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Solubilization and reconstitution characteristics of the carrier protein(s) responsible for the transport of ceftibuten, a substrate for the oligopeptide transporters, in rat renal brush-border membrane

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Abstract

Optimal procedures for the reconstitution of the transport activity of ceftibuten, a dianionic β-lactam antibiotic, from rat kidney brush-border membrane were developed. The uptake activity into reconstituted proteoliposomes appeared to be particularly sensitive to the extraction conditions, and to the lipid composition used for reconstitution. Changes in the concentration of octyl glucoside significantly affected the extraction of ceftibuten transport activity, and optimal extraction was achieved at a concentration of 60 mM. Optimal reconstitution was achieved using a lipid composition of asolectin, cholesterol and phosphatidylserine in a w/w percent ratio of 60:30:10, respectively, and with a lipid-to-protein ratio of 10. The uptake of ceftibuten into the resulting proteoliposomes showed temperature and pH dependency, was inhibitable by a range of cephem antibiotics, oligopeptides and the organic anion PAH, and was trans-stimulated by cephalexin and dipeptides. This reconstitution system will likely prove useful in future studies on the functional analysis of the peptide transport system in a purified form.

Keywords: Brush-border membrane; Ceftibuten transport; Oligopeptide; Solubilization; Reconstitution; Liposome; Renal cortex

1. Introduction

The use of various techniques such as isolated membrane vesicles, in vivo micro infusion techniques and cloning systems has contributed greatly to the characterization of the oligopeptide transport system in the renal proximal tubule [1–5]. Yet it is considered that the full isolation of the carrier proteins is a prerequisite for a complete molecular description of the function of this transport system. Since the main problem associated with the functional isolation of the carrier proteins stems from the instability of these proteins towards the harsh conditions during the processing for purification, the determination of the proper conditions for the extraction and reconstitution into an environment approximating the native membrane is an integral and important part of the strategy for purification.

Until recently, several methods for the purification of mammalian carrier proteins have been described. Yet, for the oligopeptide transport systems, only one method with a lengthy protocol was reported by Kramer et al. [6] for the purification of the small intestinal oligopeptide transporter. Since we and others [1,7,8] have reported discrepancies between the oligopeptide transport systems in the renal and the intestinal brush-border membranes, it is of importance to establish a protocol for the isolation of the kidney oligopeptide transport system(s).

This report describes the proper conditions for the solubilization and functional reconstitution of the system(s) responsible for the transport of ceftibuten, a peptide-like compound, in the renal brush-border membrane.

2. Materials and methods

2.1. Materials

Ceftibuten and compound V, 3-vinyl derivative of ceftibuten [9], were kindly donated by Shionogi (Osaka, Japan). Octyl glucoside (n-octyl β-D-glucoside) was purchased from Dojindo (Kumamoto, Japan). Asolectin (L-α-lecithin, commercial grade type II-S), phosphatidylserine

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(DL-α-phosphatidyl-L-serine dipalmitoyl), phosphatidylcholine (D-α-phosphatidylcholine dipalmitoyl), cholesterol and poly(ethylene glycol) (PEG 8000) were from Sigma (St. Louis, MO, USA). All other chemicals were of reagent grade.

2.2. Isolation of brush-border membrane

The renal brush-border membrane was isolated from the renal cortex of the male Wistar rat by the Mg^{2+}/EGTA precipitation method of Biber et al. [10] with several modifications as in our previous paper [7]. The final pellet was resuspended in a buffer composed of 100 mM mannitol, 100 mM KCl, 10 mM L-alanyl-L-proline and 10 mM Hepes-Tris (pH 7.5). The protein concentration was adjusted to 20 mg/ml, and the suspension was stored at −60°C until use.

2.3. Solubilization and reconstitution

The procedure involved solubilization of the membrane with octyl glucoside in the presence of osmolytes and substrate, precipitation of the solubilized proteins with poly(ethylene glycol) (PEG 8000) and reconstitution into liposomal preparations. Essentially, similar procedures have been successfully applied for the solubilization and functional reconstitution of the placental serotonin and taurine transporters [11,12], and the hepatic system A-mediated amino acid transporter [13]. Membranes were solubilized with octyl glucoside (unless stated otherwise, the final concentration of octyl glucoside was 60 mM) at a protein concentration of 10 mg/ml in the presence of 10 mM L-alanyl-L-proline, 1 mM DTT (dithiothreitol), 0.1 mM PMSF (phenylmethylsulfonyl fluoride), 7% glycerol, 3 mM EDTA, 100 mM KCl and 20 mM Hepes-Tris (pH 7.5). The mixture was incubated for 30 min on ice with slow stirring. It was then centrifuged at $100\,000 \times g$ for 45 min. The solubilized proteins were recovered from the clear supernatant by mixing with one volume of 30% PEG-8000 solution (in the same buffer as used for solubilization but without detergent) to yield a final PEG concentration of 15%. This concentration was found from preliminary experiments with different PEG concentrations (3–30%) to be optimal for the recovery of the transport activity. The PEG-mixture was incubated with slow stirring on ice for 15 min and then centrifuged at $220\,000 \times g$ for 30 min. The resulting pellet was rinsed four times with the reconstitution buffer (100 mM mannitol, 100 mM KCl and 20 mM Hepes-Tris, pH 7.5) and was suspended in a minimal volume of the same buffer but with the addition of DTT and PMSF at a concentration of 1 and 0.1 mM, respectively.

Liposomes (large unilamelar vesicles) for the reconstitution were prepared with various lipid compositions by the reversed phase evaporation technique [14] with minor modifications as described previously [15]. A 50 mg lipid mixture was dissolved in a sufficient amount of chloroform/methanol, 3:1 and added to a 50 ml round-bottomed flask, and solvent was removed by rotary evaporation. The thin layer of lipids was redissolved in 4 ml of diethyl ether and 1 ml of the reconstitution buffer was added. The mixture was sonicated in a bath-type sonicator (UT-204, Sharp, Osaka, Japan), under nitrogen for 5 min at 4°C. The solvent was evaporated to reverse the phases of the emulsion. The resulting preparation was then mixed with an additional volume of the reconstitution buffer (2 ml). Following vortex mixing, the suspension was evaporated to remove any traces of the organic solvent.

Reconstitution was done by mixing the PEG-precipitated proteins with a desired volume of the liposomal suspension using a dounce-type glass homogenizer. The mixture was then frozen in liquid nitrogen and thawed at room temperature. The suspension was briefly sonicated (1 min) and diluted with 6–8 volumes of the reconstitution buffer. The proteoliposomes were harvested by centrifuging the suspension at $85\,000 \times g$ for 1 h. The resulted pellet of proteoliposomes was suspended in a minimal volume of the reconstitution buffer using dounce-type homogenizer. The suspension was once again sonicated for 1 min and then used for uptake measurements.

2.4. Uptake experiment

The uptake of ceftibuten into the prepared proteoliposomes were performed at 30°C by the rapid filtration method [16]. Unless specified other wise, 40 μl of proteoliposomes was diluted with 200 μl of the uptake buffer (100 mM mannitol, 100 mM KCl, 20 mM Mes-Tris, pH 5.5) and incubated at 30°C for the indicated times. The reaction was stopped with the addition of 4 ml of an ice-cold stop solution containing 150 mM NaCl and 20 mM Hepes-Tris, pH 7.5. The mixture was filtered immediately under vacuum through a Millipore filter (HAWP, 0.45 μm, 2.5 cm diameter). The filter was then washed once with 4 ml of the ice-cold stop buffer. Ceftibuten trapped on the filter was extracted with 300 μl of the stop buffer for HPLC analysis.

2.5. Intra-vesicular space determination

Due to the fact that ceftibuten uptake did not reach equilibrium after 2 h incubation, proteoliposomes were prepared and used for the uptake study as in the routine experiment, but with the addition of ceftibuten to the reconstitution buffer at a concentration which is equal to that in the incubation medium of the uptake experiment in order to simulate the values at equilibrium conditions. The apparent intra-vesicular space was calculated from the amount of ceftibuten trapped in the vesicles.

2.6. SDS-polyacrylamide gel electrophoresis

SDS/PAGE analysis was performed with an SPG-1500 Advantec vertical electrophoresis apparatus and the

Laemmli [17] buffer system. A discontinuous slab gel (1 × 140 × 140mm) containing 10% (w/v) acrylamide and 0.3% (w/v) *N,N'*-methylenebisacrylamide was used. Prior to SDS/PAGE the proteins from the different membrane preparations were precipitated by the method of Wessel and Flugge [18] as modified by Kramer et al. [19]. The dried protein precipitates were dissolved in 40–100 μl of a buffer containing 50 mM Tris-HCl (pH 8.5), 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol and 0.002% (w/v) bromophenol blue. The mixture was vortexed vigorously for 5 min at room temperature and then was incubated at 40°C for 30 min. After the separation of protein at 25 mA, the gel was stained with Coomassie brilliant blue. Molecular mass determinations were made by using a Sigma calibration Unit (SDS-6H, Sigma).

2.7. Analytical procedures

The detection of ceftibuten was carried out by the use of high-performance liquid chromatography as described previously [9,16,20]. Protein was measured by the method of Lowry et al. [21] with bovine serum albumin as the standard. Lipid phosphorus was measured by the ashing technique of Bartlett [22].

3. Results

3.1. Solubilization with octyl glucoside

Preliminary experiments with various detergents revealed that the results with octyl glucoside were most reproducible in terms of solubilizing capacity and transport activity. Fig. 1 shows the effect of varying the octyl glucoside concentration on the total protein solubilization and the uptake activity of ceftibuten into proteoliposomes prepared with asolectin. These results show that the total membrane protein solubilization improved as the octyl glucoside concentration was increased, until it reached a plateau at approx. 50 mM octyl glucoside. However, the uptake activity of ceftibuten decreased markedly when the detergent concentration reached 100 mM. For this reason, an octyl glucoside concentration of 60 mM was adopted as an optimal concentration for the subsequent experiments.

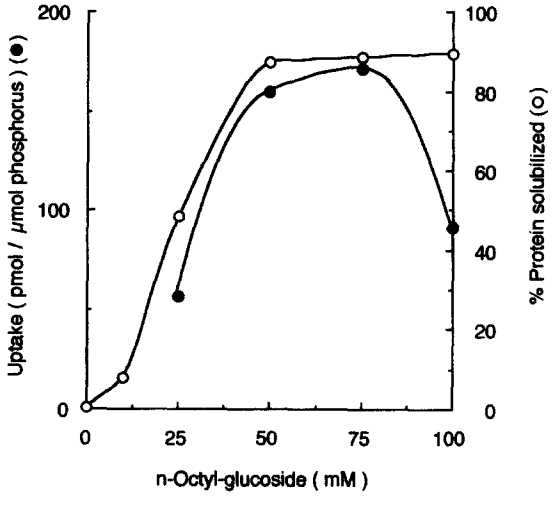

Fig. 1. Effect of detergent concentration on the solubilization and transport activity of the proteins extracted from the renal brush-border membrane. Ceftibuten (0.5 mM) steady-state (120 min) uptake activity was measured in the presence of an inward H^+ gradient (pH_{in} = 7.5, pH_{out} = 5.5) after reconstitution in asolectin vesicles. Each value is the mean with S.E.M. (n = 4 or 5). The error bars are smaller than the size of the symbols.

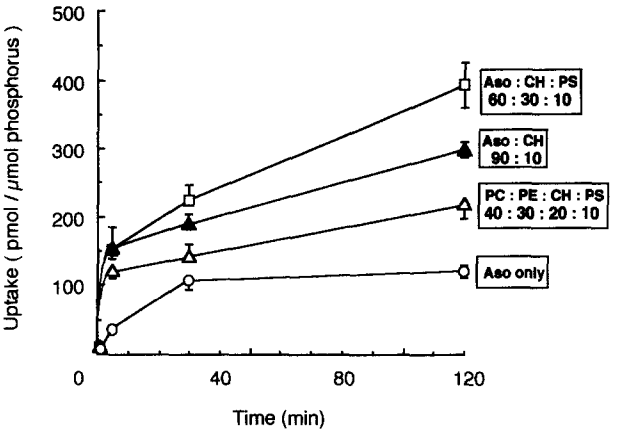

Fig. 2. Uptake of ceftibuten (0.5 mM) by proteoliposomes prepared from reconstituting the PEG-precipitated proteins with various lipid compositions. Uptake was measured in the presence of an inward H^+ gradient (pH_{in} = 7.5, pH_{out} = 5.5). Each value is the mean with S.E.M. (n = 4). The some error bars are smaller than the size of the symbols. Aso = asolectin, CH = cholesterol, PS = phosphatidylserine, and PE = phosphatidylethanolamine.

3.2. Effect of lipid composition on reconstitution of ceftibuten transport activity

Various lipid mixtures were tested for the reconstitution of ceftibuten transport activity (Fig. 2). The addition of

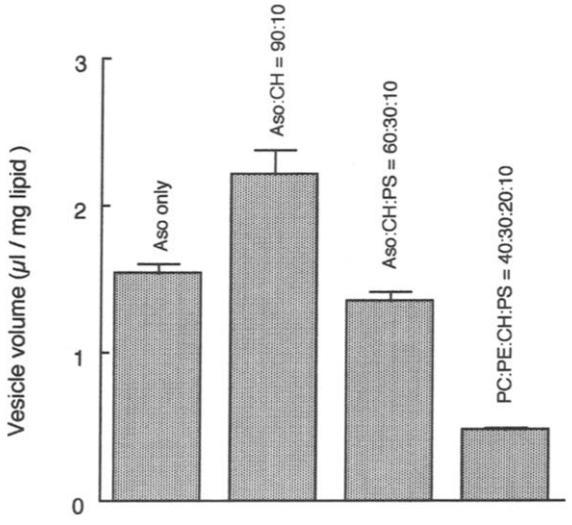

Fig. 3. Apparent intra-vesicular volume of proteoliposomes prepared from reconstituting the PEG-precipitated proteins with various lipid mixtures at a lipid-to-protein ratio of 10. Each value is the mean with S.E.M. (n = 5).

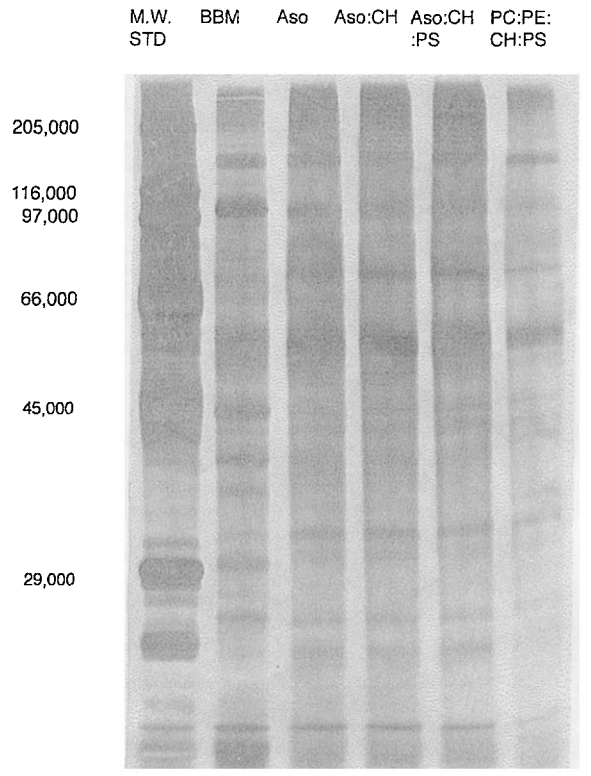

Fig. 4. SDS/PAGE analysis of proteoliposomes prepared from reconstituting the PEG-precipitated proteins with various lipid mixtures at a lipid-to-protein ratio of 10. BBM, brush-border membranes (10 μg); Aso, proteoliposomes with asolectin only (5 μg); Aso:CH, proteoliposomes with the mixture Aso/CH = 90:10 (5 μg); Aso:CH:PS, proteoliposomes with the mixture Aso/CH/PS = 60:30:10 (5 μg); PC:PE:CH:PS, proteoliposomes with the mixture PC/PE/CH/PS = 40:30:20:10 (5 μg).

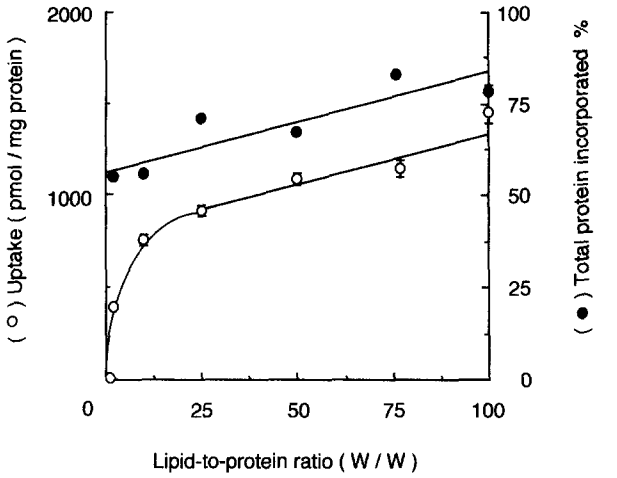

Fig. 5. Effect of lipid-to-protein ratio on the steady-state (120 min) uptake of ceftibuten (0.5 mM) by proteoliposomes prepared from reconstituting the PEG-precipitated proteins (fixed concentration) with a lipid mixture of Aso/CH/PS = 60:30:10 at different ratios. Uptake was measured in the presence of an inward H^+ gradient (pH_{in} = 7.5, pH_{out} = 5.5). Each value represents the mean with S.E.M. (n = 4). The some error bars are smaller than the size of the symbols.

cholesterol to the reconstitution mixture enhanced the initial and the maximal accumulation of ceftibuten, indicating an increase in the population of the transport competent vesicles and/or the activity of the carrier protein(s) (the number of turn over per time unit) in this liposomal preparation. However, optimal level of uptake was observed when proteoliposomes with a lipid mixture of 60% asolectin, 30% cholesterol and 10% phosphatidylserine (w/w) were used. Proteoliposomes of this composition had an initial uptake rate of 30 pmol ceftibuten/μmol phosphorus per min, with a 120 min-uptake of approx. 400 pmol ceftibuten/μmol phosphorus. These values are about 4- and 3-fold, respectively, greater than those obtained using proteoliposomes prepared from asolectin alone. As shown in Fig. 3, it is evident that the addition of choles-

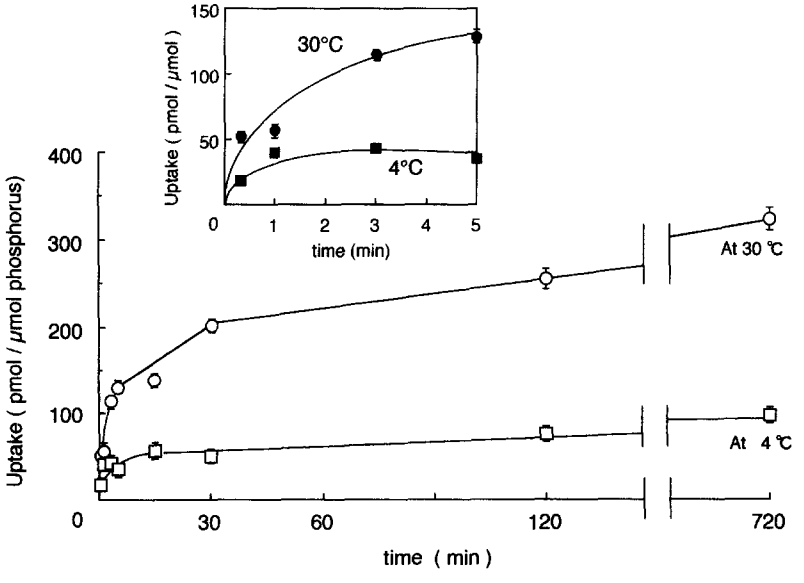

Fig. 6. Temperature dependency of the uptake of ceftibuten (0.5 mM) by proteoliposomes prepared from reconstituting the PEG-precipitated proteins in a lipid mixture of Aso/CH/PS = 60:30:10 at a lipid-to-protein ratio of 10. Uptake was measured in the presence of an inward H^+ gradient (pH_{in} = 7.5, pH_{out} = 5.5). Each value is the mean ± S.E.M. (n = 4). The inset represents the early time profiles of ceftibuten uptakes at both temperatures.

Table 1
Inhibitory effects of dipeptides, peptide-related compounds and FCCP on the uptake of ceftibuten (0.5 mM) by proteoliposomes prepared from reconstituting the PEG-precipitated proteins with a lipid mixture of Aso/CH/PS = 60:30:10 at a lipid-to-protein ratio of 10

Type of inhibitor	5-min uptake (pmol/mg protein)	120-min uptake (pmol/mg protein)
Control (without inhibitor)	675.80 ± 16.18	1386.39 ± 66.30
FCCP	563.79 ± 41.09 *	1087.05 ± 119.81 *
Compound V	207.48 ± 29.18 ***	701.73 ± 88.40 ***
L-Carnosine	371.69 ± 35.18 ***	1315.74 ± 50.71
Hippurylphenyllactic acid	522.65 ± 13.88 ***	872.28 ± 26.60 ***
L-Alanyl-L-alanine	564.03 ± 33.22 *	1092.96 ± 95.93 *
D-Alanyl-D-alanine	641.80 ± 54.71	1243.58 ± 28.16
D-Cephalexin	385.37 ± 35.65 ***	926.30 ± 41.04 ***
PAH	482.88 ± 29.37 ***	1426.59 ± 25.83
L-Valyl-L-proline	500.09 ± 19.47 ***	833.84 ± 11.46 *

FCCP was added at a concentration of 50 μM. Other inhibitors were added at a concentration of 10 mM. Data were calculated after correction for the non-specific uptake (uptake at 4°C). Each value is the mean ± S.E.M. ($n = 6$). * $P < 0.05$, *** $P < 0.001$: significantly different from control.

terol alone resulted in a significant increase in the intravesicular volume, indicating probably an enhancement in the vesicle fusion during the freeze-thaw step as reported by several authors [23,24]. However, when phosphatidylserine was added the intra-vesicular volume was brought down to the original value with asolectin alone. SDS-PAGE analysis revealed that the four liposomal preparations contained the same bands without any specific enrichment for certain proteins (Fig. 4). Therefore, it is likely that the enhancing effect of phosphatidylserine is due to a direct effect on the reconstituting capacity (the number of the carriers inserted per vesicle), and/or to an effect on the intrinsic activity of the carrier proteins (turnover number). We also tested another preparation of artificial lipid mixture (phosphatidylcholine/phosphatidylserine/phosphatidylethanolamine/cholesterol, 40:10:30:20) mentioned by the same author as the most effective combination for reconstituting nucleoside transporters. However, in the present study, this preparation failed to prove optimal for the reconstitution of ceftibuten transporter(s).

3.3. Effect of lipid-to-protein ratio on reconstitution of ceftibuten transport

Increasing the lipid-to-protein ratio from 2 to 100, while keeping the PEG-precipitated protein concentration constant, resulted in a parallel increase in the percentage of the total incorporated protein. This increase was accompanied with a non-linear increase in the 120 min-uptake of

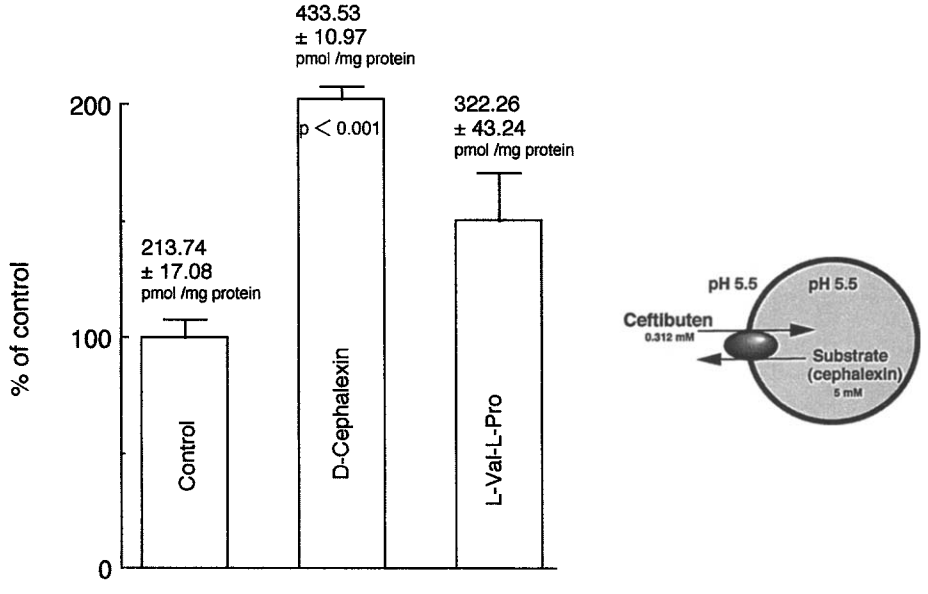

Fig. 7. Trans-stimulation effect of D-cephalexin and L-val-L-pro on the initial (5 min) uptake of ceftibuten (0.312 mM) by proteoliposomes prepared from reconstituting the PEG-precipitated proteins in a lipid mixture of Aso/CH/PS = 60:30:10 at a lipid-to-protein ratio of 10. Vesicles (20 μl) were preloaded with 5 μl of counter-substrate (25 mM) for 3 h at 30°C to give a final intra- and extra-vesicular concentration of 5 and 0.24 mM, respectively. Uptake was then initiated by incubating the preloaded vesicles with 500 μl of ceftibuten solution (0.312 mM) in the absence of H^+ gradient ($pH_{in} = pH_{out} = 5.5$). Each value is the mean with S.E.M. ($n = 5$). * $P < 0.001$, significantly different from control.

ceftibuten, reaching a plateau at a ratio of 12:1 or more (Fig. 5). A ratio of 10:1 was adopted for the subsequent routine experiments.

3.4. Functionality and substrate specificity of the established liposomal preparation

Following the establishment of the optimal conditions for solubilization and reconstitution of ceftibuten transporter(s), the functionality and substrate specificity of the carrier protein(s) were assessed. Firstly, the temperature dependence of ceftibuten uptake by the optimized preparation was examined. As shown in Fig. 6, the uptake at 4°C was significantly lower than that at 30°C, indicating an involvement of a transport process rather than a binding phenomenon. Secondly, the inhibitory effects of dipeptides, cephem antibiotics, FCCP, and PAH were examined in the presence of an inwardly directed proton gradient. As shown in Table 1, the proteoliposomal preparation of this study was characterized with an inhibition spectrum which is similar to that for the native renal brush-border membrane vesicles [20]. Furthermore, abolishing the inwardly directed proton gradient by FCCP, a protonophore, significantly inhibited the steady-state (but not the initial) uptake, indicating that the proton-mediated transport process into the proteoliposomes is very slow compared with the original membrane vesicles. Finally, to further confirm the viability of the carrier protein(s) responsible for ceftibuten transport, trans-stimulation study was made. In the presence of D-cephalexin and L-valyl-L-proline, uptake was increased 200% and 150%, respectively (Fig. 7). This result extends the evidence of the functionality of the reconstituted carrier protein(s) in this preparation.

4. Discussion

The reconstitution protocol described here is both simple and reliable. It permits the rapid reconstitution of the transport activity of ceftibuten with characteristics similar to those described in the original brush-border membrane vesicles.

4.1. Effect of solubilization conditions

The proper concentration of octyl glucoside used in this study affords the solubilization of many of the transport properties characteristic of the native membrane transporter(s). In addition, the presence of osmolytes (glycerol and mannitol) and L-alanyl-L-proline (a substrate for the peptide transporters) permits good protection for the proteins of interest. The addition of osmolytes and substrate was stemmed from the result of many trials and errors made during the search for the optimal conditions of solubilization (data not shown). There also are many examples in the literature of the protective effects of osmolytes and substrates on the carrier proteins [25,26].

4.2. Effect of lipid composition

The present study assessed the impact of lipids such as cholesterol and phosphatidylserine on the functional characteristics of the reconstituted transporter(s). Supplementing the crude asolectin mixture with cholesterol and phosphatidylserine led to greater uptake values.

As described in the result Section 3.2, the presence of cholesterol and phosphatidylserine in asolectin preparations did not lead to a dramatic alteration in the intra-vesicular space, indicating an involvement of factors other than vesicle volume for enhancing the transport activity. It is worth noting that similar lipid mixture has been previously mentioned by Hammond [27] to be convenient for the reconstitution of the nucleoside transporters in Ehrlich cell plasma membrane.

4.3. Effect of lipid-to-protein ratio

The transport activity of ceftibuten was dependent upon the amount of lipid added and increased with additional lipid to a point where all the transport competent protein(s) were inserted into the liposomes. This non-linear fashion of transport increase did not keep pace with the incorporation of the total proteins, where an almost linear correlation with lipid amount was observed, indicating that the transport competent protein(s) tend to associate with the liposomal membrane in a more specific fashion than other proteins present in the bulk solution.

4.4. Viability of the reconstituted carrier protein(s)

Proton-dependency, electrogenicity, and inhibitory effect of the derivatives on the transport observed in the native brush-border membrane vesicles were in agreement with the results obtained from reconstituted preparation of the present study. However, the uptake of ceftibuten in this study was characterized with a slower uptake rate. The slower uptake rate is likely to be due to the rigidity and impermeability of the liposomal membrane. Similar observations have been also reported for the phosphate and glucose transporters after solubilization and reconstitution from the renal brush-border membrane [28,29]. It remains unclear which peptide transporter PepT1 [30,31] or PepT2 [31] has been reconstituted in this study. However, it is probable that both of transporters were reconstituted in the proteoliposomes although this will require further investigation.

4.5. Conclusion

The present study demonstrates an efficient method for the functional solubilization and reconstitution of the carrier system(s) responsible for the transport of ceftibuten and other peptide-related compounds in the renal brush-border membrane. The simple and reliable solubilization

and reconstitution procedures presented here would therefore prove useful in future studies aiming at the purification and identification of the peptide carriers, a salient goal for understanding the transport mechanism of peptides at the molecular level.

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