Purification by p-aminobenzoic acid (PABA)-affinity chromatography and the functional reconstitution of the nateglinide/H^+ cotransport system in the rat intestinal brush-border membrane

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ABSTRACT

(-)-N-(trans-4-isopropylcyclohexanecarbonyl)-D-phenylalanine (nateglinide) is a novel oral hypoglycemic agent possessing a peptide-type bond and a carboxyl group in its structure. Although nateglinide quickly reaches the maximal serum concentration after oral administration, nateglinide itself is not transported by Pept1 or MCT1. Recently, we have shown that nateglinide transport occurs via the ceftibuten/H^+ cotransport system, which is distinct from PepT1, and that the fluorescein/H^+ cotransport system is involved in the uptake of nateglinide. The aim of this study was to characterize the functional properties of the intestinal nateglinide transporter. In the first part of this study, we demonstrated that the ceftibuten/H^+ cotransport system is identical to the fluorescein/H^+ cotransport system. We succeeded in purification of the nateglinide transporter from brush-border membranes of the rat small intestine using p-aminobenzoic acid (PABA)-affinity chromatography. We then investigated the functional properties of the nateglinide transporter using proteoliposomes prepared from the PABA-affinity chromatography elute. We demonstrated that nateglinide, ceftibuten and fluorescein are transported by the same transporter in the intestine.

(-)-N-(trans-4-isopropylcyclohexanecarbonyl)-D-phenylalanine (nateglinide) is administered to patients with type 2 diabetes (noninsulin-dependent diabetes mellitus). Although nateglinide is a non-sulfonylurea anti-diabetic agent, it stimulates insulin secretion via the same mechanism as that by which insulin secretion is stimulated by sulfonylureas. It is known that sulfonylurea therapy has several disadvantages, such as excess hypoglycemia between meals, due to the long duration of action of these agents. Nateglinide, on the other hand, can compensate for impaired insulin secretion to prevent postprandial hyperglycemia without causing prolonged hypoglycemia because it quickly reaches the maximal serum concentration and is eliminated quite rapidly after oral administration [1-3]. These characteristics of nateglinide are expected to be useful in the treatment of type 2 diabetes.

The unique feature of nateglinide is its rapid intestinal absorption. However, physicochemical characteristics of nateglinide are incompatible with rapid absorption by passive diffusion. Thus, nateglinide is thought to be absorbed via a specific transport system(s) in the intestine. Peptide transporter 1 (PEPT1/*SLC15A1*) has been shown to mediate the efficient absorption of a wide variety of oral peptide-like drugs in the small intestine [4,5]. In addition to PEPT1, at least five isoforms of MCT (MCT1, MCT2, MCT4, MCT5 and MCT8) are present in the small intestine, but only MCT1-MCT4 have been characterized in terms of their substrates and inhibitor kinetics [6,7]. Nateglinide possesses a peptide-type bond and a carboxyl group in its structure, though it is not transported by Pept1 or MCT1 [8,9]. Recently, we have

shown that nateglinide transport occurs via the ceftibuten/H^+ cotransport system, which is distinct from PepT1, and that the fluorescein/H^+ cotransport system is involved in the uptake of nateglinide [10-12]. Since kinetic analysis of nateglinide uptake by an Eadie-Hofstee plot gave a single straight line, it is possible that the ceftibuten/H^+ cotransport system is identical to the fluorescein/H^+ cotransport system. However, these transport systems have not yet been elucidated at the molecular level. It is thought that the physical isolation and the elucidation of functional properties of transport proteins are indispensable for further understanding of the transport mechanisms.

The present study was carried out to characterize the functional properties of the intestinal nateglinide transporter. We succeeded in purification of the nateglinide transporter from brush-border membranes of the rat small intestine using affinity chromatography. We then investigated the functional properties of the nateglinide transporter using prepared proteoliposomes.

Materials and methods

Chemicals. Nateglinide and ceftibuten were kindly donated by Yamanouchi (Tokyo, Japan) and Sankyo (Tokyo, Japan), respectively. Fluorescein was purchased from Nacalai Tesque (Kyoto, Japan). Salicylic acid was purchased from Wako Pure Chemical (Osaka, Japan). All other reagents were of the highest grade available and used without further purification.

Animals. Male Wistar rats, aged 6 to 7 weeks (300-350 g in weight), were obtained from NRC Haruna (Gunma, Japan). The housing conditions were described previously [13]. The experimental protocols were reviewed and approved by the Hokkaido University Animal Care Committee in accordance with the "Guide for the Care and Use of Laboratory Animals".

Preparation of brush-border membrane vesicles. Brush-border membrane vesicles were prepared from the rat intestine by the calcium precipitation method with some modification as described previously [14]. All steps were performed on ice or at 4°C. The intestine was washed with ice-cold saline and cut longitudinally. The mucosa was scraped gently with a glass microscope slide. The scrapings (4 g wet weight) were homogenized in 80 ml of ice-cold solution A (50 mM D-mannitol, 2 mM Tris / HCl, pH 7.1) with a Waring blender at 16,500 rpm for 4 min. $CaCl_2$ solution (0.5 M) was added to a final concentration of 10 mM, and the homogenate was

allowed to stand for 15 min. The homogenate was centrifuged at 6,000 x g for 15 min, and the supernatant was recentrifuged at 27,500 x g for 30 min. The resulting pellet was resuspended in 20 ml of solution B (50 mM D-mannitol, 10 mM HEPES/Tris, pH 7.5) and homogenized in a glass/Teflon Dounce-type homogenizer with 10 strokes. After a final centrifugation at 27,500 x g for 30 min, the brush-border membranes were suspended in a buffer containing 100 mM D-mannitol, 100 mM KCl and 20 mM HEPES/Tris (pH 7.5) or 100 mM D-mannitol, 100 mM KCl and 20 mM MES/Tris (pH 5.5). The level of alkaline phosphatase (a marker enzyme of the brush-border membrane) activity of the brush-border membrane was more than 12-fold higher than that of the initial homogenate. In contrast, the level of Na^+-K^+ ATPase (a marker enzyme of the basolateral membrane) activity of the brush-border membrane was the same as that of the initial homogenate. This means that brush-border membranes were enriched at least 12 fold with respect to basolateral membranes.

Study of uptake by brush-border membrane vesicles. The uptake of substrates into brush-border membrane vesicles was determined by the rapid filtration technique described previously [15,16]. In a routine assay, 40 µl of membrane vesicles (0.4-0.6 mg protein) suspension was added to 200 µl of substrate mixture kept at 25°C. The compositions of the media are described in the figure legends. At the end of the incubation period, the uptake was stopped by diluting the incubation medium with 5 ml of ice-cold 10 mM MES buffer (pH 5.5) containing 150 mM KCl. The mixture was

immediately filtered through a Millipore filter (0.45 µm in pore size, 2.5 cm in diameter; HAWP). The filter was rinsed with 5 ml of the same buffer. Substrate trapped on the filter was extracted with 300 µl of water, and the concentration of substrate was determined.

Solubilization of brush-border membrane vesicles. Brush-border membrane vesicles were suspended at a protein concentration of 8-10 mg/ml in a solution of 20 mM NaHEPES (pH 7.4) and 1 mM EDTA and were stored in liquid nitrogen until required. Solubilization of the brush-border membrane was performed by mixing the brush-border membrane vesicles 1:1 (v/v) with a solution of 1% Triton X-100 and 20 mM Tris/HCl (pH 7.4). After 25 min of incubation on ice, unextracted material was pelleted by ultracentrifugation at 150,000 x g for 25 min at 4°C. The upper layer was applied to hydroxyapatite column.

Application onto the hydroxyapatite column. Separation of Triton X-100 solubilized material by using a hydroxyapatite column (ten ml of syringe containing 2 g of dry hydroxyapatite, Bio-Gel® HTP, Bio-Rad., packed by 15 s of tapping). All steps were performed at 4°C. Each column was equilibrated with buffer A (0.1% Triton X-100, 20 mM Tris/HCl, pH 7.4). Two ml of solubilized material was applied to the column. Elution was carried out with 12 ml of phosphate buffer (25-250 mM Na_2HPO_4, NaH_2PO_4, pH 7.4 in buffer A). After pooling the eluates, the eluates were

concentrated until 200 µl. Protein concentration in the eluate was measured by the method of Lowry et al. with bovine serum albumin as a standard in the presence of 4% SDS [17]. The aliquot was used for liposomal reconstitution and further purification.

Protein characterization and determination. The purity of various active fractions was determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Prior to SDS-PAGE, protein was precipitated by the method of Wessel and Flugge [18]. The dried protein precipitates were dissolved in 10 µl of a loading buffer containing 100 mM Tris/HCl (pH 6.8), 4% SDS, 12% 2-mercaptethanol, 20% glycerol and 0.002% bromophenol blue. The mixture was denatured at 100°C for 2 min and separated on 7.5% SDS-polyacrylamide gels. The bands were visualized with Coomassie Brilliant Blue or silver nitrate staining.

Reconstitution into proteoliposomes. Reconstitution was done in a final volume of 640 µl containing 100 µl of protein fraction from the chromatography eluate, 30 µl of Triton X-100, 50 µl of the sonicated liposomes, 160 µl of 24 mM NaHEPES (pH 7.4) containing 119 mM KCl and 300 µl of Amberlite XAD®-2 beads (Fluka) in 20 mM NaHEPES, pH 7.4, containing 100 mM KCl. After 30 min of rotation at 4°C, beads were removed by short centrifugation and proteoliposomes were used for the uptake experiment.

Study of uptake by proteoliposomes. The uptake of nateglinide into the prepared proteoliposomes was performed at 20°C by the rapid filtration technique. Unless otherwise specified, 40 µl of proteoliposomes was diluted with 200 µl of the uptake buffer (100 mM D-mannitol, 100 mM KCl, 20 mM MES/Tris, pH 5.5 or 100 mM D-mannitol, 100 mM KCl, 20 mM HEPES/Tris, pH 7.4) containing 60 µM nateglinide. At the end of the incubation period, the uptake was stopped by diluting the incubation medium with 4 ml of ice-cold 20 mM HEPES buffer (pH 7.4) containing 150 mM NaCl. The contents of the tube were immediately filtered through a Millipore filter. The filter was rinsed with 5 ml of the same buffer. Substrate trapped on the filter was extracted with 300 µl of 20 mM HEPES buffer (pH 7.4) containing 150 mM NaCl, and the concentration of substrate was determined. For *trans*-stimulation studies, 40 µl of proteoliposomes was pre-incubated for 60 min at 20°C with 10 µl of an uptake buffer in the presence or absence of compounds. The transport experiment was started by mixing with 150 µl of an uptake buffer containing 500 µM nateglinide.

Immobilization of PABA to an NHS-activated column. An N-hydroxysuccinamide (NHS)-activated sepharose column (HiTrap™ NHS-activated HP, 5 ml, Amersham Biosciences) was used in this study. p-Aminobenzoic acid (PABA), the selected ligand, was coupled to the activated gel according to the

instructions of the supplier. Briefly, after washing the activated gel with six column volumes of 1 mM HCl, 5 ml of a 50 mM PABA solution in a column buffer (0.2 M $NaHCO_3$, 0.5 M NaCl, pH 8.3) was injected into the column. The reaction was run for 2 hours at room temperature. Then the remaining uncoupled NHS residues were inactivated by incubating with a blocking buffer (0.5 M monoethanolamine, 0.5 M NaCl, pH 8.3) for 1 hour at room temperature. Finally, the column was washed three times with three column volumes of wash buffer A (0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3) and wash buffer B (0.1 M acetic acid, 0.5 M NaCl, pH 4.0) alternatively. The column was then washed with a phosphate/NaN3 buffer (pH 7.0) and stored at 4°C until use. The coupling ratio was found to be approx. 96% as estimated from the amount of PABA remaining in the washing solutions after HPLC analysis.

Application onto a PABA-affinity column. All steps were performed at 4°C. The 250 mM phosphate-eluted fraction from the hydroxyapatite column was applied to a PABA column equilibrated with buffer B (0.1% Triton X-100, 10% glycerol, 20 mM Tris/HCl, pH 7.4). After washing the PABA-affinity column with four column volumes of buffer B, the bound proteins were eluted from the affinity column by 50-1000 µM NaCl in buffer B. After pooling the eluates, the eluates were concentrated until 200 µl and desalted. Desalting was performed on a 2-ml Sephadex G50 medium (Amersham Pharmacia Biotech) column equilibrated in buffer A.

Analytical procedures. Nateglinide and ceftibuten were determined using an HPLC system equipped with a JASCO 880-PU pump 870-UV UV/VIS detector. The column was a Hitachi ODS Gel #3053 (4 mm i.d. x 250 mm). In the assay for nateglinide, a mobile phase containing 50 mM H_3PO_4 (pH 2.5) : acetonitrile (55:45, v/v) was used. Column temperature and flow rate were 55°C and 0.7 ml/min, respectively. The wavelength for detection of nateglinide was 210 nm. In the assay for ceftibuten, a mobile phase containing acetonitrile/0.05M citric acid buffer with pH adjusted to 2.5 by NaOH (1:9) was used. Column temperature and flow rate were 25°C and 0.7 ml/min, respectively. The wavelength for detection of ceftibuten was 262 nm. In the assay for PABA, a mobile phase containing 50 mM NaH_2PO_4, 0.5 mM tetrabutylammonium hydrogensulfate : methanol (82:18, v/v) was used. Column temperature and flow rate were 55°C and 0.5 ml/min, respectively. The wavelength for detection of PABA was 254 nm. Peptide bands were subjected to Edman degradation procedures, and the first ten amino-terminal amino acids were elucidated. Student's t-test was used for statistical analysis, and a value of $P < 0.05$ was considered significant. Nonlinear regression analysis and least-squares fitting for the Eadie-Hofstee plot of substrate uptake were performed by using Origin® (version 6.1J).

Results

Ceftibuten/H^+ and fluorescein/H^+ cotransporters

In order to clarify whether the nateglinide/ceftibuten cotransport system is identical to the nateglinide/fluorescein cotransport system, the inhibitory effects of ceftibuten and fluorescein on the uptake of nateglinide were investigated. As shown in Fig. 1, ceftibuten significantly reduced the uptake of nateglinide. This inhibitory effect was saturable at a concentration of more than 5 mM. Moreover, the inhibitory effect of 0.5 mM fluorescein and that of 10 mM ceftibuten were almost the same. Furthermore, the effect of the combined treatment was not greater than that of ceftibuten or fluorescein alone. We then investigated the inhibitory effect of fluorescein on initial uptake of ceftibuten into brush-border membrane vesicles in the presence of an inwardly directed H^+ gradient. As shown in Fig. 2, fluorescein significantly reduced the uptake of ceftibuten. Results of Lineweaver-Burk plot analysis of ceftibuten uptake in the presence of fluorescein and an inwardly directed H^+ gradient are shown in Fig. 3. Fluorescein was demonstrated to inhibit the uptake of ceftibuten competitively.

Hydroxyapatite chromatography of intestinal brush-border membrane proteins

The first purification step consisted of chromatography on hydroxyapatite. Fig.4 shows the nateglinide transport activity of the proteoliposomes reconstituted from phosphate-eluted fractions. The proteoliposomes reconstituted from the 250 mM

phosphate-eluted fraction showed a high uptake activity. The 250 mM phosphate-eluted fraction was used for inhibition studies and further purification. Since we have reported that benzoic acid and salicylic acid significantly inhibited the uptake of nateglinide by Caco-2 cells [19], *cis*-inhibition studies were performed using benzoic acid and salicylic acid. As shown in Table 1, both of them significantly reduced the uptake of nateglinide. Moreover, ceftibuten and fluorescein significantly inhibited the nateglinide uptake. However, analysis of each fraction by SDS-PAGE revealed that many different protein bands were still present (data not shown).

Dixon plot analysis of nateglinide uptake in the presence of benzoic acid

The next purification step consisted of affinity chromatography. In order to determine the immobilized substrate, we investigated the inhibitory effect of benzoic acid on the uptake of nateglinide by rat intestinal brush-border membrane vesicles. Results of Dixon plot analysis of nateglinide uptake in the presence of benzoic acid and an inwardly directed H^+ gradient are shown in Fig. 5. Benzoic acid was demonstrated to inhibit the uptake of nateglinide competitively. The regression line obtained from the replot of slopes of the Dixon plot almost coincided with the origin (Fig. 5, inset), indicating that benzoic acid transport is mediated by a common H^+/cotransport system with nateglinide. The apparent Ki value calculated from Dixon plots for benzoic acid was 3.70 mM.

PABA-immobilized affinity chromatography of the 250 mM phosphate-eluted fraction

Since benzoic acid and nateglinide are thought to share the same transport system at intestinal brush-border membranes, we selected p-aminobenzoic acid (PABA), a derivative of benzoic acid, as a model substrate. Fig. 6A shows the nateglinide transport activity of the proteoliposomes reconstituted from NaCl-eluted fractions. The proteoliposomes reconstituted from the 500 mM NaCl-eluted fraction showed a high level of uptake activity. Analysis of each fraction by SDS-PAGE revealed the presence of proteins with apparent molecular masses of 66, 57 and 34 kDa (Fig. 6B). There was a correlation between the 57-kDa protein and the transport activity. The first ten amino-terminal amino acids of the 57-kDa protein were elucidated. The amino terminal sequence of the 57-kDa protein was determined to be GGLTVSDMEG. The identified amino acid sequence was not identical to those of previously reported transport proteins. However, some transporters had similar homology to nateglinide/H^+ cotransporter. The other major 66-kDa and 34-kDa proteins represented sucrase-isomaltase and rat maleic acid dehydrogenase, respectively.

Properties of the purified nateglinide/H^+ cotransporter

Since the final yield of the purified nateglinide/H^+ cotransporter was very small, detailed kinetic studies were difficult to perform. *cis*-Inhibition and *trans*-stimulation studies were performed using salicylic acid. Salicylic acid

significantly reduced the uptake of nateglinide (Fig. 7A). Furthermore, salicylic acid was able to induce an almost 2-fold *trans*-stimulation (Fig. 7B). To determine whether nateglinide is taken up through the ceftibuten/fluorescein transporter, *trans*-stimulation experiments were performed using proteoliposomes preloaded with ceftibuten and fluorescein. As shown in Fig. 8, by preloading with ceftibuten and fluorescein, the uptake of nateglinide was significantly enhanced.

Discussion

Oral delivery is generally the most desirable means of drug administration, mainly because of patient acceptance, convenience in administration, and cost-effective manufacturing. However, there are several problems for the development of oral delivery systems for drugs [20]. One of the major problems is the poor permeability through the intestinal mucosa. The use of transporter function offers the possibility of delivering a drug to the target organ, avoiding distribution to other organs (thereby reducing the risk of toxic effects), controlling the elimination process, and improving oral bioavailability [21,22]. Intestinal PEPT1 has been utilized to improve the intestinal absorption of poorly absorbed and pharmacologically active agents by chemically converting them to substrates for PEPT1 [5,23]. In addition to PEPT1, intestinal MCT isoforms are possible targets for drug absorption [6,7].

Since nateglinide is absorbed rapidly form the intestine, it is likely to be absorbed via a specific transporter(s) [2,3]. However, nateglinide is not transported by PEPT1 or MCT1 [8,9]. We have recently shown that nateglinide is absorbed in the intestinal brush-border membrane via the ceftibuten/H^+ cotransport system, which is distinct from PepT1, and that the fluorescein/H^+ cotransport system is involved in the uptake of nateglinide [12]. In the present study, we purified intestinal nateglinide transporter and characterized the functional properties of this transporter.

In the first part of this study, we investigated whether the ceftibuten/H^+

cotransport system is identical to the fluorescein/H^+ cotransport system [10,11]. The inhibitory effect of the combined treatment on the uptake of nateglinide into rat brush-border membrane vesicles was not greater than that of ceftibuten or fluorescein alone. Moreover, fluorescein inhibited the uptake of ceftibuten competitively. The results suggest that the ceftibuten/H^+ cotransport system is identical to the fluorescein/H^+ cotransport system and that nateglinide is transported by this transport system.

Various membrane transport proteins have been purified using hydroxyapatite in the presence of detergents. Based on this purification method for membrane proteins, we developed a purification protocol for the new nateglinide transporter. By using hydroxyapatite chromatography, we succeeded in obtaining fractions enriched in transport activity. However, many different proteins were still present in the phosphate-eluted fraction of hydroxyapatite chromatography. Further purification was needed.

The next purification step consisted of affinity chromatography. Since the results from Dixon plot analysis suggest that benzoic acid and nateglinide share the same transport system at intestinal brush-border membranes, we selected PABA, a derivative of benzoic acid, as a model substrate. By using PABA-immobilized affinity chromatography, we succeeded in obtaining fractions enriched in transport activity. SDS-PAGE analysis showed only one protein band with a molecular mass of approximately 57 kDa, correlating with the transport activity of nateglinide.

Moreover, it is unlikely that the 66-kDa protein, sucrase-isomaltase, and 34-kDa protein, rat maleic acid dehydrogenase, are involved in the transport of nateglinide. We then investigated the functional properties of the nateglinide transporter using proteoliposomes prepared from the PABA-affinity chromatography elute. Salicylic acid was found to have not only an inhibitory effect on the transport of nateglinide but also a *trans*-stimulation effect. These results indicate that we can evaluate the *trans*-stimulation effect using prepared proteoliposomes. It was found that both ceftibuten and fluorescein are able to induce *trans*-stimulation. These findings indicate that nateglinide, ceftibuten and fluorescein are transported by the same transporter in the intestine.

The absorption of drugs from the gastrointestinal tract is one of the important determinants for oral bioavailability. It is now widely recognized that drug transporters contribute to the absorption of administered drugs from the intestine and that drug targeting is an effective approach to increase the pharmaceutical activity of drugs. Utilization of PEPT1 has been considered to be a promising strategy for oral drug delivery [5,23]. However, drug-drug interactions involving PEPT1 are likely to occur due to the broad substrate specificity of PEPT1. Transporter-mediated drug-drug interactions involving drugs that have a narrow therapeutic range may have serious adverse consequences. Substrate specificity of the nateglinide transport system is more similar to that of MCTs than that of Pept1 [12]. By utilizing the nateglinide transport system as the target for oral drug delivery, it will be possible to

avoid drug-drug interactions involving PEPT1. In addition to PEPT1, intestinal MCTs may represent a novel target for improving oral bioavailability [7]. Therefore, elucidation of the molecular structure, function and regulation of MCTs is important in drug delivery and design. In this study, we have shown that a dianionic compound, ceftibuten, inhibits the transport of monocarboxylic compounds, nateglinide and fluorescein. The results of this study provide important information for further studies aimed at elucidation of the substrate recognition mechanism of MCTs.

In summary, we have demonstrated that the ceftibuten/H^+ cotransport system is identical to the fluorescein/H^+ cotransport system and that nateglinide, ceftibuten and fluorescein are transported by the same transporter in the intestine.

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Legends for figures

Fig. 1. Inhibitory effects of ceftibuten and fluorescein on the uptake of nateglinide into rat intestinal brush-border membrane vesicles. Uptake of 100 μM nateglinide into brush-border membrane vesicles was measured for 30 s with or without (control) inhibitors. Membrane vesicles were suspended in 100 mM D-mannitol, 100 mM KCl and 20 mM MES/Tris (pH 5.5) or 20 mM HEPES/Tris (pH 7.5). The substrate mixture contained 100 mM D-mannitol, 120 μM nateglinide, 100 mM KCl and 20 mM MES/Tris (pH 5.5) in the presence of various compounds. Uptake values in the absence of an inwardly directed H^+ gradient were subtracted from those in the presence of an inwardly directed H^+ gradient. Each column represents the mean with S.D. of 3 determinations. The control value for the uptake of nateglinide was 895 ± 153 pmol/mg protein/30 s. **P < .01, significantly different from the control.

Fig. 2. Inhibitory effect of fluorescein on the uptake of ceftibuten into rat intestinal brush-border membrane vesicles. Incubation conditions were identical to those described in the legend to Fig. 1. Uptake of ceftibuten was measured for 20 s. Each column represents the mean with S.D. of 3 determinations. The control value for the uptake of ceftibuten was 432 ± 50.3 pmol/mg protein/20 s. **P < .01, significantly different from the control.

Fig. 3. Lineweaver-Burk plot of H^+-driven ceftibuten uptake into rat intestinal

brush-border membrane vesicles. Incubation conditions were identical to those described in the legend to Fig. 2. Uptake of ceftibuten was measured in the presence or absence of 1 mM fluorescein. Each point represents the mean with S.D. of 3 determinations.

Fig. 4. Transport activity of nateglinide (50 μM) by proteoliposomes reconstituted from 25-250 mM phosphate-eluted fractions of hydroxyapatite chromatography. Uptake of nateglinide was measured for 3 min at 20°C. Uptake values in the absence of an inwardly directed H^+ gradient were subtracted from those in the presence of an inwardly directed H^+ gradient. Each column represents the mean with S.D. of 3 determinations.

Fig. 5. Dixon plot of the nateglinide uptake into rat intestinal brush-border membrane vesicles in the presence of benzoic acid. Uptake of 25 μM, 50 μM and 100 μM nateglinide was measured for 20 s with benzoic acid. Incubation conditions were identical to those described in the legend to Fig. 1. Each point represents the mean with S.D. of 3 determinations. Inset, replot of the slopes of the Dixon plot. The apparent Ki value was determined to be 3.70 mM by linear regression analysis from the Dixon plot.

Fig. 6. A 57-kDa protein correlates with the transport activity of nateglinide by

proteoliposomes reconstituted from the PABA-affinity chromatography elutes. (A) Transport activity of nateglinide (50 μM) by proteoliposomes reconstituted from the NaCl-eluted fractions of PABA-affinity chromatography. Incubation conditions were identical to those described in the legend to Fig. 4. (B) SDS-PAGE analysis of the NaCl-eluted fractions of PABA-affinity chromatography.

Fig. 7. *cis*-Inhibition and *trans*-stimulation of nateglinide uptake by salicylic acid. (A) Transport activity of nateglinide (50 μM) by proteoliposomes reconstituted from the 500 mM NaCl-eluted fraction of PABA-affinity chromatography was measured with or without (control) inhibitors. Incubation conditions were identical to those described in the legend to Fig. 4. (B) Proteoliposomes reconstituted from the 500 mM NaCl-eluted fraction of PABA-affinity chromatography were preincubated for 60 min with or without (control) salicylic acid. The transport experiment was started by 3-fold dilution in incubation buffer with nateglinide and allowed to proceed for 3 min. Each column represents the mean with S.D. of 3 determinations. *P< .05, **P < .01, significantly different from the control.

Fig. 8. *trans*-Stimulation of nateglinide uptake by ceftibuten and fluorescein. Incubation conditions were identical to those described in the legend to Fig. 7B. Each column represents the mean with S.D. of 3 determinations. *P< .05, **P < .01, significantly different from the control.

Table 1. Effects of various compounds on nateglinide uptake by proteoliposomes reconstituted from hydroxyapatite elute.

Compound	Concentration (mM)	Nateglinide uptake (% of control)
Control		100 ± 13.2
Benzoic acid	20	55.8 ± 12.5**
Salicylic acid	20	27.5 ± 9.15**
Ceftibuten	10	35.1 ± 20.8**
Fluorescein	1	34.7 ± 22.7**

The uptake of nateglinide (50 μM) by proteoliposomes was determined in the presence or absence of inhibitors. Incubation conditions were identical to those described in the legend to Fig. 4. Each value represents the mean with S.D. of 3 determinations. **P < .01, significantly different from the control.