British Journal of Pharmacology (2002) 137, 391–399

www.nature.com/bjp

Transport and uptake of nateglinide in Caco-2 cells and its inhibitory effect on human monocarboxylate transporter MCT1

[1]Atsuko Okamura, [1]Akiko Emoto, [1]Noriko Koyabu, [1]Hisakazu Ohtani & *,[1]Yasufumi Sawada

[1]Department of Medico-Pharmaceutical Sciences, Graduate School of Pharmaceutical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan

1 Nateglinide, a novel oral hypoglycemic agent, rapidly reaches the maximum serum concentration after oral administration, suggesting that it is rapidly absorbed in the gastrointestinal tract. The aim of this work is to clarify the intestinal absorption mechanism of nateglinide by means of *in vitro* studies.

2 We examined the transcellular transport and the apical uptake of [^{14}C]nateglinide in a human colon carcinoma cell line (Caco-2). We also examined whether nateglinide is transported *via* monocarboxylate transport-1 (MCT1) by means of an uptake study using MCT1-expressing *Xenopus laevis* oocytes.

3 In Caco-2 cells, the transcellular transport of [^{14}C]nateglinide from the apical to basolateral side was greater than that in the opposite direction. The uptake of [^{14}C]nateglinide from the apical side was concentration–dependent, H^+-dependent, and Na^+-independent. Kinetic analysis revealed that the Kt and Jmax values of the initial uptake rate of [^{14}C]nateglinide were 448 μM and 43.2 nmol mg protein^{-1} 5 min^{-1}, respectively. Various monocarboxylates, including salicylic acid and valproic acid, and glibenclamide significantly inhibited the uptake of [^{14}C]nateglinide.

4 The uptake study using MCT1-expressing oocytes showed that nateglinide inhibits the MCT1-mediated uptake of [^{14}C]L-lactic acid, though nateglinide itself is not transported by MCT1.

5 Taken together, these results suggest that the uptake of nateglinide from the apical membranes of Caco-2 cells is, at least in part, mediated by a proton-dependent transport system(s) distinct from MCT1.

British Journal of Pharmacology (2002) **137,** 391–399. doi:10.1038/sj.bjp.0704875

Keywords: Monocarboxylate transporter; nateglinide; intestinal absorption

Abbreviations: CHC, alpha-cyano-4-hydroxycinnamate; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulphonic acid; DMEM, Dulbecco's modified Eagle's medium; DMSO, Dimethyl sulphoxide; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; GER, gastric emptying rate; HBSS, Hanks balanced salt solution; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; IC_{50}, concentration giving half-maximal inhibition; MCT, monocarboxylate transporter; MES, 2-(N-morpholino)ethanesulphonic acid; OAT, organic anion transporter; PAH, p-aminohippurate; PEPT, peptide transporter; TEER, transepithelial electrical resistance; Tris, tris(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulphate

Introduction

Nateglinide (Figure 1) is a novel oral hypoglycaemic agent possessing a carboxyl group and a peptide-type bond in its structure. Although nateglinide stimulates insulin secretion *via* the same mechanism as sulphonylureas, it quickly reaches the maximal serum concentration and is eliminated quite rapidly after oral administration (Kosaka *et al*., 1997). Therefore, nateglinide can compensate for impaired insulin secretion to prevent postprandial hyperglycemia, without causing prolonged hypoglycaemia. These characteristics are expected to be useful in the treatment of noninsulin-dependent diabetes mellitus.

The pharmacokinetic features of nateglinide may be attributable to its rapid intestinal absorption. Because nateglinide is an anionic compound with pKa 3.1, it exists predominantly in ionized form at the intestinal physiological pH of 6.5. Moreover, its chloroform/water partition coefficient is reported to be 0.2 at pH 6.8 (Ajinomoto, 2000), indicating that it is scarcely lipophilic. These physicochemical features are incompatible with rapid absorption by passive diffusion, suggesting that nateglinide is absorbed *via* a specific transport system(s) in the intestine. Although nateglinide has a dipeptide-type structure (Figure 1), it has been reported not to be transported by rat peptide transporters (PEPT1 and PEPT2) (Terada *et al*., 2000). Thus, little is known about the transport mechanisms or transporters that contribute to the intestinal absorption of nateglinide.

The present study was carried out to clarify the intestinal absorption mechanism of nateglinide. We examined the transcellular transport and the uptake of [^{14}C]nateglinide in human colon carcinoma cell line Caco-2 cells. To clarify whether nateglinide is a substrate of monocarboxylate transporter MCT1, we also examined the uptake of nateglinide into MCT1-expressing *Xenopus laevis* oocytes and the inhibitory effect of nateglinide on the function of MCT1.

*Author for correspondence; E-mail: sawada@phar.kyushu-u.ac.jp

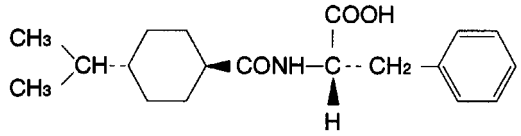

Figure 1 Chemical structure of nateglinide.

Methods

Chemicals

[^{14}C]Nateglinide (3.56 mCi mmol^{-1}), nateglinide and L-nateglinide were kindly supplied by Ajinomoto Co., Inc. (Tokyo, Japan). [^{3}H]Mannitol (SA 20 Ci mmol^{-1}) was purchased from American Radiolabeled Chemicals Inc. (MO, U.S.A.). [^{14}C]L-lactic acid (116 mCi mmol^{-1}) was purchased from ICN Biomedicals, Ltd. (CA, U.S.A.). Pravastatin sodium was kindly supplied by Sankyo Co., Inc. (Tokyo, Japan). All other chemicals used were commercial products of reagent grade.

Cell culture

Human colon carcinoma cell line (Caco-2) was obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). Cells were cultured in Dulbecco's modified Eagle's medium (GIBCO–BRL, MD, U.S.A.) containing 10% foetal calf serum, 1% non-essential amino acid, 270 μg ml^{-1} benzylpenicillin K, 100 μg ml^{-1} streptomycin sulphate at 37°C in a humidified atmosphere of 5% CO_2–95% air. The cells used for the experiment were at passages 54–70.

Transcellular transport experiment

The transcellular transport experiment was performed as described previously (Tsuji *et al*., 1994). Caco-2 cells were seeded at 1.2×10^5 cells cm^{-2} on polycarbonate membrane Transwell™ inserts (1 cm^2, 3.0 μm pore size, Corning Costar Japan, Tokyo, Japan) and cultured for 20–22 days. The cell inserts with transepithelial electrical resistance (TEER) values $>350\ \Omega$ cm^2 were washed three times with Hanks' balanced salt solution (HBSS–MES: (in mM): NaCl 136.7, KCl 5.36, $CaCl_2$ 0.952, $MgSO_4$ 0.812, KH_2PO_4 0.441, Na_2HPO_4 0.385, D-glucose 25, MES 10; pH 6.5, 37°C) before the transcellular transport study. Transport buffer was put into the receiver side (0.5 ml of HBSS-MES for the apical side, 1.5 ml of HBSS-HEPES; (in mM) NaCl 136.7, KCl 5.36, $CaCl_2$ 0.952, $MgSO_4$ 0.812, KH_2PO_4 0.441, Na_2HPO_4 0.385, D-glucose 25, HEPES 10; pH 7.4, 37°C for the basolateral side). Then transport buffer containing 15 μM [^{14}C]nateglinide or 2.6 μM [^{3}H]mannitol was put into the donor side and the cells were incubated at 37°C. At the designated time, 0.5 ml of the basolateral or 0.2 ml of the apical side solution was sampled from the receiver side, and an equivalent volume of transport buffer was added as a replacement. To assay the radiolabelled compounds, each sample was transferred into a counting vial, mixed with 4 ml of scintillation fluid (Clear-sol I, Nacalai Tesque, Kyoto, Japan) and put in a liquid scintillation counter (model LS6500, Beckman Instruments, Inc., Fullerton, CA, U.S.A.). After transport study, cells were washed three times with ice-cold buffer. The filters with monolayers were detached, and the cells on the filters were solubilized with 0.4 ml of 1 M NaOH, and neutralized with 0.4 ml of 1 M HCl. The amount of protein in the cells was measured by Lowry's method (Lowry *et al*., 1951). The transcellular transport of nateglinide or mannitol was expressed as the permeability (μl cm^{-2}), the ratio of the transported amount per surface area of insert relative to the drug concentration. The apparent permeability coefficient (μl cm^{-2} min^{-1}) is the slope obtained from the linear portion of the permeability-time plot. The real permeability coefficient (P_{trans}) of nateglinide was calculated by use of the following equation (1):

$$1/P_{app} = 1/P_{filter} + 1/P_{trans} \quad (1)$$

where P_{app} and P_{filter} are the apparent permeability coefficients estimated by transport study in the presence and absence of Caco-2 cells, respectively.

Uptake study by Caco-2 cells

The uptake experiment was performed as described previously (Tsuji *et al*., 1994). Caco-2 cells were seeded at 1.2×10^5 cells well^{-1} in 4-well multidishes (1.9 cm^2, Nunc, Denmark) and cultured for 13–15 days. For uptake experiments, the culture medium on the multidishes was removed and the cells were washed three times with 1 ml of incubation buffer (HBSS-MES, pH 6.5 at 4°C or 37°C). Nateglinide uptake experiments were performed for the designated time at 37°C in 250 μl of HBSS-MES containing 15 μM [^{14}C]nateglinide or 190 nM [^{3}H]mannitol in the absence or presence of unlabelled nateglinide or various inhibitors. Uptake experiments with L-lactic acid were performed at 37°C in 250 μl of HBSS-MES containing 20 μM [^{14}C]L-lactic acid in the absence or presence of unlabelled L-lactic acid or various inhibitors. In the energy- and temperature-dependency studies, the uptake study was performed after the preincubation for 10 min. Both the preincubation and uptake were performed at 37°C in the HBSS buffer (pH 6.5) containing 25 mM NaN_3 or 25 mM D-glucose, or at 4°C. To assay the effect of Na^+, NaCl in the buffer was replaced with choline chloride, LiCl, or N-methyl-D-glucamine chloride. The effects of extracellular pH were measured in the HBSS buffer at various pH levels adjusted by MES/Tris (pH 5.0, 6.0, 6.5 and 7.0) or HEPES/Tris (pH 7.4 and 8.0). To examine the effect of proton gradient, the uptakes of [^{14}C]nateglinide were measured in the absence or presence of 50 μM FCCP (a protonophore), 25 mM NaN_3, or both FCCP and NaN_3 at pH 6.5 after preincubation for 10 min at pH 7.4 in the HBSS buffer containing 25 mM D-glucose or 25 mM NaN_3. At appropriate time, the cells were washed three times with ice-cold buffer to stop the uptake, then dissolved in 0.3 ml of 1 M NaOH, and neutralized with 0.3 ml of 1 M HCl. To assay the radiolabelled compounds, all samples were partly transferred into counting vials, mixed with 4 ml of scintillation fluid (Clear-sol I) and put in a liquid scintillation counter (model LS6500). In the remaining portions of samples, cellular protein was measured by Lowry's method (Lowry *et al*., 1951) using bovine serum albumin as the standard. The uptake of nateglinide or L-lactic acid is presented as the uptake rate (nmol mg protein^{-1} min^{-1}) or cell/medium ratio (μl mg protein^{-1}). Cell/medium ratio was obtained by dividing the uptake amount per the cellular protein amount (d.p.m. mg protein^{-1}) by the initial drug concentration in the uptake buffer (d.p.m. μl^{-1}).

cRNA synthesis and injection to Xenopus *oocytes*

The plasmid containing human MCT1 cDNA (pCK92) was obtained from American Type Culture Collection (VA, U.S.A.). pCK92 was linearized by digestion with *Bam*HI. MCT1 cDNA was transcribed into cRNA *in vitro* using RiboMAX™ RNA production systems according to the protocol of the manufacturer (Promega) in the presence of the cap analog m7G(5′)ppp(5′)G (Ambion, Inc., TX, U.S.A.). The derived cRNA was recovered in a precipitation step and was dissolved in diethylpyrocarbonate-treated water. The quantitation and quality of cRNA were determined by UV spectrophotometry and denaturing formaldehyde-agarose gel electrophoresis.

Xenopus laevis females were obtained from Seac. Yoshitomi, Ltd. (Fukuoka, Japan). Ovary lobes were removed from the frog and treated with collagenase (type II; Sigma) for about 30–60 min at 18°C in Ca^{2+}-free buffer ((in mM): NaCl 88.0, KCl 1.0, $NaHCO_3$ 2.4, Tris-HCl 15.0, $Ca(NO_3)_2$, 0.3, $MgSO_4$ 0.82, sodium penicillin 10 μg ml^{-1}, streptomycin sulphate 10 μg ml^{-1}; pH 7.6). Healthy oocytes (stage V–VI) were selected and maintained in modified Barth's saline MBS ((in mM): NaCl 88.0, KCl 1.0, $NaHCO_3$ 2.4, Tris-HCl 15.0, $Ca(NO_3)_2$ 0.3, $CaCl_2$ 0.41, $MgSO_4$ 0.82, sodium penicillin 10 μg ml^{-1}, streptomycin sulphate 10 μg ml^{-1}; pH 7.6) at 18°C. An aliquot of 50 nl of MCT1 cRNA (1 mg ml^{-1}) or distilled water (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) was microinjected into oocytes under a microscope. The uptake experiment was performed on day 3 after injection.

Uptake experiment with oocytes

Before an uptake experiment, oocytes were washed with OR2 buffer ((in mM): HEPES 15, NaCl 82.5, KCl 2.5, $NaHPO_4$ 1, $MgCl_2$ 1; pH 7.4). For uptake experiments, groups of 9–12 oocytes were incubated in 400 μl of the uptake buffer ((in mM): MES 15, NaCl 82.5, KCl 2.5, $NaHPO_4$ 1, $MgCl_2$ 1; pH 6.0) containing radiolabelled drugs at 18°C. The oocytes were washed three times in ice-cold OR2 buffer to terminate the uptake. Washed oocytes were transferred into scintillation vials and dissolved in 200 μl of 10% SDS over 4 h. Clear-sol I (4 ml) was added and the radioactivity was determined using a liquid scintillation counter. The uptake of nateglinide or L-lactic acid by oocytes was presented as the ratio of radioactivity in the sample to the concentration in the medium (uptake; μl oocyte^{-1}). Uptake (μl oocyte^{-1}) is obtained by dividing the uptake amount per an oocyte (d.p.m. oocyte^{-1}) by the initial concentration in the uptake buffer (d.p.m. μl^{-1}).

Kinetic analysis

To determine the kinetic parameters, Kt and J_{max}, for nateglinide uptake, the following Michaelis–Menten equation (2) was fitted to the data, using nonlinear least-squares regression analysis (MULTI, Yamaoka *et al*., 1981):

$$J = J_{max} \times C/(Kt + C) \quad (2)$$

where J and C represent the uptake rate (nmol mg protein^{-1} 5 min^{-1}) and concentration of nateglinide (μM), respectively. J_{max} (nmol mg protein^{-1} 5 min^{-1}) and Kt (μM) represent the maximum uptake rate of nateglinide and the Michaelis–Menten constant.

To determine the IC_{50} value of nateglinide for the inhibition of L-lactic acid uptake, the following sigmoidal inhibition equation (3) was fitted to the data, using nonlinear least-squares regression analysis (MULTI, Yamaoka *et al*., 1981):

$$E = 100 - 100/[1 + \exp\{n_H(\ln IC_{50} - \ln C)\}] \quad (3)$$

where E, C and n_H represent the relative uptake rate of L-lactic acid (% of control), concentration of nateglinide (mM) and Hill constant, respectively.

To determine the kinetic parameters, Kt, J_{max}, and kd of L-lactic acid and K_i of nateglinide for the uptake of L-lactic acid, the following equations (4) and (5) were simultaneously fitted to the data in the absence and presence of nateglinide, respectively:

$$J = J_{max} \times C/(Kt + C) + kd \quad (4)$$

$$J = J_{max} \times C/\{Kt(1 + I/Ki) + C\} + kd \quad (5)$$

where J, C and I represent the uptake rate of L-lactic acid (nmol mg protein^{-1} min^{-1}), concentration of the substrate L-lactic acid (μM) and concentration of the inhibitor nateglinide (μM), respectively. J_{max} (nmol mg protein^{-1} min^{-1}), Kt (μM) and kd (μl mg protein^{-1} min^{-1}) represent the maximum uptake rate of L-lactic acid, the Michaelis constant and uptake rate of the nonsaturable component, respectively.

Statistical analysis

Statistical analysis was performed using Student's *t*-test or analysis of variance (ANOVA) followed by Dunnett's or Bonferroni's test. Differences between means were considered to be significant when the *P* value was less than 0.05.

Results

Transcellular transport of [^{14}C]nateglinide across Caco-2 monolayer

Figure 2 represents the time courses of the transport of [^{14}C]nateglinide across a Caco-2 monolayer. The apical-to-basolateral flux was higher than that in the opposite direction. The apparent permeability coefficients of apical-to-basolateral flux and basolateral-to-apical flux were 0.704 and 0.316 μl cm^{-2} min^{-1}, respectively. These values were significantly higher than those of [^{3}H]mannitol (0.0453 and 0.0655 μl cm^{-2} min^{-1}, respectively). The real permeability coefficients (P_{trans}) of [^{14}C]nateglinide were 0.778 and 0.333 μl cm^{-2} min^{-1}, respectively. The protein content of cells was 0.64 ± 0.014 mg protein cm^{-2} (mean ± s.e.mean, $n = 24$).

Time course of the uptake of [^{14}C]nateglinide by Caco-2 cells

The time course of the uptake of [^{14}C]nateglinide (15 μM) across the apical membrane of the Caco-2 monolayer is shown in Figure 3. The uptake increased linearly up to 5 min

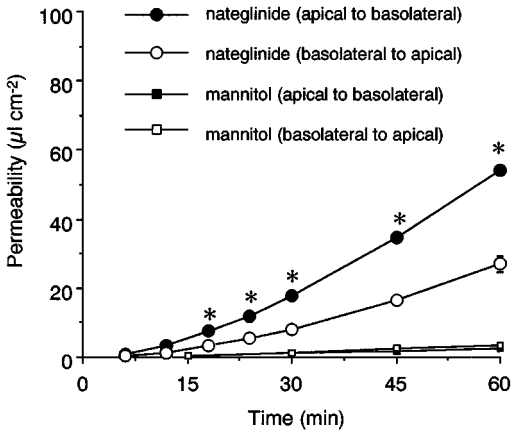

Figure 2 Transcellular transport of 15 μM [^{14}C]nateglinide and 2.56 μM [^{3}H]mannitol across Caco-2 cell monolayers. The transport of [^{14}C]nateglinide (closed circles) or [^{3}H]mannitol (closed squares) from the apical to basal side and the transport of [^{14}C]nateglinide (open circles) or [^{3}H]mannitol (open squares) from the basal to apical side were investigated at 37°C in the HBSS–MES (apical side; pH 6.5) and HBSS–HEPES (basal side; pH 7.4). Each point represents the mean±s.e.mean of three experiments. Significant differences between [^{14}C]nateglinide transport in the two directions were identified by using Student's *t*-test (*$P<0.05$).

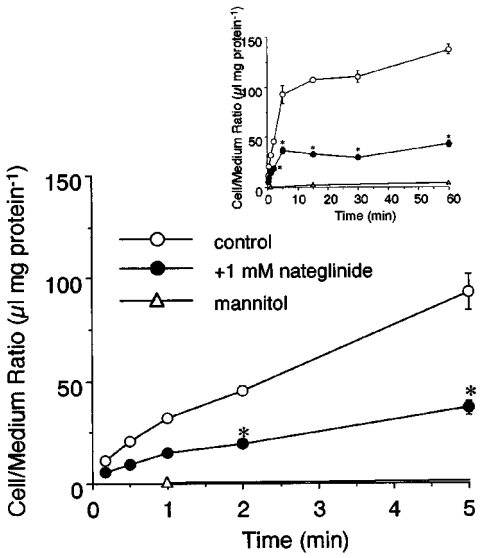

Figure 3 Time course of the uptake of [^{14}C]nateglinide by Caco-2 cells for 5 min. The inset shows the time course up to 60 min. The uptake of 15 μM [^{14}C]nateglinide (open circles), 15 μM [^{14}C]nateglinide in the presence of 1 mM unlabelled nateglinide (closed circles) or 0.19 μM [^{3}H]mannitol (open triangles) was investigated at 37°C in the 1- mM HBSS-MES buffer (pH 6.5). Each point represents the mean±s.e.mean of three experiments. Significant differences from the control were identified by using Student's *t*-test (*$P<0.05$).

and was reduced in the presence of an excess of unlabeled nateglinide (1 mM). On the other hand, the uptake of [^{3}H]mannitol, an extracellular adsorption marker, was much lower than that of [^{14}C]nateglinide.

Concentration-dependent uptake of nateglinide by Caco-2 cells

Figure 4 shows the concentration-dependent uptake of nateglinide within the concentration range up to the solubility limit (1∼1015 μM). The inset shows the Eadie–Hofstee plot. The analysis of these data using equation (2) yielded a Kt value of 448±37.0 μM and a J_{max} value of 43.2±3.02 nmol mg protein^{-1} 5 min^{-1} (estimate±s.d.).

Energy- and proton-dependent uptake of [^{14}C]nateglinide by Caco-2 cells

Figure 5 shows the effect of temperature and a metabolic inhibitor (NaN_3) on the uptake of [^{14}C]nateglinide. The uptake of [^{14}C]nateglinide was significantly reduced at 4°C. The initial uptake for 5 min was reduced to 50% of the control (37°C) after preincubation for 10 min with 25 mM NaN_3.

Figure 6 shows the effect of Na^+-substitution on the uptake of [^{14}C]nateglinide. When NaCl in the buffer was replaced with choline chloride, LiCl or N-methyl-D-glucamine chloride, the initial uptake of [^{14}C]nateglinide for 5 min was slightly, but significantly reduced to 75–89% of the control.

Figure 7 shows the effect of extracellular pH on the uptake of [^{14}C]nateglinide by Caco-2 cells. The uptake markedly increased with a decrease in extracellular pH. In the presence of 1 mM unlabelled nateglinide, the uptake of [^{14}C]nateglinide was significantly reduced as compared with that in 15 μM nateglinide at all pH values examined, and the difference was greater at acidic pH (Figure 7A). In the presence of 50 μM FCCP, a protonophore, the uptake of [^{14}C]nateglinide was significantly reduced. In the presence of both FCCP and NaN_3, the uptake of [^{14}C]nateglinide was significantly reduced as compared with that in the presence of NaN_3 alone (Figure 7B).

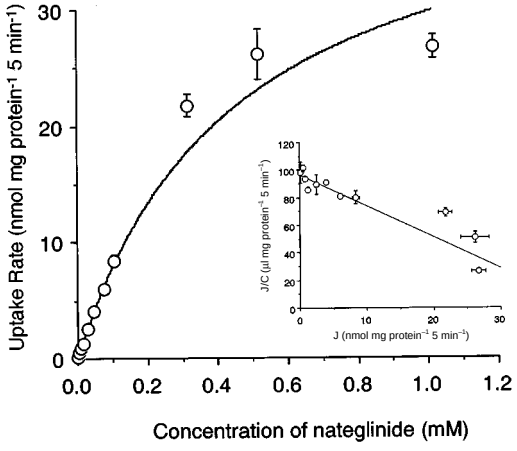

Figure 4 Concentration-dependent uptake of [^{14}C]nateglinide by Caco-2 cells. The uptake of nateglinide (1 μM–1 mM) into Caco-2 cells was performed at 37°C in the HBSS-MES buffer (pH 6.5) for 5 min. Each point represents the mean±s.e.mean of three experiments. The inset shows Eadie–Hofstee plots of the uptake of [^{14}C]nateglinide by Caco-2 cells. Equation (2) was fitted to the uptake rate (J). The Kt and J_{max} values were calculated to be 448±37.0 μM and 43.2±3.02 nmol mg protein^{-1} 5 min^{-1}, respectively (estimate±s.d.).

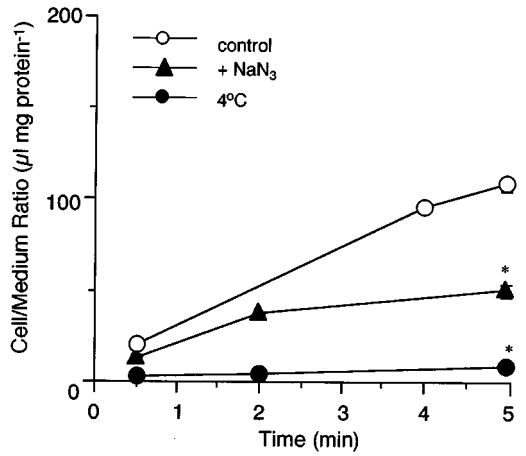

Figure 5 Energy-dependent uptake of [^{14}C]nateglinide by Caco-2 cells. Cells were preincubated for 10 min at 4°C (closed circles) or 37°C in the absence (open circles) or presence (closed triangles) of NaN_3 in the 10 mM HBSS-MES buffer (pH 6.5). Then uptake of nateglinide (15 μM) into Caco-2 cells was performed for the indicated times. Each point represents the mean ± s.e.mean of three experiments. Significant differences from the control were identified by using ANOVA followed by Dunnett's test (*$P<0.05$).

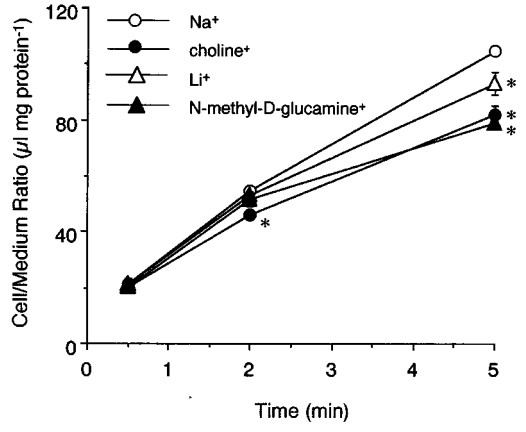

Figure 6 Effect of Na^+-substitution on the uptake of 15 μM [^{14}C]nateglinide by Caco-2 cells. The uptake of nateglinide into Caco-2 cells was performed at 37°C in the presence of 137 mM NaCl (open circles), 137 mM choline chloride (closed circles), 137 mM LiCl (open triangles) or 137 mM N-methyl-D-glucamine chloride (closed triangles) in the HBSS–MES buffer (pH 6.5). Each point represents the mean ± s.e.mean of three experiments. Significant differences from the control were identified by using ANOVA followed by Dunnett's test (*$P<0.05$).

Inhibitory effect of various compounds on the uptake of [^{14}C]nateglinide by Caco-2 cells

To characterize the carrier that is responsible for the uptake of nateglinide by Caco-2 cells, we investigated the effects of various drugs on the initial uptake of [^{14}C]nateglinide (Table 1). The effects of oral antidiabetic drugs were examined. L-Nateglinide, the enantiomer of nateglinide, inhibited the uptake as potently as did nateglinide. In the presence of 100 μM glibenclamide, the uptake was reduced to 76.5% of the control. Various amino acids and glysylsarcosine, a typical substrate of PEPT, did not significantly inhibit the uptake of [^{14}C]nateglinide.

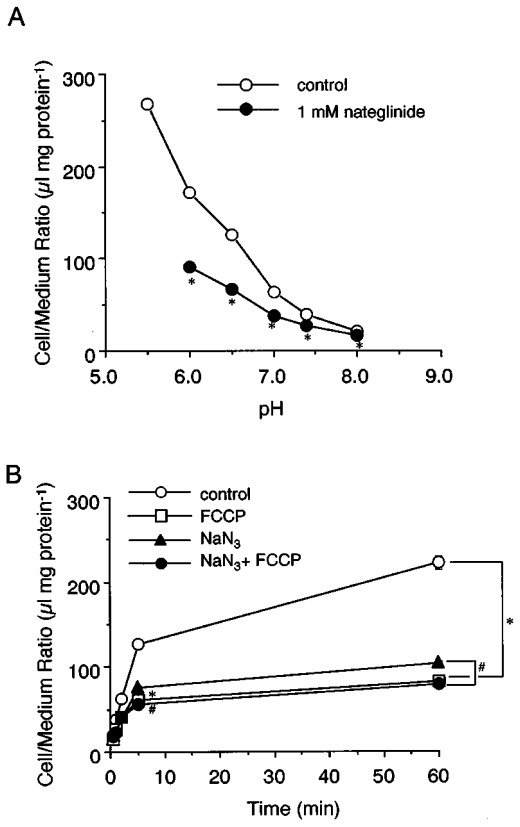

Figure 7 (A) Effect of extracellular pH on the uptake of 15 μM [^{14}C]nateglinide by Caco-2 cells. The uptake of nateglinide into Caco-2 cells was performed at 37°C for 5 min in the HBSS–MES buffer (pH 5.5–6.5) or HBSS–HEPES buffer (pH 7.0–8.0) in the absence (open circles) or presence (closed circles) of 1 mM unlabelled nateglinide. (B) Effect of FCCP on the uptake of 15 μM [^{14}C]nateglinide by Caco-2 cells. Cells were preincubated for 10 min at 37°C in the HBSS buffer (pH 7.4) in the absence (open symbols) or presence (closed symbols) of 25 mM NaN_3. Then uptake of 15 μM [^{14}C]nateglinide was performed in the new buffer (pH 6.5) as shown in the figure. Each point represents the mean ± s.e.mean of three experiments. Significant differences from the control (*) or NaN_3 (#) were identified by using ANOVA followed by Bonferroni's test (*, #; $P<0.05$). FCCP: carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

Next, the effects of various organic anions were examined. In the presence of probenecid, the uptake was reduced to 85% of the control. On the other hand, *p*-aminohippurate (PAH), taurocholate, 4.4′-diisothiocyanato-stilbene-2.2′-disulphonic acid (DIDS) and methotrexate did not significantly decrease the uptake of [^{14}C]nateglinide.

The effects of various monocarboxylates and succinic acid were examined. All the monocarboxylates examined significantly reduced the uptake of [^{14}C]nateglinide. L-Lactic acid, a typical substrate of monocarboxylate transporter 1 (MCT1), had a weak inhibitory effect. In the presence of 50 mM L-lactic acid, the uptake was reduced by 20%. Salicylic acid and valproic acid potently inhibited the uptake with IC_{50} values of 6.27 (95% confidence interval; 2.24 ~ 17.5 mM) and 16.5 mM (95% confidence interval; 4.32 ~ 63.0 mM), respectively.

The effects of α-cyano-4-hydroxycinnamate (CHC) and phloretin, which inhibit the transport of L-lactic acid by MCT, were examined. Phloretin (10 μM) did not affect the uptake of nateglinide. In the presence of 100 μM phloretin or

Table 1 Effect of various compounds on [^{14}C]nateglinide uptake by Caco-2 cells

Inhibitor	*Concentration* (mM)	*Cell/medium ratio* (% of control)
Control[a]		100±7.53
Control (0.5% ethanol)[b]		100±6.65
Control (0.5% DMSO)[c]		100±7.68
Anti-diabetic agent		
Nateglinide (in 0.5% ethanol)	1	48.9±1.98*
L-nateglinide (in 0.5% ethanol)	1	47.0±5.16*
Tolbutamide (in 0.5% DMSO)	1	97.7±6.16
Glibenclamide (in 0.5% DMSO)	0.1	76.5±2.57*
Amino acid or dipeptide		
L-alanine	10	113±3.39
L-leucine	10	113±4.39
L-lysine	10	104±2.98
L-arginine	10	121±1.98
Glutamic acid	10	102±1.52
Aspartic acid	10	95.6±2.57
L-phenylalanine	10	96.7±8.40
D-phenylalanine	10	99.3±3.79
Glycylsarcosine	10	95.0±3.24
Organic acid		
p-aminohippuric acid	10	9.67±5.10
Probenecid (in 0.5% DMSO)	1	85.9±6.13*
Taurocholate	1	103±1.41
DIDS (in 0.5% DMSO)	2	95.1±3.00
Methotrexate	5	94.9±8.52
Carboxylic acid		
Acetic acid	10	70.7±1.23*
Butyric acid	10	78.0±2.83*
n-octanoic acid	10	51.7±0.93*
Valproic acid	10	58.9±1.92*
Benzoic acid (in 0.5% DMSO)	10	78.0±2.83*
Salicylic acid	10	44.7±3.28*
Pravastatin	10	82.5±5.44*
L-lactic acid	10	100±3.63*
L-lactic acid	50	84.8±0.89*
Succinic acid	10	91.8±2.21
Inhibitors of monocarboxylate transporter		
Control[d]		100±2.34
Phloretin	0.01	100±1.52
Phloretin	0.1	92.2±1.07*
CHC	1	90.5±3.07*
CHC	10	83.3±1.04*

Uptake of [^{14}C]nateglinide (15 μM) was measured at 37°C for 5 min by incubating Caco-2 cells in the HBSS–MES buffer (pH 6.5) in the presence of each inhibitor. Each value represents the mean±s.e.mean of four experiments and is expressed as a percentage of the control. Control value is [a]82.07±6.18, [b]84.74±6.51, [c]77.55±5.16, [d]96.21±5.61 μl mg protein^{-1}. *$P<0.05$, significantly different from control by ANOVA followed by Dunnett's test. DMSO: dimethylsulphoxide, DIDS: 4,4′-diisothiocyanato stilbene-2,2′-disulphonic acid, CHC: alpha-cyano-4-hydroxy cinnamic acid.

1 mM CHC, the uptake was slightly reduced to 90% of the control. A higher concentration of CHC (10 mM) reduced the uptake to 80% of the control.

The uptake of [^{14}C]L-lactic acid by Caco-2 cells and inhibition by nateglinide

The uptake of [^{14}C]L-lactic acid increased linearly at least up to 5 min (data not shown) and the uptake in 1 min was reduced in the presence of nateglinide, CHC, phloretin, salicylic acid and valproic acid (Table 2). Nateglinide inhibited the uptake of [^{14}C]L-lactic acid in a concentration-dependent manner with an IC_{50} value of 0.264 mM (95% confidence interval; 0.108∼0.648 mM, Hill constant=0.9, Figure 8). Figure 9 shows the Eadie–Hofstee plot for the uptake of L-lactic acid in the absence or presence of 100 μM nateglinide. Based on the analysis of these data using equations (4) and (5), nateglinide was considered to competitively inhibit the uptake of L-lactic acid by Caco-2 cells. The kinetic parameters of L-lactic acid uptake (Kt, Jmax, kd) and the K_i value of nateglinide were 6.53±1.53 mM, 38.4±9.19 nmol mg protein^{-1} min^{-1}, 1.60±0.24 μl mg protein^{-1} min^{-1}, and 0.120±0.0141 mM, respectively (estimate±s.d.).

The uptake of [^{14}C]nateglinide by MCT1-expressing Xenopus laevis *oocytes*

Figure 10 shows the time courses of the uptake of [^{14}C]nateglinide and [^{14}C]L-lactic acid, a typical substrate of MCT1, by *Xenopus laevis* oocytes injected with MCT1 cRNA or water. The uptake of [^{14}C]L-lactic acid increased linearly at least up to 20 min (data not shown) and the uptake in 10 min by MCT1-expressing oocytes was 5-fold higher than that by water-injected oocytes. The uptake of [^{14}C]nateglinide by MCT1-expressing oocytes was at the same level as that by water-injected oocytes. On the other hand, nateglinide inhibited the uptake of [^{14}C]L-lactic acid in a concentration-dependent manner with an IC_{50} value of 0.187 mM (95% confidence interval; 0.0498∼0.704 mM, Hill constant=1.0, Figure 11).

Discussion

As nateglinide is absorbed rapidly from the intestine, it is likely to be absorbed *via* a specific transporter(s). Therefore, we examined the intestinal transport mechanism of nateglinide by means of *in vitro* experiments using Caco-2 cells. We report here for the first time that the uptake of nateglinide from the intestine is mediated by a saturable transport system(s). The uptake of [^{14}C]nateglinide from the apical side was not only saturable, but also H^+-dependent and Na^+-independent. Therefore, the uptake of nateglinide from the intestine is, at least in part, mediated by a proton-dependent transport system(s).

The uptake of nateglinide from the apical membrane of Caco-2 cells increased with a decrease in extracellular pH. As the saturable component also increased under acidic conditions, not merely the passive diffusion of unionized nateglinide, but also the pH-dependent transport of nateglinide is increased. In addition, FCCP significantly decreased the uptake of nateglinide. As FCCP is a metabolic inhibitor, we examined its effect in the presence of NaN_3, a cellular metabolism inhibitor. In the presence of NaN_3, FCCP further decreased the uptake of nateglinide. These results suggest that the uptake of nateglinide is associated with a proton-coupled transport system.

In the absence of Na^+, the uptake of nateglinide was reduced by 22% at most. This sodium-dependent component represents 45% of the saturable component inhibited by

Table 2 Effect of various compounds on [^{14}C]L-lactic acid uptake by Caco-2 cells

Inhibitor	*Concentration* (mM)	*Cell/medium ratio* (% of control)
Control[a]		100±9.75
Control (0.5% ethanol)[b]		100±3.61
Nateglinide (0.5% ethanol)	1	25.2±1.51*
Phloretin	0.1	54.0±10.4*
CHC	10	27.2±3.15*
Salicylic acid	10	30.2±6.25*
Valproic acid	10	51.3±5.51*

Uptake of [^{14}C]L-lactic acid (20 μM) was measured at 37°C for 1 min by incubating Caco-2 cells in the HBSS–MES buffer (pH 6.5) in the presence of each inhibitor. Each value represents the mean±s.e.mean of four experiments and is expressed as a percentage of the control. Control value is [a]7.49±0.73, [b]7.85±0.95 μl mg protein^{-1}. *$P<0.05$, significantly different from control by ANOVA followed by Dunnett's test. CHC: alpha-cyano-4-hydroxy cinnamic acid.

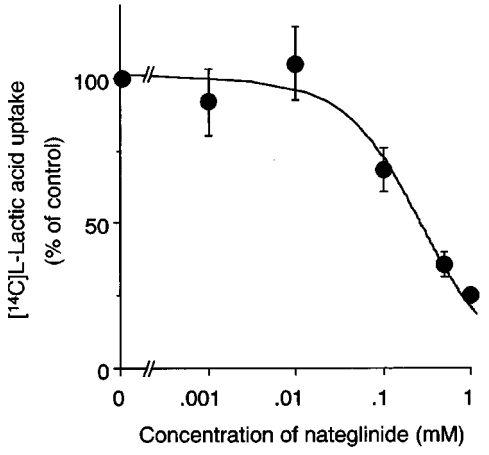

Figure 8 Concentration-dependent inhibition of nateglinide on [^{14}C]L-lactic acid uptake by Caco-2 cells. The uptake of L-lactic acid (20 μM) into Caco-2 cells was performed at 37°C, pH 6.5 for 1 min in the absence or presence of nateglinide (0.001–1 mM). Each point represents the mean±s.e.mean of three experiments. Data are presented as relative values to the uptake in the absence of nateglinide (%). Equation (3) was fitted to the uptake rate. The IC_{50} value was calculated to be 0.264 mM (95% confidence interval; 0.108∼0.648 mM, Hill constant=0.9).

1 mM nateglinide. Apparent sodium-dependency is observed not only in the case of a sodium-dependent transport system but also a proton-coupled transport system that employs a Na^+/H^+ exchanger as a source of the H^+ gradient. As the uptake of nateglinide is proton-dependent, the replacement of Na^+ may possibly decrease the proton gradient and lead to a decrease in the uptake. In any case, more than half of the saturable component is considered to be sodium-independent.

To determine the contributions of particular transporters to the uptake of nateglinide, we examined the effects of their substrates and inhibitors. The uptake of nateglinide was inhibited by various monocarboxylates including L-lactic acid, and by inhibitors of L-lactic acid transport systems such as CHC and phloretin. However, the inhibitory effects of those compounds were relatively weak (20% of the control)

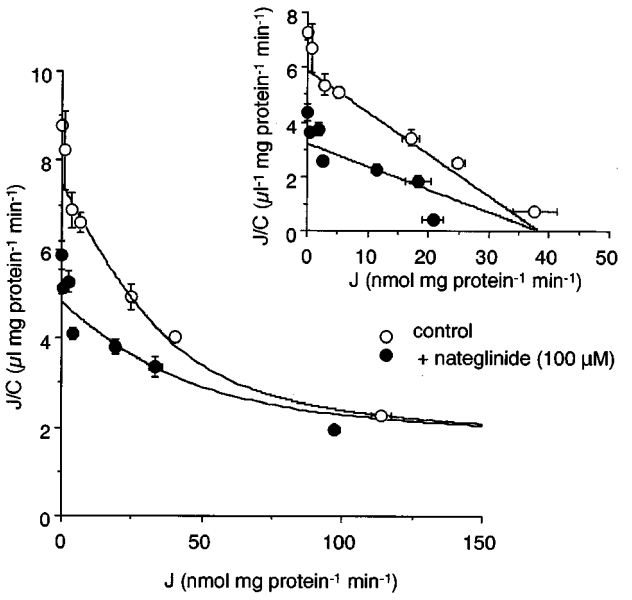

Figure 9 Eadie–Hofstee plots of the uptake of [^{14}C]L-lactic acid in the absence or presence of nateglinide (100 μM) by Caco-2 cells. The uptake of L-lactic acid (100 μM–50 mM) into Caco-2 cells was performed at 37°C in the HBSS-MES buffer (pH 6.5) for 1 min. Each point represents the mean±s.e.mean of three experiments. Equations (4) and (5) were simultaneously fitted to the uptake rate (J) in the absence and presence of nateglinide, respectively. The Kt, J_{max}, kd and K_i values were calculated to be 6.53±1.53 mM, 38.4±9.19 nmol mg protein^{-1} min^{-1}, 1.60±0.24 μl mg protein^{-1} min^{-1} and 0.120±0.0141 mM respectively (estimate±s.d.). The inset shows Eadie–Hofstee plots of the saturable component.

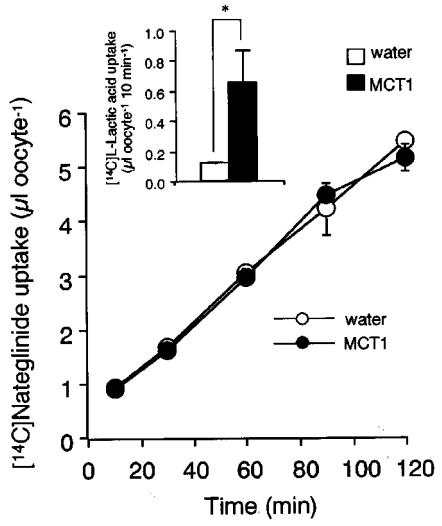

Figure 10 Time course of [^{14}C]nateglinide uptake by human MCT1-expressing oocytes. The uptake of [^{14}C]nateglinide (100 μM) was measured by incubating oocytes, injected with human MCT1 cRNA (closed circles) or water (open circles), at 18°C, pH 6.0 for the indicated times. Each point represents the mean±s.e.mean of 9–12 oocytes. The inset shows the uptake of [^{14}C]L-lactic acid (9 μM), measured by incubating oocytes injected with human MCT1 cRNA (closed bar) or water (open bar) at 18°C, pH 6.0 for 10 min. Each bar represents the mean±s.e.mean of 9–12 oocytes. The significance of the difference from water was determined by using Student's *t*-test (*$P<0.05$).

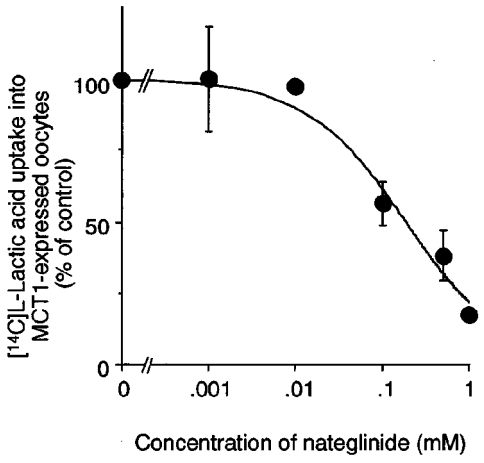

Figure 11 Concentration-dependent inhibition by nateglinide of [^{14}C]L-lactic acid uptake by human MCT1-expressing oocytes. Oocytes injected with human MCT1 cRNA or water were incubated with [^{14}C]L-lactic acid (13 μM) in the absence or presence of unlabelled nateglinide at 18°C, pH 6.0 for 10 min. Specific uptake was calculated by subtracting the radioactivity taken up into water-injected oocytes from that into MCT1 cRNA-injected oocytes. Each point represents the mean ± s.e.mean of 9–12 oocytes. Data are presented as relative uptake to that in the absence of nateglinide (% of control). Equation (3) was fitted to the uptake rate. The IC_{50} value was calculated to be 0.187 mM (95% confidence interval; 0.0498 ~ 0.704 mM, Hill constant = 1.0).

compared with the self-inhibition by 1 mM nateglinide. On the other hand, most of the saturable component of L-lactic acid uptake by Caco-2 cells was CHC-sensitive (Table 2). These results suggest that nateglinide shares a common transport system with L-lactic acid only in part (20% at the most), and is predominantly transported by other system(s).

We also examined the contribution of the monocarboxylate transporter family (MCTs), which consists of several proton-dependent monocarboxylate transporters. It has been reported that the mRNA of a variety of MCT isoforms (MCT1, 3, 4, 5, 6) is expressed in Caco-2 cells (Hadjiagapiou *et al*., 2000). The affinity for L-lactic acid and the CHC-sensitivity are different between these isoforms. For example, MCT1 exhibits high affinity for L-lactic acid with a Kt value of several mM, while MCT4 exhibits low affinity with a Kt value of several tens of mM. Both MCT1 and MCT4 are sensitive to CHC (Boer *et al*., 1998; Grollman *et al*., 2000; Fox *et al*., 2000). Therefore, we examined whether nateglinide is transported by MCT1. In the study using MCT1-expressing oocytes, MCT1-dependent uptake of nateglinide was not observed, suggesting that nateglinide is not transported by MCT1. Overall, we suggest that the uptake of nateglinide is mediated in part (20% at most) by an L-lactic acid transport system other than MCT1, and the rest is mediated by a system(s) other than the L-lactic acid transport system.

Nateglinide has a dipeptide-type structure (Figure 1). The expression of PEPT1, a proton-coupled peptide transporter, has been demonstrated in the intestine and in Caco-2 cells (Liang *et al*., 1995). However, Terada *et al*. (2000) reported that nateglinide is not transported by rat peptide transporters PEPT1 and PEPT2. Indeed, 10 mM glycylsarcosine, a typical substrate of PEPT, did not affect the uptake of [^{14}C]nateglinide (15 μM) in this study, supporting the idea that nateglinide is not transported by human PEPT1, as in the case of rat PEPT1. Therefore, PEPT1 may make little or no contribution to the intestinal absorption of nateglinide.

We also examined the contribution of organic anion transport system (OATs), because nateglinide is an anionic compound. PAH, a typical substrate of OATs, at the concentration of 10 mM did not affect the uptake of [^{14}C]nateglinide (15 μM) in this study. Indeed, it has been reported that nateglinide inhibits rat organic anion transporter 1 (rOAT1) but is not a substrate (Uwai *et al*., 2000). Moreover, the expression of OATs in the intestine has not been reported so far. In the case of the organic anion transporting polypeptide family (OATPs), several isoforms such as OATP-B, -D and -E have been found in the intestine (Tamai *et al*., 2000). In this study, however, DIDS and probenecid, typical inhibitors of OATPs, had virtually no inhibitory effect on the uptake of nateglinide. Therefore, OATPs are also not responsible for the intestinal absorption of nateglinide, even if nateglinide is a substrate of OATP. Anion-exchangers have been reported to be DIDS-sensitive (Ogihara *et al*., 1999). However, DIDS did not affect the uptake of nateglinide, suggesting that anion-exchangers are not responsible for the intestinal absorption of nateglinide.

In conclusion, these results suggest that nateglinide is transported in an absorptive direction across Caco-2 cell monolayers, and its uptake from apical membranes is, at least in part, mediated by a proton-dependent transporter(s). Furthermore, nateglinide potently inhibits MCT1, although it is not itself transported by MCT1.

References

AJINOMOTO. (2000). *Interview form of 'Fastic'*.

BOER, S., SCHNEIDER, H.-P., BROER, A., RAHMAN, B., HAMPRECHT, B. & DEITMER, J.W. (1998). Characterization of the monocarboxylate transporter 1 expressed in *Xenopus* oocyte by change in cytosolic pH. *Biochem. J.,* **333,** 167–174.

FOX, J.E.M., MEREDITH, D. & HALESTRAP, A.P. (2000). Characterization of human monocarboxylate transporter 4 substantiates its role in lactic acid efflux from skeletal muscle. *J. Physiol.,* **529,** 285–293.

GROLLMAN, E.F., PHILP, N.J., MCPHIE, P., WARD, R.D. & SAUER, B. (2000). Determination of transport kinetics of chick MCT3 monocarboxylate transporter from retinal pigment epithelium by expression in genetically modified yeast. *Biochemistry,* **39,** 9351–9357.

HADJIAGAPIOU, C., SCHMIDT, L., DUDEJA, P.K., LAYDEN, T.J. & RAMASWAMY, K. (2000). Mechanism(s) of butyrate transport in Caco-2 cells: role of monocarboxylate transporter 1. *Am. J. Physiol. Gastrointest. Liver Physiol.,* **279,** G775–G780.

KOSAKA, K., URAE, A., AMAMOTO, T., KENSYOH, A. & KATOH, N. (1997). A phase I study of AY4166 in healthy male volunteers – the effects of food on the bioavailability of AY4166. *Clin. Pharmacol. Therapy [Yakuri to Rinsyou],* **7,** 615–634.

LIANG, R., FEI, Y.J., PRASAD, P.D., RAMAMOORTHY, S., HAN, H., YANG-FENG, T.L., HEDIGER, M.A, GANAPATHY, V. & LEIBACH, F.H. (1995). Human intestinal H^+/peptide cotransporter: cloning, functional expression, and chromosomal localization. *J. Biol. Chem.,* **270,** 6456–6463.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.,* **193,** 265 – 275.

OGIHARA, T., TAMAI, I. & TSUJI, A. (1999). Structural characterization of substrates for the anion exchange transport in Caco-2 cells. *J. Pharm. Sci.,* **88,** 1217 – 1221.

TAMAI, I., NEZU, J., UCHINO, H., SAI, Y., OKU, A., SHIMANE, M. & TSUJI, A. (2000). Molecular identification and characterization of novel members of the human organic anion transporter (OATP) family. *Biochem. Biophys. Res. Commun.,* **273,** 251 – 260.

TERADA, T., SAWADA, K., SAITO, H., HASHIMOTO, Y. & INUI, K. (2000). Inhibitory effect of novel oral hypoglycemic agent nateglinide (AY4166) on peptide transporters PEPT1 and PEPT2. *Eur. J. Pharmacol.,* **392,** 11 – 17.

TSUJI, A., TAKANAGA, H., TAMAI, I. & TERASAKI, T. (1994). Transcellular transport of benzoic acid across Caco-2 cells by pH-dependent and carrier-mediated transport mechanism. *Pharm. Res.,* **11,** 30 – 37.

UWAI, Y., SAITO, H., HASHIMOTO, Y. & INUI, K. (2000). Inhibitory effect of anti-diabetic agents on rat organic anion transporter rOAT1. *Eur. J. Pharmacol.,* **398,** 193 – 197.

YAMAOKA, K., TANIGAWARA, Y., NAKAGAWA, T. & UNO, T. (1981). A pharmacokinetic analysis program (MULTI) for microcomputer. *J. Pharmacobio.-Dyn.,* **4,** 879 – 885.

(Received April 16, 2002
Revised June 12, 2002
Accepted July 12, 2002)