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Intestinal uptake of dipeptides and β-lactam antibiotics. I. The intestinal uptake system for dipeptides and β-lactam antibiotics is not part of a brush border membrane peptidase

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The uptake of β-lactam antibiotics into small intestinal enterocytes occurs by the transport system for small peptides. The role of membrane-bound peptidases in the brush border membrane of enterocytes from rabbit and pig small intestine for the uptake of small peptides and β-lactam antibiotics was investigated using brush border membrane vesicles. The enzymatic activity of aminopeptidase N was inhibited by β-lactam antibiotics in a non-competitive manner whereas dipeptidylpeptidase IV was not affected. The peptidase inhibitor bestatin led to a strong competitive inhibition of aminopeptidase N whereas the uptake of cephalexin into brush border membrane vesicles was only slightly inhibited at high bestatin concentrations (> 1 mM). Modification of brush border membrane vesicles with the histidine-modifying reagent diethyl pyrocarbonate led to a strong irreversible inhibition of cephalexin uptake whereas the activity of aminopeptidase N remained unchanged. A modification of serine residues with diisopropyl fluorophosphate completely inactivated dipeptidylpeptidase IV whereas the transport activity for cephalexin and the enzymatic activity of aminopeptidase N were not influenced. With polyclonal antibodies raised against aminopeptidase N from pig renal microsomes the aminopeptidase N from solubilized brush border membranes from pig small intestine could be completely precipitated; the binding protein for β-lactam antibiotics and oligopeptides of apparent M_r 127000 identified by direct photoaffinity labeling with [^{3}H]benzylpenicillin showed no crossreactivity with the aminopeptidase N anti serum and was not precipitated by the anti serum. These results clearly demonstrate that peptidases of the brush border membrane like aminopeptidase N and dipeptidylpeptidase IV are not directly involved in the intestinal uptake process for small peptides and β-lactam antibiotics and are not a constituent of this transport system. This suggests that a membrane protein of M_r 127000 is (a part of) the uptake system for β-lactam antibiotics and small peptides in the brush border membrane of small intestinal enterocytes.

Introduction

The small intestine plays an important role for homeostasis. Ingested biopolymers like proteins, carbohydrates and lipids are digested to smaller fragments by the action of digestive enzymes. Proteins are hydrolyzed to oligopeptides and amino acids by enzymes secreted by the stomach and the pancreas. The resulting oligopeptides are further degraded to di- and tripeptides and free amino acids by the action of membrane-bound peptidases located in the brush border membrane of the enterocyte [1–3]. The role of membrane-bound peptidases in the absorption of peptides is far from clear. In contrast to the large protein molecules, amino acids and oligopeptides are efficiently absorbed by the small intestine with the aid of specific transport systems for amino acids [4–8] and small peptides [9–12]. Di- and tripeptides resistant to hydrolysis are taken up by a H^+-dependent transport system which is stimulated by an inwardly directed H^+-gradient [13–16]; orally active α-amino-β-lactam antibiotics share the intestinal uptake system for di- and tripeptides [17–24]. A putative pro-

Abbreviations: DEP, diethyl pyrocarbonate; DFP, diisopropyl fluorophosphate; Hepes, *N*-(2-Hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; HPLC, high pressure liquid chromatography; SDS, sodium dodecylsulfate; Triton X-100, octylphenol polyethyleneglycol ether, $n = 10$.

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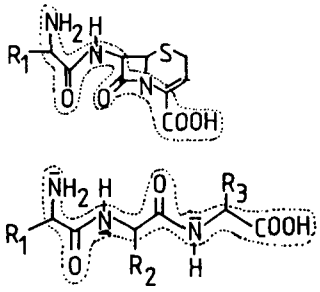

Fig. 1. Structures of α-amino-β-lactam antibiotics and tripeptides.

tein component of this transport system with an apparent molecular weight of 127000 has been identified by photoaffinity labeling using photoreactive derivatives of β-lactam antibiotics and dipeptides [21–24]. Since α-amino-β-lactam-antibiotics are derivatives and structural analogues of tripeptides (Fig. 1) it was necessary to elucidate whether the photolabeled 127 kDa polypeptide is a specific transport protein or a brush border membrane peptidase. Among the peptidases located in the intestinal brush border membrane, aminopeptidase N (aminopeptidase M (EC 3.4.11.2)) and dipeptidylpeptidase IV (EC 3.4.14.-), have subunits of similar molecular weight as the binding protein for β-lactam antibiotics and dipeptides. In the present study we have therefore investigated whether aminopeptidase N and dipeptidylpeptidase IV are directly involved in the uptake process of orally active β-lactam antibiotics.

Part of this work has been presented in preliminary form [50].

Materials and Methods

Materials

[*phenyl*-4(n)-^{3}H]Benzylpenicillin (specific radioactivity 8–31 Ci/mMol) was obtained from Amersham (Amersham Buchler GmbH & Co., Braunschweig, F.R.G.). β-Lactam antibiotics, L-leucyl-*p*-nitroanilide, glycyl-L-prolyl-*p*-nitroanilide, bestatin and marker proteins for electrophoresis were from Sigma (Sigma München, F.R.G.). Triton X-100, diethyl pyrocarbonate (DEP), diisopropyl fluorophosphate (DFP) on Kieselgur, phenylmethylsulfonyl fluoride (PMSF) and materials for electrophoresis were purchased from Serva (Serva Heidelberg, F.R.G.). Merckotest Kit 531 for the determination of aminopeptidase M activity and solvents for HPLC were obtained from Merck (Merck GmbH Darmstadt, F.R.G.). Cellulose nitrate filters (type HAWP 0.45 μm, 25 mm diameter) for transport studies were obtained from Millipore (Millipore Eschborn, F.R.G.).

Methods

Preparation of brush border membrane vesicles from rabbit and pig small intestine

White rabbits (3–3.5 kg body weight) (Tierzucht Kastengrund, Hoechst Aktiengesellschaft, Frankfurt, F.R.G.) maintained on standard diets and tap water ad libitum were killed by intraveneous injection of 0.5 ml of T-61® (2.5 mg tetracain HCl, 100 mg embutramid and 25 mg mebezonium iodide, Hoechst Aktiengesellschaft, Frankfurt, F.R.G.). The small intestine was immediately removed, rinsed with ice-cold phosphate buffered saline and cut into pieces of 30 cm length which were kept frozen at −80°C. For the preparation of brush border membrane vesicles the intestinal segments were thawed at 30° C and the mucosa was scraped off. The brush border membrane vesicles were prepared by the Mg^{2+}-precipitation method as described [25]. The activities of the brush border marker enzymes aminopeptidase N (EC 3.4.11.2) and γ-glutamyltransferase (EC 2.3.2.2) were determined using the Merckotest kits 3359 and 3394 (Merck GmbH, Darmstadt, F.R.G.). Protein was determined according to Bradford [26] using the Bio-Rad assay (Bio-Rad, München, F.R.G.). The functional integrity of the vesicles was determined by measurement of the Na^+-dependent D-glucose uptake. Brush border membrane vesicles from pig small intestine were prepared according to the same procedure starting from deep-frozen (−80° C) jejunal segments of pig small intestine. In the final preparation step the vesicles were loaded with 10 mM Tris-Hepes buffer (pH 7.4)/300 mM mannitol and were stored at a protein concentration of 10–20 mg/ml in liquid nitrogen up to 4 weeks without loss of transport function or enzymatic activity.

Enzymatic measurement of aminopeptidase N and dipeptidylpeptidase IV

For the determination of aminopeptidase N acticity, 100 μl of a vesicle suspension (40–110 μg of protein) were added to 1.1 ml of 50 mM Tris-HCl buffer (pH 7.5)/250 mM NaCl containing L-leucine-*p*-nitroanilide as substrate (4 mM for standard tests). At 25° C the increase in absorbance at 405 nm was continuously measured during a 3 min-period in a Shimadzu UV-160 spectrophotometer (Shimadzu, Büro BEKRA, Mainz, F.R.G.).

For the enzymatic measurement of dipeptidylpeptidase IV activity an analogous procedure was used with glycyl-L-prolyl-*p*-nitroanilide as substrate. All enzymatic determinations were performed in triplicate with at least three different membrane preparations.

Treatment of brush border membrane vesicles with DEP

Brush border membrane vesicles were treated with DEP as described previously [27,28]. Membrane vesicles were incubated for 10 min with freshly prepared DEP

solutions (0.1 ml DEP + 11.3 ml 20 mM potassium phosphate buffer (pH 6.4)/280 mM mannitol) in the concentration range 1–20 mM. The reaction was terminated by addition of cold 10 mM Tris-Hepes buffer (pH 7.4)/300 mM mannitol. After centrifugation and collection of the vesicles the washing procedure was repeated. The resulting membrane pellet was resuspended in 10 mM Tris-Hepes buffer (pH 7.4)/300 mM mannitol.

Treatment of brush border membrane vesicles with DFP

A 50 mM stock solution of DFP was prepared by mixing of 100 mg of 6% DFP on Kieselgur (Serva GmbH, Heidelberg, F.R.G.) with 660 µl of dimethylsulfoxide. After 20 min of vortexing the suspension was centrifuged and the clear supernatant representing a 50 mM solution of DFP in dimethylsulfoxide was used for further studies. For inhibition experiments brush border membrane vesicles suspended in 20 mM Tris-Hepes buffer (pH 7.4)/300 mM mannitol were mixed with the appropriate volumes of DFP stock solutions, whereas for control the vesicles were only mixed with dimethylsulfoxide. After the desired incubation time the vesicle suspension was diluted with ice-cold 10 mM Tris-Hepes buffer (pH 7.4)/300 mM mannitol and after centrifugation the vesicles were resuspended in 10 mM Tris-Hepes buffer (pH 7.4)/300 mM mannitol.

Uptake measurements

The uptake of cephalexin by intestinal brush border membrane vesicles was measured using the membrane filtration method [29,30] as described [22,24,27]. The determination of cephalexin taken up by the vesicles was performed by HPLC analysis [22,24,27] with a 250 × 4 mm column filled with LiChrosorb RP 18 (7 µm, Merck GmbH, Darmstadt, F.R.G.) using a mixture of 73% (v/v) solvent A (30 mM sodium phosphate buffer (pH 6.2)/10 mM tetraethylammonium chloride) and 27% (v/v) solvent B (400 g of solvent A and 468 g acetonitrile).

Preparation of polyclonal antibodies against aminopeptidase N from pig kidney microsomes

0.45 mg of lyophilized aminopeptidase N from pig kidney microsomes (Serva GmbH, Heidelberg, F.R.G.) were dissolved in 0.45 ml of 0.9% sodium chloride solution. After mixing with 0.45 ml of Freund's complete adjuvans polyclonal antibodies against pig renal aminopeptidase N were raised in rabbits by subcutaneous injection. 10 and 20 days after the initial immunization two booster injections were performed (0.225 mg of aminopeptidase N dissolved in 0.225 ml of 0.9% sodium chloride solution/0.225 ml Freund's incomplete adjuvans). 10 days after the second booster injection 20 ml of blood was collected and after clotting of blood the anti serum against aminopeptidase N was prepared by centrifugation. The clear antiserum was aliquoted and stored at −80 °C.

Immunoblotting

Electrophoretic transfer of polypeptides separated by SDS-polyacrylamide gel electrophoresis to nitrocellulose sheets was performed in a Bio-Rad Trans-blot cell (Bio-Rad, München, F.R.G.) at 80 V for 4 h using 0.2 M glycine-Tris buffer (pH 8.3)/20 methanol as transfer buffer. Detection of the subunits of aminopeptidase N was performed according to the Bio-Rad protocol with a 1 : 100 dilution of antiserum using protein A-horseradish peroxidase conjugate for detection of antigen-IgG complexes. For detection of radioactivity the immunoblotted stripes were cut into 2 mm pieces; after incubation with 0.25 ml of solubilizer Biolute S (Zinsser Analytic GmbH, Frankfurt, F.R.G.), 4 ml of scintillator Quickszint 501 (Zinsser Analytic GmbH, Frankfurt , F.R.G.) were added and radioactivity was measured by liquid scintillation counting in a Packard TriCarb 2000 counter.

Photoaffinity labeling

Photoaffinity labeling of brush border membrane vesicles from pig small intestine with [^{3}H]benzylpenicillin was performed as described in previous papers [24,31]. For solubilisation the washed brush border membrane vesicles were incubated at 4 °C for 60 min with 1% Triton X-100. By centrifugation at 48 000 × g for 30 min the solubilized proteins were separated from nonsolubilized material and the clear supernatant was used for further experiments with antiserum. The proteins were precipitated according to Wessel and Flügge [32] and submitted to SDS-polyacrylamide gel electrophoresis.

SDS-polyacrylamide gel electrophoresis

SDS-gel electrophoresis was performed on discontinuous SDS slab gels (200 × 140 × 1.5 mm) using a Pharmacia L2/4B apparatus (Pharmacia GmbH, Freiburg, F.R.G.). Fixing and staining of the gels was performed as described [33] and radioactivity in the gels was measured by liquid scintillation counting after slicing of the gels into 2 mm pieces and digestion of peptides with Biolute S (Zinsser Analytic GmbH, Frankfurt, F.R.G.).

Results

Effect of β-lactam antibiotics on the enzymatic activities of aminopeptidase N and dipeptidylpeptidase IV of rabbit small intestinal brush border membrane vesicles

β-Lactam antibiotics inhibit the uptake of orally active α-amino-β-lactam antibiotics into intestinal brush border membrane vesicles in a competitive manner [18,24,34]. In order to investigate whether the uptake

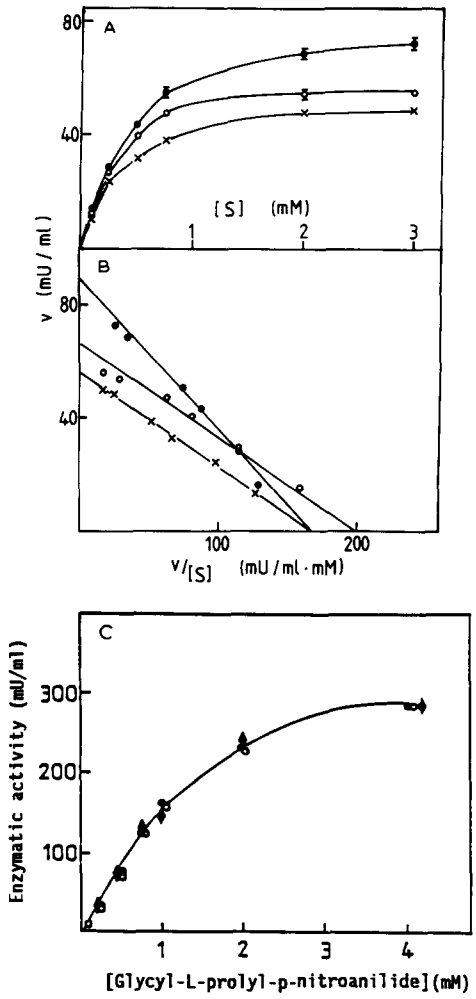

Fig. 2. Influence of β-lactam antibiotics on the enzymatic activity of aminopeptidase N and dipeptidylpeptidase IV in rabbit small intestinal brush border membrane vesicles. The activity of aminopeptidase N (30 μg brush border membrane protein for each determination) was measured at 30 °C in the absence (●) and in the presence of 10 mM benzylpenicillin (○) or 10 mM cephalexin (×) with 0.1, 0.25, 0.5, 0.75, 2 and 3 mM L-leucyl-*p*-nitroanilide as substrate (A). (B) Eadie-Hofstee-diagram of the inhibition of aminopeptidase N in the absence (●) and the presence of 10 mM benzylpenicillin (○) and 10 mM cephalexin (×). (C) The activity of dipeptidylpeptidase IV (48.5 μg of brush border membrane protein for each determination) was measured at 30 °C in the absence (●) and the presence of 10 mM (○) and 25 mM (◆) benzylpenicillin.

system for β-lactam antibiotics and dipeptides is related to or even identical with membrane bound peptidases, the effect of β-lactam antibiotics on the enzymatic activity of aminopeptidase N and dipeptidylpeptidase IV was investigated. Fig. 2A shows the concentration-dependent inhibition of aminopeptidase N activity by benzylpenicillin and cephalexin using L-leucyl-*p*-nitroanilide as substrate. Both compounds do not lead to a competitive inhibition of aminopeptidase N in contrast to the competitive inhibition of the peptide transport system by benzylpenicillin and cephalexin [24]. The kinetic analysis demonstrated an uncompetitive inhibition of aminopeptidase N by cephalexin, whereas benzylpenicillin showed a mixed inhibition type (Fig. 2B). The enzymatic activity of dipeptidylpeptidase IV using glycyl-L-prolyl-*p*-nitroanilide as chromogenic substrate was not affected by β-lactam antibiotics and no inhibition could be observed (Fig. 2C).

Effect of substrates and inhibitors for aminopeptidase N and dipeptidylpeptidase IV on the uptake of cephalexin by rabbit small intestinal brush border membrane vesicles

L-Leucyl-*p*-nitroanilide, a substrate of aminopeptidase N, strongly inhibited the uptake of cephalexin by brush border membrane vesicles (Fig. 3); a half-maximal inhibition of cephalexIn uptake was achieved with about 0.5 mM L-leucyl-*p*-nitroanilide. Glycyl-L-prolyl-*p*-nitroanilide, a substrate of dipeptidylpeptidase IV, did not significantly inhibit the uptake of cephalexin by brush border membrane vesicles (Fig. 3). Since β-lactam antibiotics inhibit aminopeptidase N and vice versa L-leucyl-*p*-nitroanilide inhibits cephalexin uptake, the effect of bestatin – an inhibitor of aminopeptidase N – both on aminopeptidase N activity and on the uptake of cephalexin by brush border membrane vesicles was investigated. Figs. 4A and 4B show that bestatin strongly inhibited aminopeptidase N. The kinetic analysis revealed a competitive inhibition of aminopeptidase N by bestatin; a half-maximal inhibition was achieved with about 10 μM bestatin.

In contrast, for a significant inhibition of cephalexin-uptake into brush border membrane vesicles much higher concentrations of bestatin were necessary (Fig. 4C). A half-maximal inhibition of cephalexin uptake (2 mM) was achieved by approx. 10 mM bestatin. This great difference of three orders of magnitude in the inhibitory effect of bestatin on the enzymatic activity of

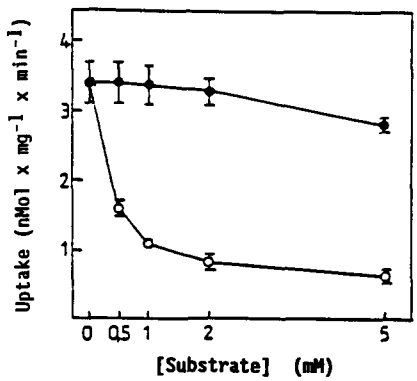

Fig. 3. Influence of substrates of aminopeptidase N and dipeptidylpeptidase IV on the uptake of cephalexin into brush border membrane vesicles from rabbit small intestine. Brush border membrane vesicles (100 μg, 20 μl) loaded with 10 mM Tris-Hepes buffer (pH 7.4)/300 mM mannitol were mixed with 180 μl of 20 mM Tris-citrate buffer (pH 6.0)/140 mM KCl containing 2 mM cephalexin and the indicated concentrations either of L-leucyl-*p*-nitroanilide (○) or glycyl-L-prolyl-*p*-nitroanilide (●). Initial uptake of cephalexin was measured after 1 min of incubation.

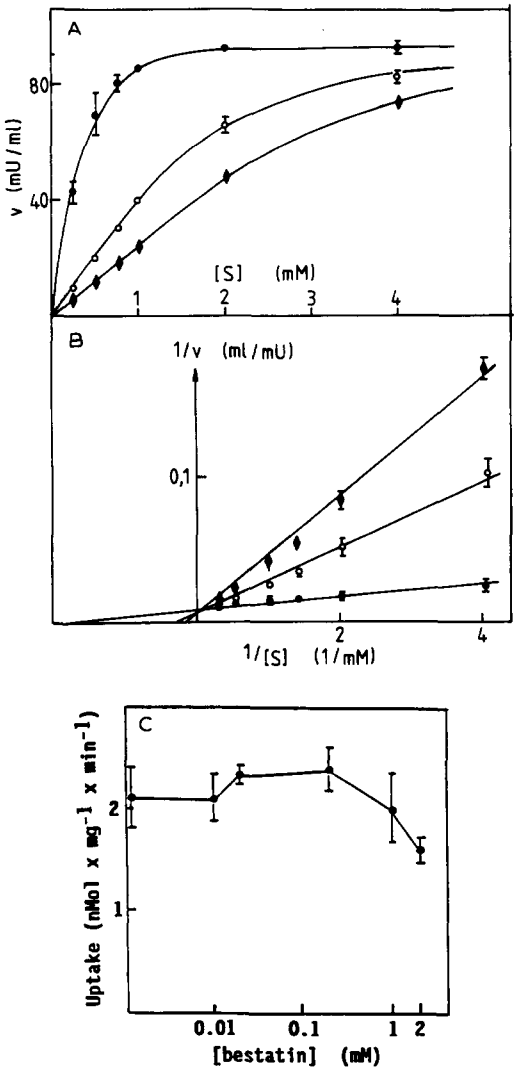

Fig. 4. Influence of bestatin on the enzymatic activity of aminopeptidase N (1A and B) and on the uptake of cephalexin (C) in brush border membrane vesicles from rabbit small intestine. (A) The activity of aminopeptidase N (30 μg of brush border membrane protein for each determination) was measured at 30 °C with 0.25, 0.5, 0.75, 1, 2 and 4 mM L-leucyl-*p*-nitroanilide as substrate in the absence (●) and in the presence of 10 μM (○) and 20 μM (◆) bestatin. (B) Lineweaver-Burk diagram of the inhibition of aminopeptidase N by bestatin. (C) Brush border membrane vesicles (100 μg, 20 μl) loaded with 10 mM Tris-Hepes buffer (pH 7.4)/300 mM mannitol were mixed with 180 μl of 20 mM Tris-citrate buffer (pH 6.0)/140 mM KCl containing 2 mM cephalexin and the indicated concentrations of bestatin. Initial uptake of cephalexin was measured after 1 min of incubation.

aminopeptidase N and on the transport activity argues against an involvement of aminopeptidase N in the uptake process of β-lactam antibiotics and dipeptides across the intestinal brush border membrane.

Effect of DEP and DFP on the enzymatic activities of aminopeptidase N and dipeptidylpeptidase IV and on the uptake of cephalexin by intestinal brush border membrane vesicles

The intestinal uptake system for orally active α-amino-β-lactam antibiotics could be irreversibly inhibited by treatment of brush border membrane vesicles with DEP, a histidine-modifying reagent [27]. Therefore, in a further series of experiments the effect of DEP-treatment both on the uptake of cephalexin and on the enzymatic activities of aminopeptidase N and dipeptidylpeptidase IV was investigated. Table I shows that increasing concentrations of DEP resulted in an increasing inhibition of H^+-dependent cephalexin uptake; the enzymatic activity of aminopeptidase N however, was not significantly inhibited by DEP-treatment of the vesicles. 5 mM DEP led to a slight inhibition of dipeptidylpeptidase IV and aminopeptidase N.

Since dipeptidylpeptidase IV belongs to the serine-proteases, the effect of serine-modifying agents like the organic fluorophosphate DFP or PMSF [35] on enzymatic and transport activity was investigated. Treatment of rabbit brush border membrane vesicles with PMSF neither had an influence on the uptake of cephalexin nor on the activity of aminopeptidase N; in contrast to the renal dipeptidylpeptidase IV [36], the activity of the intestinal enzyme was slightly inhibited by PMSF. When brush border membrane vesicles from rabbit or pig small intestine were treated with DFP, a strong inhibition of dipeptidylpeptidase IV occurred. After 6 h of incubation with DFP the dipeptidylpeptidase IV was completely inactivated, whereas the activity of aminopeptidase N remained unchanged (Fig. 5A). The uptake of cephalexin into brush border membrane vesicles, however, was not influenced by DFP-treatment (Fig. 5B).

TABLE I

Influence of diethyl pyrocarbonate treatment on the enzymatic activity of aminopeptidase N and dipeptidylpeptidase IV and on the uptake of cephalexin into brush border membrane vesicles from rabbit small intestine

Brush border membrane vesicles were incubated at 20 °C for 10 min with 0, 0.5, 1, 2, and 5 mM diethyl pyrocarbonate. After washing twice with 10 mM Tris-Hepes buffer (pH 7.4)/300 mM mannitol the enzymatic activities of aminopeptidase N and dipeptidylpeptidase IV (50 μg brush border membrane protein for each determination) were measured. For transport studies 20 μl (100 μg) of brush border membrane vesicles were mixed with 180 μl of Tris-citrate buffer (pH 6.0)/140 mM KCl containing 2 mM cephalexin and cephalexin uptake was measured after 1 min of incubation. Values are given as the mean ± S.D. from three determinations.

[Diethyl pyrocarbonate] (mM)	0	0.5	1	2	5
Aminopeptidase N (mU/ml)	308.3 ±1.3	301.4 ±3.6	295.9 ±2.4	295.1 ±1.5	272.8 ±2.0
Dipeptidylpeptidase IV (mU/ml)	63.8 ±0.05	63.0 ±0.1	62.2 ±0.8	57.4 ±0.4	39.6 ±0.8
Cephalexin uptake (% of control)	100	88.04 ±2.3	55.4 ±4.5	48.2 ±0.85	47.0 ±3.3

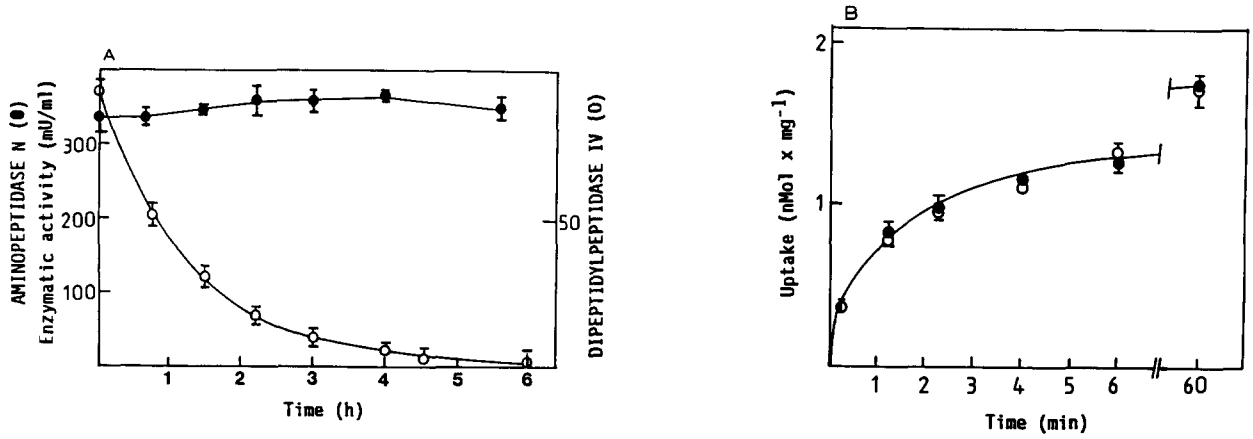

Fig. 5. Influence of treatment of brush border membranes with DFP on the enzymatic activity of dipeptidylpeptidase IV and aminopeptidase N (A) and on the uptake of cephalexin into brush border membrane vesicles from rabbit small intestine (B). (A) Brush border membrane vesicles from pig small intestine were incubated at 4 °C with 2.5 mM DFP in 10 mM Tris-Hepes buffer (pH 7.4)/300 mM mannitol/5% DMSO. After the indicated incubation periods aliquots (80 μg of brush border membrane protein for each determination) were removed for the measurement of the enzymatic activities of dipeptidylpeptidase IV and aminopeptidase N. (B) Brush border membrane vesicles from rabbit small intestine were incubated at 4 °C for 3 h with 2.5 mM DFP in 10 mM Tris-Hepes buffer (pM 7.4)/300 mM mannitol/5% DMSO or with buffer alone. After washing membrane vesicles (100 μl, 20 μl) were mixed with 180 μl of 20 mM citrate-Tris buffer (pH 6.0)/140 mM KCl containing 2 mM cephalexin. Uptake was measured at 30 °C after the indicated incubation times. ●, uptake into control vesicles; ○, uptake into treated vesicles.

Immunoprecipitation of proteins from solubilized brush border membrane vesicles from pig small intestine with anti serum raised against aminopeptidase N after photoaffinity labeling with [^{3}H]benzylpenicillin

The experiments described above suggest that the transport system for β-lactam antibiotics and dipeptides is different from aminopeptidase N and dipeptidylpeptidase IV. The subunits of dipeptidylpeptidase IV have a slightly smaller molecular weight than the photolabeled 127 kDa polypeptide and migrate on SDS gels in a band of lower molecular weight than the radioactively labeled protein. The subunits of aminopeptidase N however, have been ascribed to a prominent band on SDS-gels which also contains the photolabeled 127 kDa polypeptide. An unequivocal discrimination between aminopeptidase N and the photolabeled binding protein for β-lactam antibiotics and dipeptides is possible with antibodies either against aminopeptidase N or the purified binding protein of M_r 127000. Purified aminopeptidase N from pig renal microsomes is commercially available and antibodies raised against the renal enzyme crossreact with the intestinal aminopeptidase N [37]. Antibodies raised against pig renal microsomal aminopeptidase N did not inhibit the activity of aminopeptidase N from pig small intestine both in membrane vesicles as in the solubilized state. However, the aminopeptidase N activity was completely precipitated with antiserum, whereas preimmune serum had no effect (Fig. 6). Incubation of pig small intestinal brush border membrane vesicles with anti-aminopeptidase N antibodies did not inhibit the H^+-dependent uptake of cephalexin.

The antibodies prepared against pig renal aminopeptidase N crossreact with the aminopeptidase N from pig small intestine but do not crossreact with aminopeptidase N from rabbit small intestinal brush border membranes. For an immunological characterization of the binding protein for β-lactam antibiotics and dipeptides the photoaffinity labeling studies were therefore performed with brush border membrane vesicles

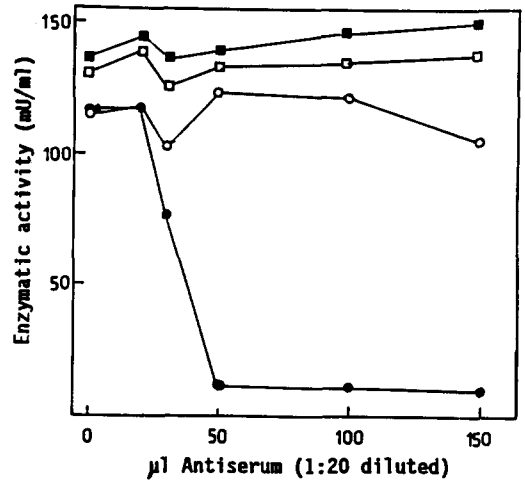

Fig. 6. Immunoprecipitation of aminopeptidase N from solubilized brush border membrane vesicles from pig small intestine. 2 mg of brush border membrane vesicles from pig small intestine were solubilized in 1200 μl 10 mM Tris-HCl buffer (pH 7.4)/1% Triton X-100 for 30 min at 4 °C. After centrifugation 110 μl of the clear supernatant were mixed with 440 μl 10 mM Tris-HCl buffer (pH 7.4) and the indicated volumes of 1:20 diluted preimmune serum or anti serum raised against aminopeptidase from pig renal microsomes. After 1 h incubation at 20 °C either 100 μl of the incubation mixtures were directly used for the determination of aminopeptidase N activity or the activity was determined in the resulting supernatants (100 μl) after centrifugation at 15000 × *g* for 10 min. □, + preimmune serum; ■, + preimmune serum after centrifugation; ○, + antiserum; ●, + anti serum after centrifugation.

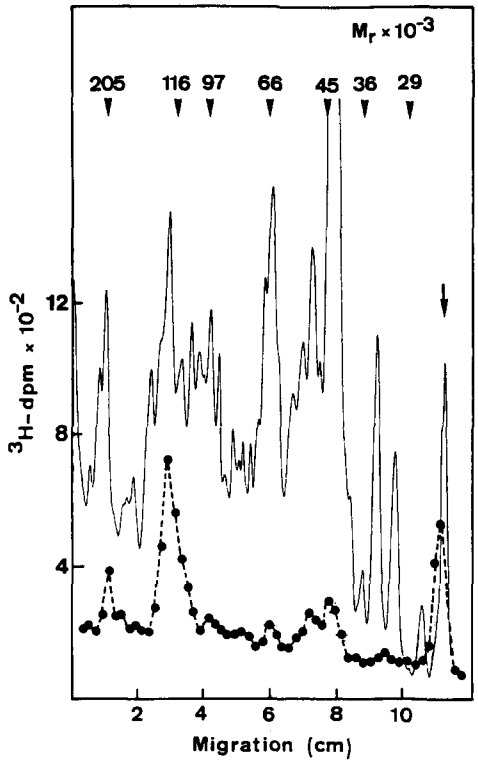

Fig. 7. Distribution of radioactivity after SDS-gel electrophoresis of brush border membrane vesicles from pig small intestine after photoaffinity labeling with [^{3}H]benzylpenicillin. 200 μg of pig small intestinal brush border membrane vesicles loaded with 10 mM Tris-Hepes buffer (pH 7.4)/300 mM mannitol were photolabeled with 1.94 μM (3.75 μCi) [^{3}H]benzylpenicillin in 10 mM sodium phosphate buffer (pH 7.4)/140 mM KCl. After washing membrane proteins were separated by SDS-gel electrophoresis using 7.5% gels. The solid line shows the distribution of polypeptides after staining with Serva Blue R 250, whereas the dotted line shows the distribution of radioactivity.

from pig small intestine. Fig. 7 shows that a polypeptide of apparent molecular weight 127000 was labeled as with rabbit and rat intestinal brush border membrane vesicles. In contrast to rabbit and rat the photolabeled 127 kDa polypeptide was found in a minor band. The distribution of radioactively labeled polypeptides in pig membrane vesicles is quite different from the densitometric scan of stained polypeptides indicating a specific labeling of membrane proteins by photoreactive β-lactam antibiotics. As in the rabbit and the rat [22,24] the labeling of the 127 kDa polypeptide could be specifically inhibited by the presence of β-lactam antibiotics and dipeptides whereas amino acids, sugars and bile acids had no effect (data not shown); the specificity of the 127 kDa binding protein in pig intestinal brush border membranes is therefore similar to that found in rabbit and rat.

In order to determine whether the binding protein for β-lactam antibiotics and dipeptides of molecular weight 127000 is identical with aminopeptidase N or not, an immunological characterization of brush border membrane proteins was performed with antibodies against aminopeptidase N. Brush border membrane vesicles from pig small intestine were photolabeled with [^{3}H]benzylpenicillin and subsequently solubilized with Triton X-100; both the photolabeled 127 kDa polypeptide and the aminopeptidase N activity were found in the soluble fraction (Fig. 8, upper panel). After addition of anti-aminopeptidase N, an immunoprecipitate was formed containing the complete aminopeptidase N activity (Fig. 8, mid panel) whereas no signifi-

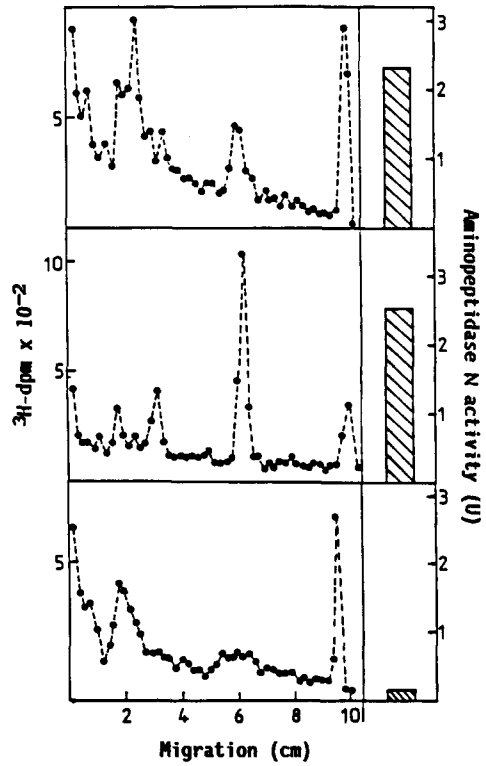

Fig. 8. Immunoprecipitation of aminopeptidase N from solubilized brush border membranes of pig small intestine after photoaffinity labeling. 1 mg of brush border membrane vesicles from pig small intestine loaded with 10 mM Tris-Hepes buffer (pM 7.4)/300 mM mannitol were photolabeled with 1.11 μM (10 μCi) [^{3}H]benzylpenicillin in 20 mM Tris-citrate buffer (pH 6.0)/140 mM KCl. After washing membrane vesicles were solubilized by resuspending of the vesicles in 500 μl 1% Triton X-100 at 4°C and incubation for 30 min. After centrifugation at 48000 × g for 30 min the clear supernatant was used for the following experiments: (a) 100 μl (200 μg of brush border membrane protein) were used for SDS-gel electrophoresis (upper panel). (b) To 365 μl (corresponding to 730 μg of brush border membrane protein) were added 45 μl of antiserum raised against aminopeptidase N from pig renal microsomes. After incubation for 1 h at 20°C the immunoprecipitate formed was separated from solubilized proteins by centrifugation at 15000 × g for 8 min. The resulting precipitate was redissolved in 200 μl of 1% Triton X-100 and after removal of 25 μl for the determination of aminopeptidase N, proteins were precipitated and submitted to SDS-gel electrophoresis (mid panel). From the supernatant after immunoprecipitation 50 μl (corresponding to 50 μg of brush membrane protein) were submitted to SDS-gel electrophoresis after precipitation of proteins (lower panel). The activity of aminopeptidase N is given as overall activity (units) in the solubilized membrane (upper panel), the immunoprecipitate (mid panel) and the supernatant after immunoprecipitation (lower panel).

cant aminopeptidase N activity was found in the supernatant (Fig. 8, lower panel). The photolabeled 127 kDa polypeptide, however, remained in the supernatant and was not precipitated by antiserum against aminopeptidase N (Fig. 8, lower panel). SDS polyacrylamide gel electrophoresis of the immunoprecipitate revealed 3 radioactively labeled bands with apparent molecular weights 130000, 95000 and 50000 (Fig. 8, mid panel). These labeled bands correspond to the three subunits of aminopeptidase N from pig small intestine with apparent molecular weights of 130000, 97000 and 49000 [38,39]; the two lower molecular weight polypeptides are formed from the 130000 subunit by the action of digestive enzymes [40]. By immunoblot analysis with anti-aminopeptidase N also three radioactively labeled polypeptides of molecular weight 130000, 95000 and 50000 were detected in brush border membrane vesicles from pig small intestine on one- and two-dimensional SDS-gels. These studies clearly demonstrate that the photolabeled binding protein for β-lactam antibiotics and dipeptides is a membrane protein different from aminopeptidase N and dipeptidylpeptidase IV.

Discussion

During digestion dietary proteins are enzymatically hydrolyzed to amino acids and oligopeptides in the stomach and the small intestine. The resulting amino acids are resorbed by specific Na^+-dependent transport systems [4–8]. Oligopeptides are further hydrolyzed to amino acids and di- and tripeptides. Di- and tripeptides can reach blood in intact form [41–43] and are actively transported into intestinal mucosa cells by a specific transport system which is different from the uptake systems involved in the intestinal absorption of free amino acids. The role of membrane-bound peptidases for the absorption of peptides is still unclear and four models have been proposed [12]:

(a) Hydrolysis of oligopeptides by membrane-bound peptidases and uptake of the resulting amino acids by different amino acid carriers.
(b) Hydrolysis of oligopeptides by membrane-bound peptidases and subsequent uptake of the resulting amino acids by a carrier not available for free amino acids.
(c) Transport of oligopeptides by the dipeptide carrier and subsequent hydrolysis of peptides by cytosolic peptidases.
(d) A multifunctional system with a transport site for peptides at the outside of the brush border membrane and and an enzymatic site facing the inside of the plasma membrane.

The arrival of intact di- and tripeptides in portal blood can not be explained by the action of one of the models a–c alone, whereas the experimental findings could be explained either by model d or by a combined action of models a–c. Fogel and Adibi [44] have found that brush-border hydrolases are not involved in the disappearance of dipeptides from the intestinal lumen whereas the disappearance of a tetrapeptide could be completely blocked by inhibition of brush border aminopeptidase [45]. An involvement of brush-border peptidases in the uptake proces for dipeptides and β-lactam antibiotics could hitherto not be ruled out for the following findings:

(1) β-lactam antibiotics are analogues of tripeptides. Therefore the possibility exists that the binding protein for β-lactam antibiotics and dipeptides identified with photoreactive derivatives of β-lactam antibiotics and dipeptides is a peptidase.

(2) The binding protein for β-lactam antibiotics and dipeptides with a molecular weight of 127000 has a similar molecular weight as the subunits of aminopeptidase N and dipeptidylpeptidase IV.

(3) An involvement of aminopeptidase N – a transmembrane protein [46] – in the uptake process for amino acids has been suggested; according to this hypothesis amino acids can be hydrolyzed from tetrapeptides by aminopeptidase N and are transported through the plasma membrane by the aminopeptidase N itself without the involvement of the transport systems for free amino acids [47,48].

For the investigation of the mechanisms responsible for the intestinal uptake process of peptides and β-lactam antibiotics it was important to determine whether brush border membrane peptidases are directly involved in this transport process. β-Lactam antibiotics inhibited aminopeptidase N in a noncompetitive manner and did not inhibit dipeptidylpeptidase IV. In contrast, the uptake of cephalexin and dipeptides was competitively inhibited by β-lactam antibiotics. The peptidase inhibitor bestatin led in the μM range to a very strong inhibition of aminopeptidase N whereas for a slight inhibition of cephalexin transport bestatin must be used in the mM range. Similar different effects on the enzymatic activities of aminopeptidase N and dipeptidylpeptidase IV on one side and the transport activity for cephalexin on the other side were observed after treatment of brush border membrane vesicles with group-specific reagents. The uptake system for β-lactam antibiotics and dipeptides was sensitive to treatment with the histidine-modifying reagent DEP, whereas DEP did not influence the activity of aminopeptidase N and only slightly inhibited dipeptidylpeptidase IV. A modification of serine-residues with organic fluorophosphates had no influence on the transport system and the activity of aminopeptidase N whereas dipeptidylpeptidase IV was completely inhibited by DFP. With antibodies raised against aminopeptidase N it was proven that the photolabeled binding protein for β-lactam antibiotics and dipeptides of molecular weight 127000 is different from aminopeptidase N. The studies performed clearly

give evidence that the brush border enzymes aminopeptidase N and dipeptidylpeptidase IV are not directly involved in the transport process of oligopeptides and β-lactam antibiotics across the brush border membrane of small intestinal mucosa cells. This suggests that the photolabeled 127 kDa protein is a component of the intestinal transport system for oligopeptides and β-lactam antibiotics. The studies with anti-aminopeptidase N antibodies ruled out the possibility that the transport activity and the peptidase activity are located on a single multi-site, multi-functional protein. This does not exclude the possibility that peptide hydrolyzing enzymes of the intestinal brush border membrane are associated with the peptide transport system to form a multifunctional protein complex. Preliminary cross-linking experiments, however, gave no evidence for such a membrane protein complex. Further studies are under way to investigate the topology of the peptide transport system. The accompanying paper describes the purification of the putative transport system for oligopeptides and β-lactam antibiotics from rabbit small intestine [49].

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