable (Fig. 3). The kinetic parameters for the binding of these peptides were estimated by nonlinear least-squares analysis (28). The maximal internalization ($B_{max}$), the half-saturation constant ($K_d$), and the nonsaturable internalization were 1.13 ± 0.23 pmol/mg protein, 0.47 ± 0.43 µM, and 3.13 ± 0.19 µl/mg protein, respectively.

Effect of cell surface deglycosylation on internalization of $^{125}$I-001-C8 into Caco-2 cells. To investigate the chemical nature of the membrane-associated anionic sites, the apical surface of Caco-2 cells was treated, before exposure of the cell to $^{125}$I-001-C8, with heparinase I, heparitinase, chondroitinase ABC, hyaluronidase, or neuraminidase to remove heparin, heparan sulfates, chondroitin sulfates, hyaluronic acid, or sialic acid, respectively (Table 2). The internalization of $^{125}$I-001-C8 was significantly decreased by heparinase I, heparitinase, and chondroitinase ABC. The effects of these enzymes in combination were approximately additive. Digestion with hyaluronidase had little effect, whereas neuraminidase treatment increased the internalization. We also examined the influence of heparin, which was found to cause a significant decrease in internalization. None of these enzymes affected internalization of [3H]PEG 900.

Confocal microscopic analysis of internalization of 001-C8-NBD in Caco-2 cells. Figure 4 shows the time-dependent internalization into Caco-2 cells of 001-C8 labeled with the fluorescent probe NBD (001-C8-NBD). Faint fluorescence was detected at an incubation time of 10 min. At 60 min, significant fluorescence appeared within the cells, excluding the nuclei. The granular staining indicated the sequestration of 001-C8-NBD within endocytotic vesicles. Tetrathymelrhodamine-
Table 1. Effects of temperature, an endocytosis inhibitor, and various cationic peptides on internalization of 125I-001-C8 into Caco-2 cells

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Internalization, % of control</th>
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<tbody>
<tr>
<td>Control</td>
<td>100.0 ± 4.6</td>
</tr>
<tr>
<td>4°C</td>
<td>60.9 ± 9.6*</td>
</tr>
<tr>
<td>+ Dansylcadaverine (500 µM)</td>
<td>55.6 ± 2.4*</td>
</tr>
<tr>
<td>+ Poly-L-lysine (1 mM)</td>
<td>47.3 ± 1.3*</td>
</tr>
<tr>
<td>+ Protamine (1 mM)</td>
<td>49.5 ± 2.2*</td>
</tr>
<tr>
<td>+ E-2078 (1 mM)</td>
<td>61.7 ± 6.3*</td>
</tr>
<tr>
<td>+ Ebiratide (1 mM)</td>
<td>67.1 ± 2.9*</td>
</tr>
<tr>
<td>+ 001-C8 (1 mM)</td>
<td>29.6 ± 0.8*</td>
</tr>
<tr>
<td>+ Poly-L-glutamic acid (1 mM)</td>
<td>130.7 ± 31.5</td>
</tr>
<tr>
<td>+ Tyrosine (1 mM)</td>
<td>115.7 ± 6.9</td>
</tr>
<tr>
<td>+ Glycylglycine (25 mM)</td>
<td>113.2 ± 7.8</td>
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Values are means ± SE of 3 or 4 experiments. Internalization of 125I-labeled 001-C8 at 60 min under indicated conditions was determined as described in Fig. 1 legend. Each point is mean ± SE of 3 or 4 experiments. Internalization was determined as described in Fig. 1 legend. For the study with dansylcadaverine, cells were preincubated for 30 min at 37°C in incubation solution containing 500 µM dansylcadaverine. *Statistically significantly different from control (Student’s t-test, P < 0.05).

dextran was used as a marker for lysosomal distribution and compared with the distribution of 001-C8-NBD (Fig. 5). A part of NBD-derived fluorescence was detected in the same granule with tetramethylrhodamine-dextran. Accordingly, a part of 001-C8-NBD is suggested to be localized in lysosomes.

Effect of an endocytosis inhibitor and a cationic protein on internalization of 001-C8-NBD into Caco-2 cells. We assessed the effects of an endocytosis inhibitor, dansylcadaverine, and a cationic protein, protamine, on the internalization of 001-C8-NBD into Caco-2 cells (Fig. 6). Dansylcadaverine (500 µM) strongly decreased the granular distribution of 001-C8-NBD within the cells. Protamine (1 mM) also strongly reduced the internalization.

Fig. 3. Concentration dependence of internalization of 125I-001-C8 into cultured monolayers of Caco-2 cells. Caco-2 cells were incubated with 125I-001-C8 for 60 min at 37°C with increasing concentrations of unlabeled 001-C8 (0.1 µM to 1 mM). Internalization was determined as described in Fig. 1 legend. Each point is mean ± SE of 3 or 4 experiments.

Table 2. Effects of cell surface glycosylation on internalization of 125I-001-C8 and [3H]PEG 900 into Caco-2 cells

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Internalization, % of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100.0 ± 2.0</td>
</tr>
<tr>
<td>+ Heparinase I</td>
<td>82.1 ± 1.0*</td>
</tr>
<tr>
<td>+ Heparitinase</td>
<td>89.1 ± 1.1*</td>
</tr>
<tr>
<td>+ Chondroitinase ABC</td>
<td>79.0 ± 4.8*</td>
</tr>
<tr>
<td>+ Hyaluronidase</td>
<td>97.3 ± 3.4</td>
</tr>
<tr>
<td>+ Neuraminidase</td>
<td>115.9 ± 5.9*</td>
</tr>
<tr>
<td>+ Heparinase I, + heparitinase</td>
<td>76.8 ± 4.5*</td>
</tr>
<tr>
<td>+ Heparitinase, + chondroitinase ABC</td>
<td>69.9 ± 6.8*</td>
</tr>
<tr>
<td>+ Heparinase I, + chondroitinase ABC</td>
<td>61.5 ± 4.7*</td>
</tr>
<tr>
<td>+ Heparinase I, + heparitinase, + chondroitinase ABC</td>
<td>60.9 ± 3.0*</td>
</tr>
<tr>
<td>+ Heparin</td>
<td>60.3 ± 4.0*</td>
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Values are means ± SE of 3 or 4 experiments. Caco-2 cells were preincubated with various enzymes (4 U/ml) or heparin (1 mg/ml) at 37°C for 2 h, and internalization of 125I-001-C8 and [3H]PEG 900 at 60 min was determined as described in Fig. 1 legend. PEG, polyethylene glycol. *Statistically significantly different from control (Student’s t-test, P < 0.05). ND, not determined.

DISCUSSION

The purpose of the present study was to examine AME in intestinal epithelial cells using a cationic model peptide, 001-C8. We had shown previously that, among various peptide derivatives examined in brain capillary endothelial cells, 001-C8 was internalized most efficiently (21), but it remained unknown whether such a process is also involved in intestinal epithelial cells. The Caco-2 cell line was used as an in vitro model because of its close morphological and functional similarity to the intestinal epithelium (15).

The six criteria for AME provided in our study were as follows: 1) A cationic nature of the model peptide 001-C8, having cationic charges at physiological pH with an estimated isoelectric point of 12.5, is considered to be an important determinant for AME at the blood-brain barrier. 001-C8 was basically designed to cross the blood-brain barrier via AME. 2) Arginine and methylarginine included in 001-C8 are important determinants of affinity to the sites of adsorption on the cell surface, in the case of AME at the blood-brain barrier. 3) Internalization is inhibited by various cationic compounds such as poly-L-lysine, protamine, E-2078, and ebiratide, which can be inhibitors of AME. 4) Internalization of the peptide is labile to enzymatic digestion of the cell surface, which removes membrane-associated anionic polysaccharides. 5) Internalization and granular distribution of the peptide-derived fluorescence can be confirmed by morphological observation. 6) K_d of 001-C8 is comparable with previously published K_d values of ligands for AME, which are two to three orders higher than the values of ligands for RME. All of the results provided in the present study are explained by AME, which is an endocytosis mechanism that is affected by the cationic
and anionic nature of peptides and certain components on the cell surface, respectively.

$^{125}$I-001-C8 was stable within Caco-2 cells throughout the incubation period (Fig. 2). It is also stable in intestinal tissue homogenate, giving a half-life of about 100 min in 1 mg protein/ml of rat intestinal homogenate (16). The metabolically stable nature of $^{125}$I-001-C8 may be ascribed to the $N$-methylation of Tyr and Arg, substitution with the D-isomer of Leu, and modification of the carboxy-terminal structure. A minor degradation product detected in the internalized fraction was identified as free $^{125}$I on the basis of its retention time of 5 min in HPLC assay (Fig. 2). This property of 001-C8 is suitable for study of AME.

We evaluated the internalization of 001-C8 caused by endocytosis both functionally and morphologically. Because of the basic nature (isoelectric point = 12.5) of 001-C8, measurement of cell-associated total radioactivity cannot distinguish internalization by AME from surface binding. To separate cell surface-bound (acid-sensitive) from internalized (acid-resistant) $^{125}$I-001-C8, an acid-wash technique was used. The results suggest that at least one-half of the cell-associated total radioactivity was internalized (Fig. 1). To confirm that acid-wash-resistant binding reflects endocytosis rather than cytosolic distribution caused by diffusion or paracellular transfer, we used confocal microscopy. The granular distributions of the staining and the absence of staining in nuclei in optical sections of the center of the cells strongly indicate the sequestration of 001-C8 within endocytotic vesicles (Fig. 4). Endocytosis was also suggested by the observation that the internalization was reduced by dansylcadaverine and at low temperatures (Table 1). The incomplete inhibitory effect (see Table 1) may be ascribed to the incomplete removal of cell surface-bound $^{125}$I-001-C8 by acid washing in the present procedure, although an involvement of uptake mechanisms other than AME cannot be exclusively ruled out. However, involvement of the oligopeptide transporter PepT1, which is expressed at the brush border of absorptive epithelial cells and accepts not only di- or tripeptides but also peptide-mimetic $\beta$-lactam antibiotics and angiotensin-converting enzyme inhibitors as substrates (10, 17, 27), is unlikely, because glycylglycine at 25 mM did not affect the internalization. Furthermore, since the internaliza-

![Fig. 4. Confocal photomicrographs showing internalization of fluorescence-derived 001-C8 (001-C8-4-nitrobenz-2-oxa-1,3-diazole (001-C8-NBD)) into cultured monolayers of Caco-2 cells. Caco-2 cells grown on a glass coverslip were loaded with 001-C8-NBD, mounted on the microstage, and incubated at 37°C. Digital confocal microscopy images were taken at 10 (A), 20 (B), and 60 (C) min. Cell image obtained by the trans-detector is shown in D. Scale bar, 25 µm.](image)

![Fig. 5. Subcellular distribution of rhodamine-dextran and internalization of 001-C8-NBD into cultured monolayers of Caco-2 cells. Caco-2 cells were preloaded with tetramethylrhodamine-dextran (10 mg/ml) overnight and chased for 30 min at 37°C to mark secondary lysosomes (arrowheads). Cells were then further incubated with 001-C8-NBD. Digital fluorescence images of tetramethylrhodamine (A) and NBD (C) were independently taken with the use of respective optical filter blocks, and both images were merged electronically (B). Scale bar, 25 µm.](image)
tion of 001-C8 (7.24 ± 0.41 µl/mg protein) was significantly higher than that of PEG 900 (0.81 ± 0.06 µl/mg protein), which is a measure of fluid-phase endocytosis as well as extracellular attached water space. 001-C8 is likely to be internalized into Caco-2 cells via an endocytosis-type mechanism(s) other than fluid-phase endocytosis.

The internalization of 001-C8 was inhibited by cationic peptides including protamine, poly-L-lysine, E-2078, and ebiratide (Table 1; Fig. 6). E-2078 and ebiratide have been reported to be transported through the blood-brain barrier via AME based on isolated brain capillary studies (19, 23) or the use of cultured brain capillary endothelial cells (24). The $K_d$ value obtained by nonlinear least-squares analysis may also be considered a criterion for AME. The observed $K_d$ value in this study (0.47 µM) (Fig. 3) is comparable to those for substrates reported to be taken up via the AME mechanism in brain capillary endothelial cells, including cationized BSA (0.8 µM) (6), histone (15.2 µM) (14), E-2078 (4.62 µM) (23), and ebiratide (15.9 µM) (24). In contrast, the $K_d$ values for RME reported for insulin (2.3 nM) (2), transferrin (5.6 nM) (13), and atrial natriuretic factor (0.4 nM) (20) and that for receptor binding of epidermal growth factor (0.35 nM) (8) are several thousand times smaller than those for AME. These data indicate that 001-C8 is taken up into Caco-2 cells via the AME mechanism.

The internalization of $^{125}$I-001-C8 was significantly decreased by predigestion of the apical membrane of the Caco-2 cells with heparinase I, heparitinase, or chondroitinase ABC (Table 2). Excess heparin also decreased the internalization. These results suggest that the anionic site(s) on the apical membrane involved in the internalization of 001-C8 is one of a variety of acid mucopolysaccharides, including heparin, heparan sulfate, and chondroitin sulfate, in the endocytosis of 001-C8 by Caco-2 cells. Apparently incomplete inhibition by enzyme treatments may be ascribed to the incomplete digestion by enzymes and/or an involvement of multiple anionic components as the target for AME.

Partial identification of endocytotic granules was performed by double staining of the cells with tetramethylrhodamine-dextran as a marker for secondary lysosomes (Fig. 5). It was revealed that a part of the NBD fluorescence was colocalized with rhodamine-dextran, suggesting a minor contribution of lysosomes to this pathway.

In conclusion, the present study demonstrates that a basic peptide, 001-C8, is taken up by enterocyte-like Caco-2 cells via the AME system. Because of its stability in the cell, 001-C8 may be passed into the systemic circulation via adsorptive-mediated transcytosis (AMT) in vivo. Our preliminary data (unpublished observations) using filter-grown Caco-2 monolayers and in situ vascular perfusion of the intestine indicate the existence of such an AMT mechanism.

REFERENCES


