Biochimica et Biophysica Acta, 1030 (1990) 50–59
Elsevier

BBAMEM 75047

Intestinal absorption of dipeptides and β-lactam antibiotics. II. Purification of the binding protein for dipeptides and β-lactam antibiotics from rabbit small intestinal brush border membranes *

Werner Kramer, Ulrike Gutjahr, Frank Girbig and Irina Leipe

Hoechst Aktiengesellschaft, Frankfurt am Main (F.R.G.)

(Received 21 January 1990)
(Revised manuscript received 14 June 1990)

Key words: Peptide transport; Carrier protein; β-Lactam antibiotic; Brush border membrane vesicle; Photoaffinity labeling; (Rabbit small intestine)

By photoaffinity labeling of brush border membrane vesicles from rabbit small intestine with photoreactive derivatives of β-lactam antibiotics and dipeptides, a binding protein for dipeptides and β-lactam antibiotics with an apparent molecular weight of 127000 was labeled. The labeled 127 kDa polypeptide could be solubilized with the non-ionic detergents Triton X-100, *n*-octyl glucoside or CHAPS. If the vesicles were solubilized prior to photoaffinity labeling, no clear incorporation of radioactivity into the 127 kDa polypeptide occurred indicating a loss of binding ability upon solubilization. By affinity chromatography of solubilized brush border membrane proteins on an agarose wheat germ lectin column, the binding protein for dipeptides and β-lactam antibiotics of M_r 127000 was retained on the column. With *N*-acetyl-D-glucosamine the photolabeled binding protein for β-lactam antibiotics and dipeptides was eluted together with the brush border membrane-bound enzyme aminopeptidase N. Separation from aminopeptidase N and final purification was achieved by anion-exchange chromatography on DEAE-sephacel. Polyclonal antibodies against the purified binding protein were raised in guinea pigs. The photolabeled 127 kDa protein could be precipitated from solubilized brush border membranes with these antibodies. Incubation of brush border membrane vesicles with antiserum prior to photoaffinity labeling significantly reduced the extent of labeling of the 127 kDa protein. Treatment of brush border membrane vesicles with antiserum significantly inhibited the efflux of the α-aminocephalosporin cephalexin from the brush border membrane vesicles compared to vesicles treated with preimmune serum. These studies indicate that the binding protein for dipeptides and β-lactam antibiotics of apparent molecular weight 127000 in the brush border membrane of rabbit small intestinal enterocytes is directly involved in the uptake process of small peptides and orally active β-lactam antibiotics across the enterocyte brush border membrane.

Introduction

Small peptides are absorbed from the small intestine by a carrier-mediated uptake system [1–5]. Peptide-derived drugs like the orally active α-amino-β-lactam antibiotics and enzyme inhibitors like the angiotensin converting enzyme inhibitor captopril share this nutrient transport system [6–10]. The uptake of dipeptides and α-amino-β-lactam antibiotics across the brush border membrane of enterocytes in the small intestine is stimulated by an inwardly directed H^+-gradient ($[pH]_{out} < [pH]_{in}$) [8,9,11–16]. A histidine-residue of the transport protein is involved in the translocation process of a carrier-bound orally active α-amino-β-lactam antibiotic across the brush border membrane [16,17].

During our efforts to investigate the molecular mechanisms responsible for the intestinal absorption of small peptides and β-lactam antibiotics, photoaffinity labeling studies with photoreactive derivatives of β-lactam antibiotics and dipeptides identified a membrane polypeptide of apparent molecular weight 127000 which specifically binds dipeptides and β-lactam antibiotics

* Dedicated to Professor Hansgeorg Gareis on the occasion of his 60th birthday.

Abbreviations: CHAPS, 3-(3-cholamidopropyl)dimethylammonio-3-propane sulfonate; EDTA, ethylene diamine tetraacetic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; HPLC, high pressure liquid chromatography; *n*-octyl glucoside, octyl β-D-glucopyranoside; PMSF, phenylmethyl sulfonyl fluoride; SDS, sodium dodecylsulfate; Triton X-100, octylphenol polyethyleneglycol ether, $n = 10$; Triton X-114, octylphenyl polethyleneglycol ether, $n = 7–8$.

Correspondence: W. Kramer, Hoechst Aktiengesellschaft, PGE Stoffwechsel Pharma Forschung, Postfach 80 03 20, D-6230 Frankfurt am Main 80, F.R.G.

[15,17–20]. This binding protein for dipeptides and β-lactam antibiotics is different from the brush border membrane-bound peptidases aminopeptidase N and dipeptidylpeptidase IV and a direct involvement of these enzymes in the uptake process for dipeptides and β-lactam antibiotics was ruled out [21].

In the present study we describe the purification of the binding protein for β-lactam antibiotics and dipeptides from rabbit small intestinal brush border membrane vesicles. Experiments with polyclonal antibodies raised against the purified protein indicate that the binding protein for β-lactam antibiotics and dipeptides with apparent molecular weight 127000 is directly involved in the intestinal uptake process of dipeptides and orally active α-amino-β-lactam antibiotics.

Parts of this work has been published in preliminary form [44].

Materials and Methods

Materials

[phenyl-4-(n)-^{3}H]Benzylpenicillin (specific radioactivity 8-31 Ci/mMol) was obtained from Amersham (Amersham Buchler GmbH & Co KG, Braunschweig, F.R.G.). β-Lactam antibiotics, *N*-acetyl-D-glucosamine and marker proteins for molecular weight determinations in SDS-polyacrylamide gel electrophoresis were from Sigma (Sigma, München, F.R.G.). Triton-X-100 and materials for electrophoresis were from Serva (Serva Heidelberg, F.R.G.). Wheat germ lectin agarose, protein A-Sepharose CL-4B, activated CH Sepharose 4B, epoxy-activated Sepharose 6B and DEAE-Sephacel were purchased from Pharmacia (Pharmacia LKB Biotechnologie, Freiburg, F.R.G.). Nitrocellulose sheets, protein A-horseradish peroxidase conjugate and chemicals for immunoblotting were from Bio-Rad (Bio-Rad GmbH, München, F.R.G.). Solvents for HPLC were from Merck (Merck, Darmstadt, F.R.G.), scintillators Quickszint 501 and Unisolve 1 and the tissue solubilizer Biolute S were obtained from Zinsser (Zinsser Analytic GmbH, Frankfurt, F.R.G.). Cellulose nitrate filters (type HAWP 0.45 μm, 25 mm diameter) for transport studies were from millipore (Millipore GmbH, Eschborn, F.R.G.). All other materials were of analytical grade and were obtained from commercial sources.

Methods

Preparation of brush border membrane vesicles from rabbit small intestine

Brush border membrane vesicles from rabbit small intestine were prepared by the Mg^{2+}-precipitation method as described previously [15,16,20,22]. The purity of the brush border membrane vesicles was determined by measurement of the activities of the brush border marker enzymes leucine aminopeptidase (EC 3.4.11.2) and γ-glutamyltransferase (EC 2.3.2.2). Protein was determined according to Bradford [23] using the Bio-Rad kit (Bio-Rad, München, F.R.G.). The functional integrity of the brush border membrane vesicles was tested by measurement of the Na^+-dependent D-glucose uptake. The vesicles loaded with 10 mM Tris-Hepes buffer (pH 7.4)/300 mM mannitol were stored at a protein concentration of 10–20 mg/ml in liquid nitrogen up to 4 weeks without loss of transport or enzyme activity.

Transport measurements

Transport studies with brush border membrane vesicles were performed by the membrane filtration method [24,25] as described [15,16,20,21,26]. For efflux experiments the brush border membrane vesicles, preloaded with 10 mM Tris-Hepes buffer (pH 7.4)/300 mM mannitol, were equilibrated for 60 min with a solution of 2 mM cephalexin in 10 mM Tris-Hepes buffer (pH 7.4)/300 mM mannitol. After equilibration with cephalexin an equal volume of buffer (10 mM Tris-Hepes (pH 7.4)/300 mM mannitol/2 mM cephalexin) without, with antiserum or with preimmune serum were added. After incubation for 60 min at 20 °C efflux was started by 10-fold dilution of the vesicle suspension. Efflux was stopped after 0, 1, 2, 3, 4 and 60 min by addition of 1 ml of ice-cold stop solution (10 mM Tris-Hepes buffer (pH 7.4)/140 mM NaCl). This mixture was immediately filtered through a prewetted cellulose nitrate filter and washed once with 5 ml of ice-cold stop solution. The cephalexin entrapped by the vesicles was eluted with 300 μl of water and quantification of cephalexin was performed by HPLC analysis as described [15,16,20,21,26]. As eluent 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 solvent A and 468 g acetonitrile) was used.

Photoaffinity labeling

For photoaffinity labeling brush border membrane vesicles, suspended in 10 mM sodium phosphate buffer (pH 7.4)/140 mM NaCl, or solubilized brush border membrane proteins (protein concentration 1–4 mg/ml) were incubated with the indicated amounts of [^{3}H]benzylpenicillin for 5 min in the dark and subsequently the vesicle suspension was irradiated for 2.5 min at a wavelength of 254 nm at 20 °C in a Rayonet RPR 100 photochemical reactor equipped with 16 RPR 2530 Å lamps. After photoaffinity labeling the membrane suspension was diluted with ice cold buffer (10 mM Tris-Hepes (pH 7.4)/300 mM mannitol/4 mM PMSF/4 mM EDTA/4 mM iodacetamide) and subsequently centrifuged at 48000 × *g* for 30 min. The resulting pellet was either analyzed by SDS-gel electrophoresis after precipitation of proteins [27] or used for chromatography.

Purification of the binding protein for dipeptides and β-lactam antibiotics from rabbit small intestinal brush border membrane vesicles

Brush border membrane vesicles (5 mg) were solubilized for 30 min at 4° C in 1 ml of 1% Triton X-100-solution. Nonsolubilized material was removed by centrifugation at 48000 × *g* for 30 min. The resulting supernatant was loaded on to an affinity column (1 cm diameter, 5 ml of agarose wheat germ lectin) equilibrated with 10 mM Tris-HCl buffer (pH 7.4)/100 mM NaCl/0.1% Triton X-100 with a flow rate of 10 ml/h. Eluted proteins were monitored by ultraviolet absorption at 280 nm and 1.5 ml fractions were collected. After 12 fractions the adsorbed proteins were eluted with 25 ml of a solution of 100 mM *N*-acetyl-D-glucosamine in 10 mM Tris-HCl buffer (pH 7.4)/100 mM NaCl/0.1% Triton X-100. Aliquots from each fraction were removed for the determination of aminopeptidase N activity, (and radioactivity if photolabeled brush border membrane vesicles were used) and the determination of the polypeptide pattern by SDS polyacrylamide gel electrophoresis. The fractions of the elution peak were stored at −20° C until further chromatographic purification.

The fractions containing the *N*-acetyl-D-glucosamine-eluted proteins from the affinity column from three individual chromatographies (2–2.5 mg of protein) were pooled and loaded on to a DEAE-Sephacel column (1 cm diameter, 30 cm length) equilibrated with 10 mM Tris-HCl buffer (pH 7.0)/100 mM NaCl/0.1% Triton X-100 at a flow rate of 7 ml/h. Bound proteins were eluted with a linear sodium chloride gradient (130 ml 10 mM Tris-Hepes buffer (pH 7.0)/0.1% Triton X-100, 100–275 mM NaCl). Fractions of 1,5 ml were collected and 150 µl from each fraction were removed for the determination of aminopeptidase N activity. Further 150 µl of each fraction were analyzed by SDS-polyacrylamide gel electrophoresis to determine the distribution of photolabeled polypeptides in the different fractions. The fractions containing the binding protein for dipeptides and β-lactam antibiotics of molecular weight 127000 were pooled and kept at −80° C. The yield of purified binding protein was 300–500 µg.

Preparation of polyclonal antibodies against the binding protein for dipeptides and β-lactam antibiotics from rabbit intestinal brush border membranes

The immunisation of guinea pigs with the purified dipeptide and β-lactam antibiotic binding protein was kindly performed by Dr. Horst Neubauer and Mr. Jochen Duhmke (Hoechst Aktiengesellschaft, Frankfurt, F.R.G.). The pooled fractions from DEAE-chromatography containing the binding protein for dipeptides and β-lactam antibiotics (approx. 30 ml containing 300–500 µg of protein) were dialyzed against 1 l of water (three changes within 36 h) and subsequently lyophilized. The dry residue was redissolved in 0.5 ml of 0.9% sodium chloride solution and subsequently 0.5 ml of Freund's complete adjuvans was added. Polyclonal antibodies were raised in guinea pigs by subcutaneous injection of 600 µl (300 µg of protein) of this solution. 10 and 20 days after the initial immunization two booster injections were performed (200 µg of protein dissolved in 200 µl 0.9% sodium chloride solution/200 µl Freund's incomplete adjuvans).

14 days after the second booster injection 4 ml of blood was collected from each animal by retroocular puncture. After clotting serum was collected by centri-

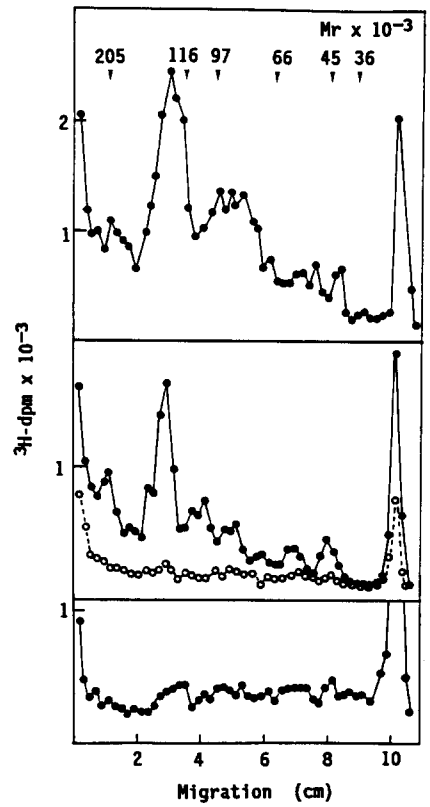

Fig. 1. Solubilisation of membrane proteins from rabbit small intestinal brush border membranes before and after photoaffinity labeling. Solubilization after photoaffinity labeling (upper and mid panel). 400 µg of brush border membrane vesicles were photolabeled for 150 s with 2.5 µM (20 µCi) [^{3}H]benzylpenicillin in 10 mM potassium phosphate buffer (pH 7.4)/150 mM NaCl. After washing 200 µg of vesicles were submitted to SDS-gel electrophoresis (upper panel). 200 µg of membrane vesicles after photoaffinity labeling were solubilized with 1% Triton X-100. After separation from nonsolubilized material by centrifugation the proteins in the supernatant were precipitated and submitted to SDS-gel electrophoresis (mid panel, curve with closed circles; the curve with open circles shows the distribution of proteins from nonsolubilized material). Solubilization prior to photoaffinity labeling (lower panel). 300 µg of brush border membrane vesicles were solubilized with 200 µl of 1% Triton X-100 in 10 mM potassium phosphate buffer (pH 7.4)/150 mM NaCl for 30 min at 4° C. After centrifugation the clear supernatant was incubated for 10 min with 2.5 µM (10 µCi) [^{3}H]benzylpenicillin and subsequently irradiated at 254 nm for 150 s. After precipitation of proteins and SDS-gel electrophoresis the distribution of radioactivity was determined. Total acrylamide concentration was 7.5%.

fugation and after aliquoting the serum samples were stored at −80 °C.

Immunoprecipitation of the binding protein for dipeptides and β-lactam antibiotics from rabbit intestinal brush border membranes

After photoaffinity labeling of brush border membrane vesicles the membranes were diluted with 10 mM Tris-Hepes buffer (pH 7.4)/300 mM mannitol. After centrifugation at 48000 × *g* for 30 min the resulting pellet was resuspended in 10 mM Tris-Hepes buffer/300 mM mannitol. Membrane proteins were solubilized with 1% Triton X-100 at 4 °C for 30 min at a protein concentration of 1 mg/ml. After centrifugation at 48000 × *g* the clear supernatant was divided into three aliquots. One part was used as a control after precipitation of proteins and subsequent SDS-polyacrylamide gel electrophoresis. The other aliquots were mixed either with preimmune serum or with antiserum raised against the dipeptide and β-lactam antibiotic binding protein. After incubation at 20 °C for 1 h the probes were centrifuged at 15000 × *g* for 10 min and the supernatants were carefully removed and analyzed by SDS-gel electrophoresis. The immunoprecipitates were resuspended in 300 µl of 0.9% sodium chloride solution and after centrifugation the resulting pellets were analyzed by SDS-gel electrophoresis. In other experiments the binding protein for dipeptides and β-lactam antibiotics was removed from solubilized brush border membranes with immunoglobulin-coated protein A-Sepharose beads [27]. 50 µl of a 30% suspension of protein A-Sepharose beads in 10 mM Tris-HCl buffer (pH 7.4)/100 mM NaCl/0.1% Triton X-100 were incubated for 12 h with 30 µl of either antiserum or preimmune serum. Afterwards the beads were washed three times with the abovementioned buffer. The immunoglobulin-coated beads were then added to 200 µl (200 µg) of solubilized brush border membrane proteins prepared from photolabeled membrane vesicles. After 1 h of incubation at 20 °C the beads were sedimented by centrifugation. After washing of the beads the adsorbed proteins were eluted with sample buffer for SDS-gel electrophoresis; the proteins in the corresponding supernatants were precipitated and subsequently submitted to SDS-gel electrophoresis.

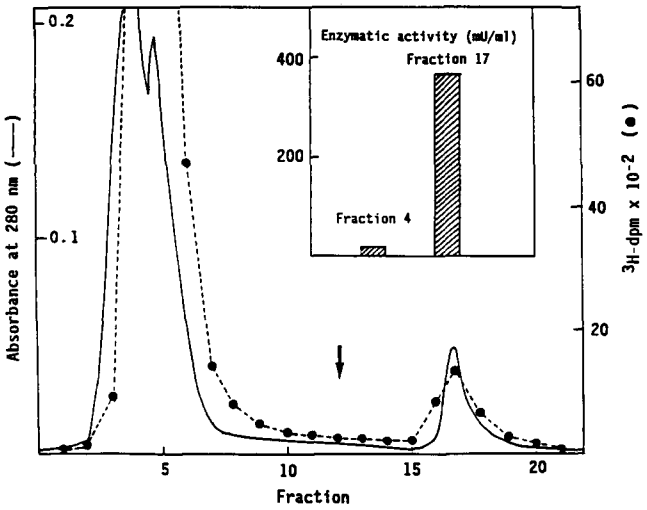

Fig. 2. Affinity chromatography of solubilized intestinal brush border membrane proteins on a agarose wheat germ lectin column after photoaffinity labeling. 5 mg of brush border membrane vesicles were photolabeled with 5 µM (100 µCi) [^{3}H]benzylpenicillin in 1 ml of 20 mM Tris-citrate buffer (pH 6.0)/280 mM mannitol for 150 s at 254 nm. After washing with 30 ml of 10 mM Tris-Hepes buffer (pH 7.4)/300 mM mannitol/4 mM PMSF/4 mM EDTA/4 mM iodacetamide and centrifugation the resulting pellet was resuspended and solubilized in 1 ml of 1% Triton X-100 solution for 30 min at 4 °C. After centrifugation at 48000 × *g* the clear supernatant was applied to an agarose wheat germ lectin column. The arrow indicates the application of 100 mM *N*-acetyl-D-glucoseamine for elution of bound proteins. 150 µl of each fraction were removed for the determination of radioactivity and 150 µl of each fraction were used for the determination of the enzymatic activity of aminopeptidase N, ——, UV absorption at 280 nm; ●- - - - -●, distribution of radioactivity. The inset shows the activity of aminopeptidase N in fraction 4 of the flow through and fraction 17 of the eluate.

SDS-gel electrophoresis

The proteins in the individual chromatography fractions and solubilized membrane vesicles were precipitated according to Wessel and Flügge [28]. SDS-gel electrophoresis was performed on discontinuous SDS slab gels (200 × 140 × 1.5 mm) as previously described [29]. The proteins were dissolved in 70 µl of SDS sample buffer (62.5 mM Tris-HCl (pH 6.8)/2% SDS/10% glycerol/5% 2-mercaptoethanol/0.001% bromophenol blue). After centrifugation at 15000 × *g* for 5 min the clear supernatants were submitted to SDS-polyacrylamide gel electrophoresis on 7.5–9% gels. After fixing and staining [29] the gels were scanned with a densitometer CD 50 (DESAGA, Heidelberg, F.R.G.). For the quantification of the polypeptides of molecular weight 127000 in the different fractions after DEAE-chromatography, the bands of the 127 kDa proteins were scanned; the area of the respective peaks was used as a measure of the relative amount of the 127 kDa protein in the different fractions.

Detection of radioactivity

Radioactivity in the chromatography fractions was determined by liquid scintillation counting after mixing of the aqueous samples with 4 ml of scintillator Unisolve I. For the detection of radioactively labeled polypeptides on SDS gels, the gel tracks were cut into 2 mm slices. After digestion of the polypeptides with 250 µl of Biolute S, 4 ml of scintillator Quickszint 501 was added and radioactivity was measured by liquid scintillation counting using a Tricarb 2000 counter (Packard Instruments).

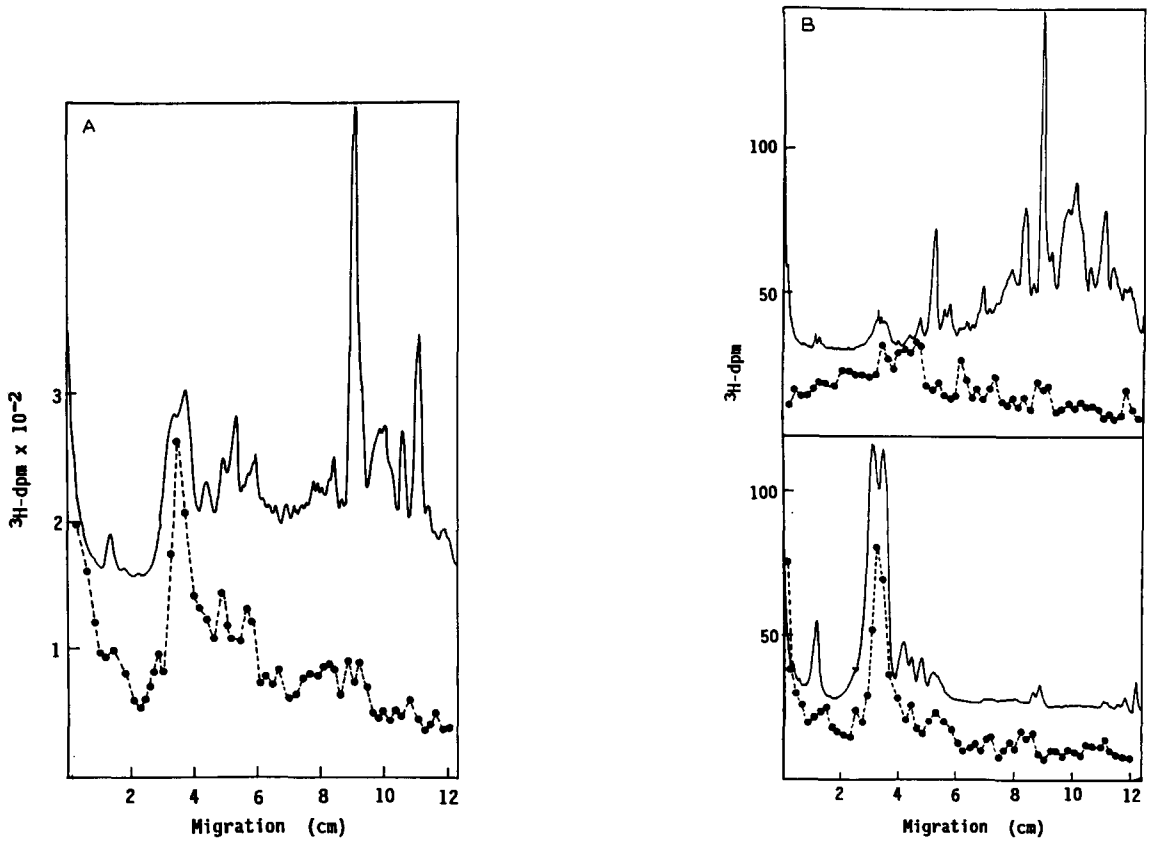

Fig. 3. SDS-polyacrylamide gel electrophoresis of photolabeled membrane proteins from solubilized brush border membrane proteins after photoaffinity labeling separated by agarose wheat germ lectin chromatography. The labeling conditions are described in Fig. 2. (A) Supernatant after solubilization of photolabeled brush border membrane vesicles (200 μg of protein). (B) Fractions after wheat germ lectin chromatography. Upper panel: Pooled fractions 3–6 from agarose wheat germ lectin chromatography (300 μl). Lower panel: Pooled fractions 16–20 from agarose wheat germ lectin chromatography (300 μl). The drawn lines show the distribution of Serva Blue R 250 stained proteins, whereas the dotted lines show the distribution of radioactivity.

Results

Solubilisation of the binding protein for dipeptides and β-lactam antibiotics from rabbit small intestinal brush border membrane vesicles

Fig. 1 shows that the binding protein for dipeptides and β-lactam antibiotics of molecular weight 127000 labeled with photoreactive β-lactam antibiotics could be completely solubilized from the brush border membrane vesicles with nonionic detergents like Triton X-100, *n*-octyl glucoside or with CHAPS and was found in the supernatant; no significant amounts of the 127 kDa binding protein remained in the nonsolubilizable material. If the brush border membrane vesicles were at first treated with Triton X-100 and the solubilized membrane proteins subsequently submitted to photoaffinity labeling, no clear incorporation of the photoprobe into the 127 kDa binding protein occurred (Fig. 1, lower panel). This finding indicates that the binding protein for β-lactam antibiotics and dipeptides loses its ability for specific binding of its substrates upon solubilization.

Purification of the binding protein for dipeptides and β-lactam antibiotics from rabbit small intestinal brush border membranes

For the isolation of the 127 kDa binding protein for dipeptides and β-lactam antibiotics, we initially tried affinity chromatography using immobilized ligands. The orally active cephalosporin cephalexin was coupled either by its amino group or by its carboxyl group to activated sepharose gels containing a 6-carbon spacer arm. With the affinity matrix containing cephalexin coupled via the α-amino group, a slight retardation of a polypeptide of molecular weight 127000 could be achieved after application of solubilized brush border membranes on the column, whereas with a carboxyl-linked cephalexin matrix no proteins were retarded. However, with this approach only tiny amounts of the 127 kDa polypeptide (< 1 μg) were obtained. Therefore, an alternative purification procedure was necessary. From investigations concerning the topology of the β-lactam antibiotic and dipeptide binding protein we knew that the photolabeled 127 kDa polypeptide in rabbit small

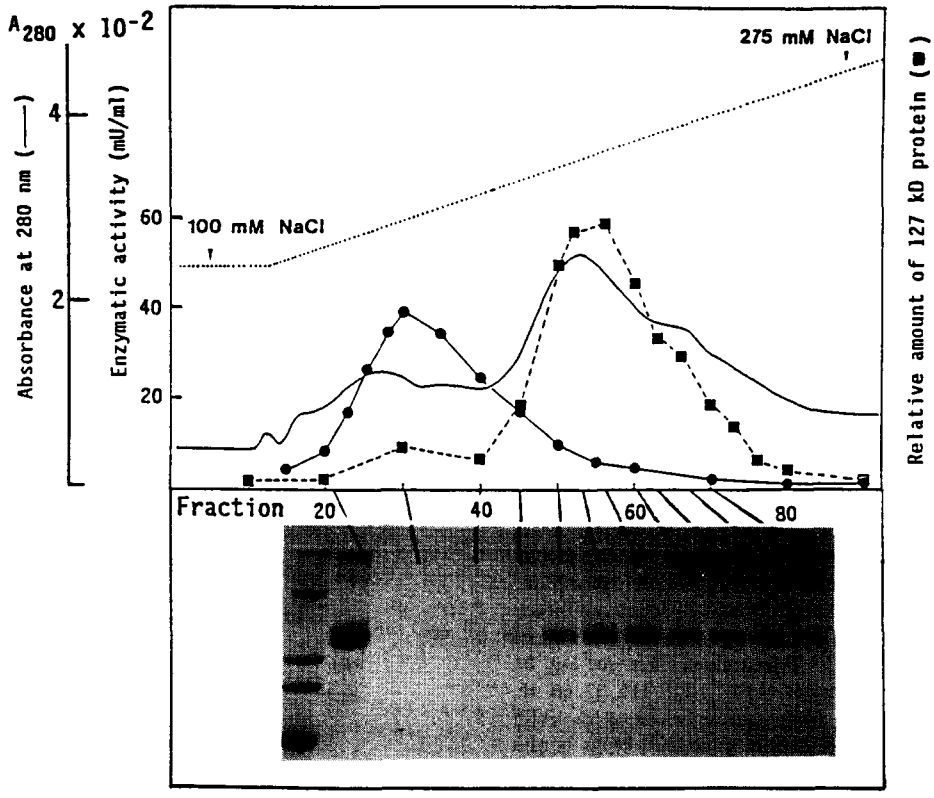

Fig. 4. DEAE-ion exchange chromatography of brush border membrane proteins retained by agarose wheat germ lectin. The fractions of the elution peak from agarose wheat germ lectin chromatography from three individual runs were pooled (9 ml) and applied on a DEAE-Sephacel column. Proteins were eluted with a linear NaCl-gradient. From each fraction 150 µl were removed for the measurement of aminopeptidase N activity and 150 µl were used for SDS-polyacrylamide gel electrophoresis of each fraction. After electrophoresis the relative amount of the 127 kDa protein was determined by densitometry of the stained gels. ———, UV-absorption at 280 nm; ●———●, activity of aminopeptidase N; ■- - - - -■, intensity of the 127 kDa protein. The lower graph shows the distribution of polypeptides in the different fractions after DEAE-chromatography after SDS-polyacrylamide gel electrophoresis. On the left side of the gel marker proteins were applied, the neighbouring track contains the starting material applied on the DEAE-column.

intestinal brush border membranes is a glycoprotein. Consequently, affinity chromatography using immobilized lectins was performed. Brush border membrane vesicles were photolabeled with [^{3}H]benzylpenicillin and subsequently the membrane proteins were solubilized with Triton X-100; the solubilized membrane proteins were loaded on to an agarose wheat germ lectin column. Most of the applied protein appeared in the void volume (Fig. 2, first peak). With *N*-acetyl-D-glucosamine in the elution buffer, the proteins retarded on the column were eluted (Fig. 2, second peak). The brush border membrane-bound enzyme aminopeptidase N was also completely retarded by the affinity matrix and was also eluted with *N*-acetyl-D-glucosamine. Noncovalently bound radioactivity was eluted with peak 1; after application of *N*-acetyl-D-glucosamine, radioactively labeled proteins were eluted (Fig. 2, peak 2) indicating a covalent attachment of the photoprobe to proteins. The protein composition of the different fractions was analyzed by SDS-gel electrophoresis. Fig. 3 shows that the polypeptides in the molecular weight range 100 000–130 000 were nearly completely retained by the wheat germ lectin column; in the fractions of the flow through peak 1 no significant amount of polypeptides in this molecular weight range were detectable (Fig. 3B, upper panel). SDS-gel electrophoresis of the fractions eluted by *N*-acetyl-D-glucosamine and analysis of the radioactively labeled polypeptides in the different fractions showed that the photolabeled 127 kDa polypeptide is found in the elution peak (Fig. 3B, lower panel). Attempts to separate the labeled 127 kDa polypeptide from aminopeptidase N by elution with a linear gradient of *N*-acetyl-D-glucosamine did not result in a separation of both proteins. The proteins in the eluates from wheat germ lectin agarose chromatography were further fractionated by ion exchange chromatography. Fractions of elution peak 2 from agarose wheat germ lectin affinity chromatography of three individual chromatographic runs were loaded on a DEAE-sephacel column equilibrated with 10 mM Tris-HCl buffer (pH 7.0)/100 mM NaCl/0.1% Triton X-100; proteins were eluted with a linear NaCl gradient. Fig. 4 shows that

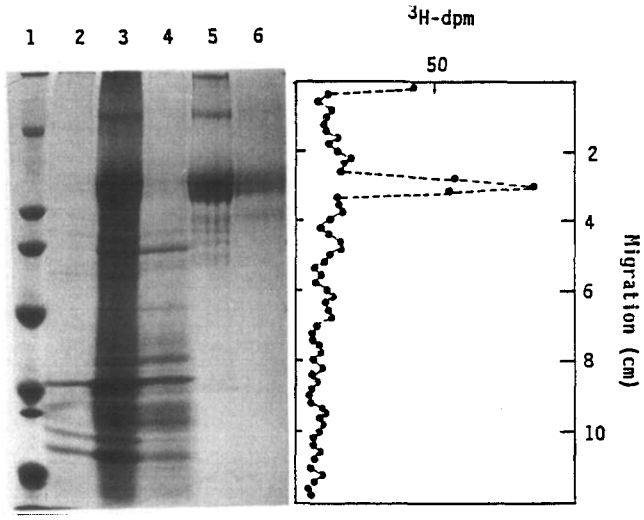

Fig. 5. SDS-polyacrylamide gel electrophoresis at the different stages of purification of the binding protein for dipeptides and β-lactam antibiotics from rabbit small intestinal brush border membrane vesicles. The binding protein for dipeptides and β-lactam antibiotics was purified as described starting from 5 mg of membrane vesicles which have been photolabeled with [^{3}H]benzylpenicillin (see Fig. 2). Lane 1: Molecular weight standards ($M_r \times 10^{-3}$: 205, 116, 97, 66, 45, 36, 29, 24). Lane 2: Fraction 3 from agarose wheat germ lectin chromatography. Lane 3: Supernatant from solubilized brush border membranes after centrifugation at 48000 × g. Lane 4: Fraction 4 from agarose wheat germ lectin chromatography. Lane 5: Fraction 17 from agarose wheat germ lectin chromatography. Lane 6: Fraction 60 from DEAE-chromatography. After slicing of the gel the 127 kDa protein still contained covalently linked radioactivity.

under these conditions the aminopeptidase N activity could be separated from the binding protein for dipeptides and β-lactam antibiotics of M_r 127000. Fig. 5 shows the polypeptide patterns at the different stages of purification after SDS-gel electrophoresis; the purified binding protein for dipeptides and β-lactam antibiotics still contained the radioactive label of the photoprobe initially used to label the brush border membrane vesicles. The purified 127 kDa binding protein always appeared as a broad band on SDS gels; this broadening is caused by the microheterogeneity of the 127 kDa protein in its carbohydrate composition [30–32]. Dependent upon the preparation, the broad band of the 127 kDa polypeptide can split into two bands of apparent molecular weights 127000 and 124000. By repeated experiments it was shown that the appearance of this doublet increases with time after isolation of the binding protein suggesting a limited proteolysis of the glycoprotein.

Immunoprecipitation of the binding protein for dipeptides and β-lactam antibiotics from brush border membranes

The purified binding protein for dipeptides and β-lactam antibiotics was used for the preparation of polyclonal antibodies in guinea pigs. Dot blot analysis showed that the antibodies crossreact with the purified Triton-solubilized 127 kDa protein. However, if brush border membranes or the purified binding protein were submitted to SDS-gel electrophoresis followed by electrophoretic transfer to nitrocellulose sheets, no clear immunological reaction between the 127 kDa protein and the antiserum could be observed. A detailed analysis of this phenomenon revealed that the raised antibodies recognize the 127 kDa protein in intact brush border membranes or after solubilization with nonionic detergents, whereas after denaturation of the proteins with SDS no immunocomplex is formed. For an immunological characterization of the photolabeled binding protein for dipeptides and β-lactam antibiotics therefore immunoprecipitation experiments instead of immunoblot analysis were performed.

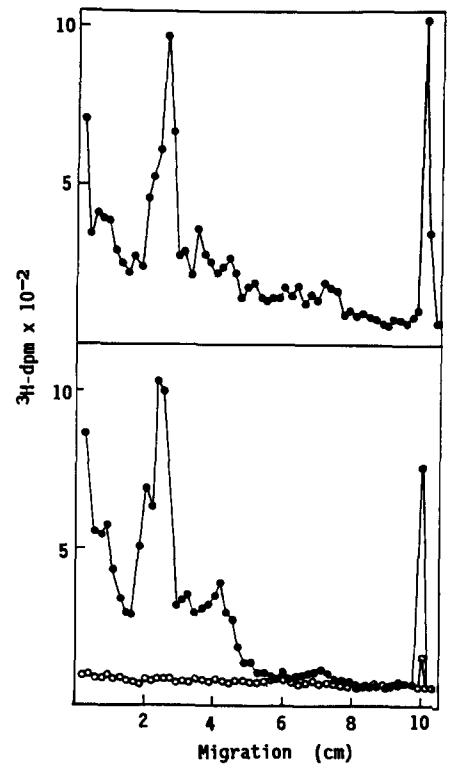

Fig. 6. Immunoprecipitation of the photolabeled binding protein for dipeptides and β-lactam antibiotics from solubilized brush border membranes. 2 mg of brush border membrane vesicles were photolabeled with 2.5 μM (50 μCi) [^{3}H]benzylpenicillin in 20 mM Tris-citrate buffer (pH 6.0)/280 mM mannitol. After washing the membrane proteins were solubilized with 2 ml of 1% Triton X-100. After centrifugation the clear supernatant was used for immunoprecipitation experiments. Upper panel: Distribution of radioactivity in 200 μg of solubilized membrane proteins after SDS-gel electrophoresis. Lower panel: 200 μl of the supernatant were incubated either with 40 μl of preimmune serum or 40 μl of antiserum for 1 h at 20 °C. After centrifugation at 15000 × g for 20 min the supernatants were carefully removed and the pellets were resuspended in 200 μl of 0.9% NaCl-solution. After centrifugation the pellets were submitted to SDS-gel electrophoresis. ●———●, incubation with antiserum; ○———○, incubation with preimmune serum.

Brush border membrane vesicles were photolabeled and subsequently solubilized with Triton X-100. After separation from nonsolubilizable material by centrifugation, the clear supernatant was incubated either with preimmune serum or with antiserum; after centrifugation the resulting supernatants and pellets were analyzed by SDS-gel electrophoresis. As expected, preimmune serum was unable to precipitate the 127 kDa polypeptide (Fig. 6, lower graph, 0); the labeled polypeptide completely remained in the supernatant. In contrast, with anti-127 kDa protein-antiserum a complete precipitation of the photolabeled 127 kDa polypeptide was obtained (Fig. 6, lower graph, •). In further experiments the IgG-globulins in the preimmune serum and the antiserum were attached to protein A-sepharose beads [28]. By incubation of solubilized photolabeled brush border membranes with these IgG-coated beads from antiserum, the labeled 127 kDa polypeptide could be completely removed from the solubilized membrane proteins whereas protein A-sepharose beads incubated with preimmune serum could not bind the 127 kDa protein. If brush border membrane vesicles were incubated either with preimmune serum or with antiserum and subsequently submitted to photoaffinity labeling, the extent of labeling of the 127 kDa protein was

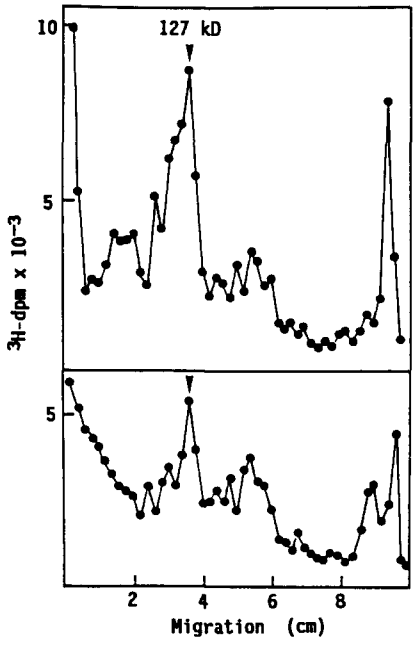

Fig. 7. Effect of preimmune serum and antiserum raised against the binding protein for dipeptides and β-lactam antibiotics on photoaffinity labeling of brush border membrane vesicles with [^{3}H]benzylpenicillin. 250 μg of brush border membrane vesicles were incubated at 20 °C for 1 h either with 20 μl of preimmune serum (upper panel) or 20 μl of antiserum (lower panel). After dilution with 1 ml of ice cold buffer the vesicles were sedimented by centrifugation. After resuspension in 200 μl of 10 mM Tris-Hepes buffer (pH 7.4)/300 mM mannitol the membrane vesicles were photolabeled with 2.5 μM (10 μCi) [^{3}H]benzylpenicillin and submitted to SDS-gel electrophoresis after washing.

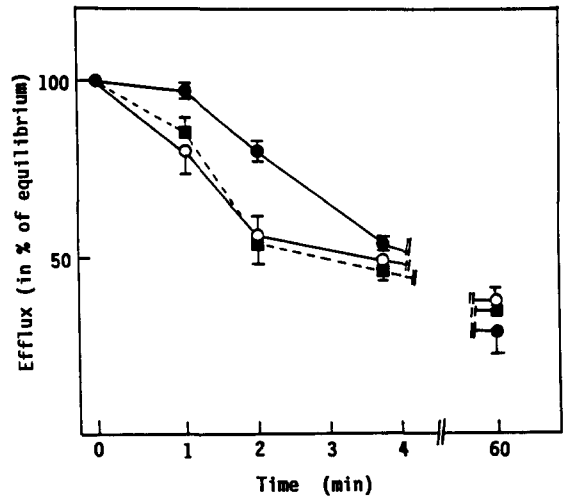

Fig. 8. Effect of preimmune serum and antiserum raised against the binding protein for dipeptides and β-lactam antibiotics on the efflux of cephalexin from brush border membrane vesicles. 5.5 mg of brush border membrane vesicles were incubated with 2 mM cephalexin in 10 mM Tris-Hepes buffer (pH 7.4)/300 mM mannitol in a total volume of 680 μl for 1 h at 20 °C. 220 μl of this suspension were mixed either with 70 μl of buffer, 70 μl of preimmune serum or 70 μl of antiserum. Efflux was initiated by mixing of 90 μl of these incubation mixtures with 850 μl of 10 mM Tris-Hepes buffer (pH 7.4)/300 mM mannitol. After the indicated times 150-μl aliquots were removed for the determination of cephalexin remaining within the vesicles. ■- - - - -■, incubation with buffer; ○———○, incubation with preimmune serum; ●———●, incubation with antiserum.

significantly lower in membrane vesicles pretreated with antiserum (Fig. 7, lower graph) compared to vesicles treated with preimmune serum (Fig. 7, upper graph).

Incubation of brush border membrane vesicles which have been equilibrated with cephalexin, with antiserum led to a decreased efflux of cephalexin from these vesicles compared to vesicles which have been incubated with preimmune serum or with buffer alone (Fig. 8). The uptake of cephalexin into brush border membrane vesicles also could be inhibited by antiserum against the 127 kDa protein in some experiments. However, only with the antibodies obtained 34 days after the initial immunization a significant inhibitory effect on cephalexin uptake could be achieved whereas the antibodies obtained from the same animals 12 or 20 weeks after the initial immunization showed no effect on transport; the 127 kDa binding protein however could also be precipitated from solubilized brush border membranes with these antibodies.

Discussion

The initial characterization of the transport system for dipeptides and β-lactam antibiotics in the brush border membrane of small intestinal cells has been accomplished by uptake studies into brush border membrane vesicles [2,4,8,9,15,16,33–43] and by photoaffinity labeling experiments using photolabile derivatives of

cephalosporins, penicillins and dipeptides [15,18–21]. These studies indicated that a membrane protein with an apparent molecular weight of 127000 in the brush border membrane of enterocytes from rabbit, rat and pig small intestine specifically binds dipeptides and orally active β-lactam antibiotics. It was found that all β-lactam antibiotics whether they are enterally absorbed or not inhibit both the uptake of orally active α-amino-β-lactam antibiotics by brush border membrane vesicles and the photolabeling of the 127 kDa protein [15,20].

For a clear understanding of the molecular mechanisms responsible for the intestinal absorption of peptides and β-lactam antibiotics, the purification and characterization of the putative transport protein is of particular importance. Photoaffinity labeling studies and phase separation experiments with Triton X-114 have shown that the binding protein for dipeptides and β-lactam antibiotics is an integral membrane protein. For the isolation of this protein the brush border membrane proteins must be solubilized. A prerequisite for the purification of a transport protein by affinity chromatography with an immobilized substrate is that the transport protein retains its ability for specific binding of its substrates upon solubilization. Photoaffinity labeling of solubilized brush border membrane proteins did not result in a clear and high labeling of the 127 kDa binding protein. This indicates that the affinity of the transport protein is abolished by solubilization; therefore a purification of this protein by affinity chromatography with immobilized ligands is not very promising. Nevertheless we have tried affinity chromatography with cephalexin affinity matrices. If cephalexin was linked by its amino group to the matrix, tiny amounts of the 127 kDa protein could be obtained, but for a further characterization of the binding protein for dipeptides and β-lactam antibiotics a more efficient purification procedure is necessary.

From two-dimensional electrophoresis of photoaffinity-labeled brush border membranes and from experiments with sugar-cleaving enzymes it was evident that the 127 kDa protein is a glycoprotein. Consequently, affinity chromatography with immobilized lectins was tried as a first purification step for the putative intestinal dipeptide transporter. With soybean lectin sepharose or lentil-lectin-sepharose no polypeptides of molecular weights 100000–130000 could be retained. With wheat germ lectin agarose however, proteins in the molecular weight region 100000–130000 were selectively adsorbed to the affinity matrix. With *N*-acetyl-D-glucosamine the photolabeled 127 kDa protein and the brush border-bound enzyme aminopeptidase N were eluted. A separation of the binding protein for dipeptides and β-lactam antibiotics of molecular weight 127000 from aminopeptidase N of identical molecular weight was achieved by subsequent anion exchange chromatography; from 15 mg membrane protein of rabbit intestinal brush border membranes 300–400 μg of pure binding protein for dipeptides and β-lactam antibiotics were obtained. The isolated 127 kDa protein was used to prepare polyclonal antibodies; with these antisera raised in guinea pigs the photolabeled 127 kDa binding protein could be selectively precipitated from solubilized brush border membrane proteins. If brush border membrane vesicles were incubated with antiserum against the 127 kDa protein prior to photoaffinity labeling, a clear decrease in the extent of labeling of the 127 kDa polypeptide occurred compared to vesicles incubated with preimmune serum; this indicates that that the antibodies can partially block the binding of β-lactam antibiotics to the putative transport protein. The binding activity of the purified binding protein for dipeptides and β-lactam antibiotics could not be assayed in a conventional binding assay since the 127 kDa binding protein loses its specificity for binding upon solubilization. In order to investigate whether the isolated 127 kDa binding protein is not only involved in binding but also in transport of β-lactam antibiotics and dipeptides across the intestinal brush border membrane, the effect of antiserum and preimmune serum on the transport of cephalexin by intestinal brush border membrane vesicles was investigated. Incubation of brush border membrane vesicles with antiserum led to a significant slower efflux of cephalexin from the vesicles compared to vesicles incubated with preimmune serum or buffer alone. These findings support the hypothesis that the binding protein for dipeptides and β-lactam antibiotics of molecular weight 127000 in the brush border membrane of small intestinal enterocytes is directly involved in the transport process of small peptides and β-lactam antibiotics across the brush border membrane and is (a component of) the intestinal peptide transport system. Preliminary reconstitution experiments revealed that the binding activity and the transport activity can be restored by incorporation of solubilized brush border membrane proteins into proteoliposomes.

Acknowledgements

The authors express their gratitude to Dr. Horst Neubauer and Mr. Jochen Duhmke for the immunization experiments.

References

1 Matthews, D.M. (1975) Physiol. Rev. 55, 537–608.
2 Sigrist-Nelson, K. (1975) Biochim. Biophys. Acta 394, 220–226.
3 Matthews, D.M. and Adibi, S.A. (1976) Gastroenterology 71, 151–161.
4 Ganapathy, V. and Leibach, F.H. (1982) Life Sci. 30, 2137–2146.
5 Addison, J.M., Burston, D., Dalrymple, J.A., Matthews, D.M., Payne, J.W., Sleisenger, M.H. and Wilkinson, S. (1975) Clin. Sci. Mol. Med. 49, 313–322.

6 Kimura, T., Endo, H., Yoshihawa, M., Muranishi, S. and Sezaki, K. (1978) J. Pharm. Dyn. 1, 262–267.
7 Nakashima, E., Tsuji, A., Mizuo, H. and Yamana, T. (1984) Biochem. Pharmacol. 33, 3345–3352.
8 Okano, T., Inui, K., Takano, M. and Hori, R. (1986) Biochem. Pharmacol. 35, 1781–1786.
9 Okano, T., Inui, K., Maegawa, H., Takano, M. and Hori, R. (1986) J. Biol. Chem. 261, 14130–14134.
10 Hu, M. and Amidon, G.L. (1988) J. Pharm. Sci. 77, 1007–1011.
11 Takuwa, N., Shimada, T., Matsumoto, H. and Hoshi, T. (1985) Biochim. Biophys. Acta 814, 186–190.
12 Ganapathy, V. and Leibach, F.H. (1985) Am. J. Physiol. 249, G153–G160.
13 Ganapathy, V. and Leibach, F.H. (1983) J. Biol. Chem. 258, 14189–14192.
14 Ganapathy, V., Burckhardt, G. and Leibach, F.H. (1984) J. Biol. Chem. 259, 8954–8959.
15 Kramer, W. (1987) Biochim. Biophys. Acta 905, 65–74.
16 Kramer, W., Girbig, F., Petzoldt, E. and Leipe, I. (1988) Biochim. Biophys. Acta 943, 288–296.
17 Kramer, W. (1988) Naunyn-Schmiedeberg's Arch. Pharmacol. 337, R 49.
18 Kramer, W. (1987) Naunyn-Schmiedeberg's Arch. Pharmacol. 361, R46.
19 Kramer, W. (1987) Biol. Chem. Hoppe-Seyler 368, 1261.
20 Kramer, W., Girbig, F., Leipe, I. and Petzoldt, E. (1988) Biochem. Pharmacol. 37, 2427–2435.
21 Kramer, W., Dechent, C., Girbig, F., Gutjahr, U. and Neubauer, H. (1990) Biochim. Biophys. Acta 1030, 41–49.
22 Burckhardt, G., Kramer, W., Kurz, G. and Wilson, F.A. (1983) J. Biol. Chem. 258, 3618–3622.
23 Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
24 Hopfer, U., Nelson, K., Perrotto, J. and Isselbacher, K.J. (1973) J. Biol. Chem. 248, 25–32.
25 Berner, W., Kinne, R. and Murer, H. (1976) Biochem. J. 160, 467–474.
26 Kramer, W., Leipe, I., Petzoldt, E. and Girbig, F. (1988) Biochim. Biophys. Acta 939, 167–172.
27 Ruetz, S., Hugentobler, G. and Meier, P.J. (1988) Proc. Natl. Acad. Sci. USA 85, 6147–6151.
28 Wessel, D. and Flügge, U.I. (1984) Anal. Biochem. 138, 141–146.
29 Kramer, W., Burckhardt, G., Wilson, F.A. and Kurz, G. (1983) J. Biol. Chem. 258, 3623–3628.
30 Kraml, J., Kolinska, J., Kadlecova, L., Zakostelecka, M. and Lajda, Z. (1983) FEBS Lett. 151, 193–196.
31 Yu, J. and Steck, T.L. (1975) J. Biol. Chem. 250, 9170–9175.
32 Lienhard, G.E., Crabb, J.H. and Ransome, K.J. (1983) Biochim. Biophys. Acta 769, 404–410.
33 Kimura, T., Yamamoto, T., Ishizuka, R. and Sezaki, H. (1985) Biochem. Pharmacol. 34, 81–84.
34 Rajendran, V.M., Ansari, S.A., Harig, J.M., Adams, M.B., Khan, A.H. and Ramaswamy, K. (1985) Gastroenterology 89, 1298–1304.
35 Himukai, M., Kameyama, A. and Hoshi, T. (1983) Biochim. Biophys. Acta 732, 659–667.
36 Himukai, M., Kano-Kameyama, A. and Hoshi, T. (1982) Biochim. Biophys. Acta 687, 170–178.
37 Berteloot, A., Khan, A.S. and Ramaswamy, K. (1982) Biochim. Biophys. Acta 686, 47–54.
38 Berteloot, A., Khan, A.S. and Ramaswamy, K. (1982) Biochim. Biophys. Acta 649, 179–188.
39 Rajendran, V.M., Berteloot, A. and Ramaswamy, K. (1985) Am. J. Physiol. 248, G682–G686.
40 Rajendran, V.M., Berteloot, A., Ishikawa, Y., Khan, A.H. and Ramaswamy, K. (1984) Biochim. Biophys. Acta 778, 443–448.
41 Ganapathy, V., Mendicino, J.F. and Leibach, F.H. (1981) J. Biol. Chem. 256, 118–124.
42 Ganapathy, V., Mendicino, J.F. and Leibach, F.H. (1981) Life Sci. 29, 2451–2457.
43 Miyamoto, Y., Ganapathy, V. and Leibach, F.H. (1986) J. Biol. Chem. 261, 16133–16140.
44 Kramer, W. (1989) Biol. Chem. Hoppe-Seyler 370, 624.