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**Title:**

**Intestinal first-pass metabolism via carboxylesterase in rat jejunum and ileum**

**Authors:**

Kenji Masaki, Mitsuru Hashimoto and Teruko Imai

Graduate School of Pharmaceutical Sciences, Kumamoto University, 5-1 Oe-Honmachi,  
Kumamoto, 862-0973, Japan.

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**Running title:**

Intestinal first-pass hydrolysis via carboxylesterase

**Corresponding author:**

Teruko Imai, Ph.D., Professor

Graduate School of Pharmaceutical Sciences, Kumamoto University

5-1 Oe-Honmachi, Kumamoto, 862-0973, Japan.

Phone: +81-96-371-4626, Fax: +81-96-371-4626

E-mail: iteruko@gpo.kumamoto-u.ac.jp

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## ABSTRACT

In order to determine the activity of a major intestinal esterase in the first-pass hydrolysis of *O*-isovaleryl-propranolol (isovaleryl-PL), a model ester-compound, rat intestinal jejunum and blood vessels were perfused simultaneously after inhibition of a carboxylesterase (CES) by bis-*p*-nitrophenyl phosphate (BNPP). BNPP specifically inhibits about 90% of CES activity without influencing aminopeptidase activity or the transport of L-leu-*p*-nitroanilide and *p*-nitroaniline, non-ester compounds. When isovaleryl-PL was perfused into the jejunal lumen after BNPP treatment, its absorption clearance ( $7.60 \pm 0.74$   $\mu\text{L}/\text{min}$ ) increased about three-fold compared with control, while its degradation clearance ( $32.5 \pm 5.40$   $\mu\text{L}/\text{min}$ ) decreased to 23% of control. Therefore, CES seems to be mainly responsible for the intestinal first-pass hydrolysis of isovaleryl-PL. This finding is consistent with the results from studies of *in vitro* BNPP inhibition in mucosal S9 fraction.  $V_{\max}$  values for valeryl-PL, isovaleryl-PL and *p*-nitrophenyl acetate in the jejunal S9 fraction were 1.7–2.5 fold higher than that in the ileal S9 fraction, which agreed with the jejunum/ileum ratio (about 1.5-fold) of mRNA expression levels for the CES2 isozymes, AB010635 and AY034877. These findings indicated that CESs expressed in the intestine markedly contribute to first-pass hydrolysis in both jejunum and ileum.

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## INTRODUCTION

The small intestine is well recognized as having numerous functions, such as absorption, metabolism and exsorption (Lin et al., 1999). A wide spectrum of metabolic activities occur there, due to the presence of various phase I and II enzymes for oxidation, hydrolysis and conjugation, although these enzymes are found at lower levels than in the liver (Pang, 2003). Intestinal metabolism plays an important role in the bioavailability of oral therapeutic drugs. The P-450 isoforms in the intestine are well documented, and it has been shown that CYP3A4 in enterocytes is responsible for decreased oral absorption (Paine et al., 1996; Lin et al., 1999). The absorption of ester-containing drugs is also limited by hydrolase (Masaki et al., 2006; Okudaira et al., 2000; Prueksaritanont et al., 1998; Ruiz-Balaguer et al., 2002).

Carboxylesterase (CES; EC 3.1.1.1) is an important enzyme for the hydrolysis of xenobiotics and numerous endogenous compounds in the small intestine (Satoh & Hosokawa, 1998). Yoshigae et al. (1998) and Prueksaritanont et al. (1996) demonstrated that CESs are primarily responsible for the hydrolysis of xenobiotics such as propranolol (PL) ester derivatives, acetylsalicylic acid and *p*-nitrophenyl acetate (PNPA) in mammalian intestinal microsomes. The mammalian CESs comprise a multigene family, and isozymes are classified into five main groups and subgroups (Satoh & Hosokawa, 2006). CES1 and CES2 group enzymes are mainly involved in the hydrolysis of xenobiotics, and CES2 enzymes are particularly abundant in the gastro-intestinal tract. In the rat, two major CES2 isozymes (AB010635 and AY034877) and a minor CES2 isozyme (D50580) have been found in the small intestine (Sanghani et al., 2002; Furihata et al., 2005).

The introduction of an ester bond into the molecular structure of a drug is useful in

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improving membrane permeability by increasing the lipophilicity of the parent compound (Mizen & Burton, 1998). For these ester-containing drugs, such as prodrugs, intestinal hydrolysis is one of the major determinants of their pharmacokinetics and pharmacodynamics. Various hydrolases intrinsically expressed in the brush border membrane (BBM), cytosol and microsomes of enterocytes will hydrolyze ester compounds during the process of absorption. Several hydrolases, such as alkaline phosphatase, aminopeptidase and retinyl ester hydrolase are expressed in BBM, where all compounds are first transported (Fleisher et al., 1985; Rigtrup & Ong, 1992). However, *in vitro* drug metabolism is generally studied first in the 9000g supernatant (S9) and microsomal fraction of mucosal cells, this being a useful screening technique prior to whole animal studies, which are considerably more lengthy and expensive. CES is located in the endoplasmic reticulum through the binding of its C-terminal with the KDEL receptor (Sato & Hosokawa, 1998). Therefore, CES activity is highest in the microsomal fraction and makes the predominant contribution to the esterase activity of S9 and microsomal fractions.

We previously reported the nearly complete intestinal first-pass hydrolysis of *O*-isovaleryl-propranolol (isovaleryl-PL), a model ester compound, in rat jejunum *in situ* (Masaki et al., 2006; Yoshigae et al., 1998). In the *in situ* single-pass perfusion model, the intestinal architecture is maintained with respect to metabolism, absorption, and exsorption (Pang et al., 1986; Tamura et al., 2003; Zimmerman et al., 2000). Various hydrolases expressed in the BBM first hydrolyze isovaleryl-PL, after which isovaleryl-PL taken up into the cell is hydrolyzed by microsomal and cytosolic esterases during absorption. However, the major hydrolase involved in the hydrolysis of isovaleryl-PL has not yet been identified. It is important in both drug development and in the clinical

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application of a drug to determine the major enzyme involved in intestinal hydrolysis during *in vivo* absorption of ester-containing drugs.

In the present study, *in situ* rat jejunal single-pass perfusion was performed in the presence and absence of bis-*p*-nitrophenyl phosphate (BNPP) in order to determine the contribution of CESs to the intestinal hydrolysis of isovaleryl-PL. BNPP specifically combines with CES and suppresses hydrolase activity by noncompetitive inhibition without any modulation of  $\alpha$ -chymotrypsin, trypsin, acetylcholinesterase, or non-specific serum cholinesterase (Mentlein et al., 1988; Block & Arndt, 1978; Heymann & Krisch, 1967). In addition, we investigated the hydrolyzing capacity and expression level of rat intestinal CES isozymes in the jejunum and the ileum.

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## MATERIALS AND METHODS

### Materials

PL ester derivatives were synthesized from PL hydrochloride (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and acid chloride (Tokyo Kasei Kogyo Co. Ltd., Tokyo, Japan) according to previously described methods (Shameem et al., 1993). The identity and purity of the synthesized PL ester derivatives were confirmed by infrared, NMR, atomic analysis, and HPLC. L-Leucyl-*p*-nitroanilide hydrochloride (L-Leu-*p*-NA), *p*-nitroaniline, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) and 2-(*N*-morpholino)ethanesulfonic acid (MES) were purchased from Wako Pure Chemical Industries. Nobo heparin was provided by Leo Pharmaceutical Products, Ltd. (Ballerup, Denmark). Bovine serum albumin (BSA, Fraction V) and fluorescein isothiocyanate dextran 4000 (FD-4) were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). Bis-*p*-nitrophenyl phosphate (BNPP), *p*-nitrophenol, *p*-nitrophenyl acetate (PNPA) and testosterone were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). All other chemicals were of analytical grade.

### Animals

Male Wistar rats (250–300 g, 8 weeks of age) were housed in an air-conditioned room with free access to commercial chow and tap water, and fasted for 15 h prior to the experiment.

### *In Situ* Intestinal Single-Pass Perfusion

The perfusion studies were carried out as previously reported (Masaki et al., 2006). Briefly, rats were anesthetized by intraperitoneal injection of 2% sodium pentobarbital

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(0.5 mL) and a small intestinal loop (upper jejunum, about 10 cm) was isolated. Both ends of the jejunal loop were cannulated with Teflon tubes (3 mm i.d.). The superior mesenteric artery and the portal vein were cannulated with polyethylene tubes (PE10 and PE15, respectively) for vascular perfusion. The cannulated intestinal segment was isolated from other portions and suspended in a serosal bath containing 150 mL of Krebs-Henseleit bicarbonate buffer (KHBB, pH 7.4) warmed at 37°C with a water jacket.

Single-pass perfusion of the blood vessel was initiated just after isolation of the intestine and continued throughout the experiment. KHBB containing 3% BSA and 10 mM D-glucose was used as the vascular perfusate at 3.0 mL/min. The jejunal loop was perfused with MES buffer (pH 6.5) containing test compounds at 0.3 mL/min. FD-4 (0.1 mg/mL), a nonabsorbable marker, was added to the luminal perfusate. The volume of the luminal perfusate was corrected from the dilution of FD-4. The perfusates from the intestinal segment and the vascular outflow were collected at 10- and 5-min intervals, respectively, for 60 min. The extraction solvent was immediately added to the perfusate samples. It had been confirmed in a previous experiment that the degradation of the test compounds during the sampling period was negligible.

Three series of *in situ* perfusion studies were performed, one with BNPP (400  $\mu$ M) alone, one with L-Leu-*p*-NA (500  $\mu$ M), and one with isovaleryl-PL (300  $\mu$ M). In the second and third series, the rats were divided into two groups, which were pre-perfused either with MES buffer alone or with MES buffer plus BNPP (400  $\mu$ M) for 40 min (control and treated groups, respectively).

Determination of isovaleryl-PL concentrations in the vascular and luminal sides was carried out as previously reported (Masaki et al., 2006). For determination of BNPP and *p*-nitroaniline, an aliquot of the vascular samples (6 mL) was adjusted to pH 4.0 by



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addition of phosphate solution buffer saturated with NaCl (6 mL) and extracted with 10 mL of ethyl acetate. The organic phase was evaporated to dryness. The resulting residue was redissolved in 200  $\mu$ L acetonitrile before injection of 30  $\mu$ L aliquots onto the HPLC column. The luminal samples (100  $\mu$ L) were deproteinized with 1 mL acetonitrile. The supernatant (30  $\mu$ L) was injected onto an HPLC column after centrifugation. In the above extraction process, no degradation of the test compounds in the samples was detectable. FD-4 (0.1 mg/mL) in the luminal sample was determined by fluorescence spectrometry (F-4500; Hitachi High-Technologies Co., Tokyo, Japan).

#### **Hydrolysis of PL Ester Derivatives, *p*-Nitrophenyl Acetate (PNPA) and L-Leucyl-*p*-Nitroanilide (Leu-*p*-NA) in the Jejunal and Ileal Mucosal S9 Fraction**

Rats were sacrificed by exsanguination from the abdominal aorta under ether anesthesia. Both jejunum and ileum were removed and washed with ice-cold 1.15% KCl. The intestinal mucosa was stripped, minced, and homogenized with three volumes of 50 mM HEPES buffer (pH 7.4) containing 1.15% KCl using a Potter-Elvehjem Teflon pestle under ice-cold conditions. The homogenates were centrifuged at 9000g for 20 min at 4°C to obtain the supernatant (S9) fraction. Protein contents were determined by the method of Bradford using BSA as the standard (Bradford, 1976). These preparations were stored at -80°C until use.

Both jejunal and ileal S9 were diluted with pH 7.4 HEPES buffer (50 mM) to the required concentration. The S9 solutions were preincubated at 37°C for 5 min, and the reactions started by addition of racemic PL ester derivatives (butyryl-, valeryl-, isobutyryl- and isovaleryl-PL; final concentration 100  $\mu$ M) dissolved in dimethyl sulfoxide (DMSO). After incubation, the formation of PL was measured using previously

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reported methods (Masaki et al., 2006). The initial hydrolytic activity was measured under reaction conditions such that less than 20% of substrate was hydrolyzed. Hydrolysis of PNPA and Leu-*p*-NA in the S9 solution (1 mL) was initiated by the addition of 5  $\mu$ L of PNPA or Leu-*p*-NA dissolved in DMSO (final concentration 500  $\mu$ M or 1 mM, respectively). The formation of *p*-nitrophenol and *p*-nitroaniline from PNPA and Leu-*p*-NA was determined by the initial linear increase in absorbance at 405 nm. The final concentration of DMSO was maintained at 0.5%, which had no effect on hydrolase activity.

Kinetic parameters for the hydrolysis of PL derivatives and PNPA (final concentrations 2–200 and 25–500  $\mu$ M, respectively),  $K_m$  and  $V_{max}$ , were calculated by fitting the data to the Michaelis–Menten equation by nonlinear least-squares analysis, MULTI program (Yamaoka et al., 1981). The hydrolase activity of intestinal S9 was inhibited using BNPP, a specific carboxylesterase inhibitor, by incubating the intestinal S9 with BNPP (1–100  $\mu$ M) for 5 min following a 5-min preincubation. The degree of inhibition was calculated as a percentage of control activity.

### HPLC Analysis

PL, PL ester derivatives, BNPP and *p*-nitroaniline concentrations were determined by HPLC (pump: PU-980; Jasco International Co. Ltd., Tokyo, Japan; fluorescence detector: FP-1520S; Jasco International; ultraviolet detector: UV-970; Jasco International; data application apparatus: C-R7A; Shimadzu Co., Kyoto, Japan). For determination of racemic PL and its derivatives, BNPP and *p*-nitroaniline, a LiChrosorb RP-select B column (7  $\mu$ m, 250  $\times$  4 mm i.d.; Merck Ltd., Tokyo, Japan) was used with a mobile phase of acetonitrile/20 mM  $KH_2PO_4$  [1:1 (v/v)] at a flow rate of 1.0 mL/min. For the

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determination of PL enantiomer concentrations, a Chiralcel OD column ( $250 \times 4$  mm i.d.; Daicel Chemical Industries, Ltd., Kyoto, Japan) was used with a mobile phase of *n*-hexane/isopropanol/diethylamine [90:10:1 (v/v/v)] at flow rate of 1.0 mL/min. PL and PL derivatives were detected at excitation and emission wavelengths of 285 and 340 nm, respectively. BNPP and *p*-nitroaniline were detected at UV wavelengths of 286 and 405 nm, respectively. The quantitative limitation thresholds of PL, PL derivatives, BNPP and *p*-nitroaniline were injected amounts of 15, 30, 200 and 190 pmol, respectively.

#### **Total RNA Preparation from Rat Jejunal and Ileal Mucosa and Reverse Transcription-Polymerase Chain Reaction**

Total RNA was extracted from jejunal and ileal mucosa using ISOGEN (Nippon Gene Co., Ltd., Toyama, Japan). To prevent contamination with genomic DNA, the extracts were treated with DNase I (Invitrogen Co., Carlsbad, CA, USA). RNA concentration and purity were determined spectrophotometrically. One microgram of total RNA was reverse-transcribed using 5 pmol of oligo (dT) primer (Toyobo Co., Ltd., Osaka, Japan), 2 mM dNTP, and RNase H-free ReverTra Ace (Toyobo Co., Ltd., Osaka, Japan) with one cycle of reverse transcription reaction (42°C for 1 h). Reverse transcription samples were subsequently subjected to reverse transcription–polymerase chain reaction (RT–PCR). PCR was performed with Platinum *Taq* DNA polymerase (Invitrogen). The PCR conditions and the sequences of the forward and reverse primers are listed in Table 1. PCR was performed in the linear range to amplify segments of AB010635, AY034877, hydrolase B/C and GAPDH. The end-point of PCR cycles was determined by using mRNA from a rat jejunum which contained the highest esterase activity as a standard sample. Amplified PCR products were separated on 1.5% agarose gel and stained with

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ethidium bromide.

## Data Analysis

The *in situ* data was kinetically analyzed as described in a previous report (Masaki et al., 2006). The appearance rate ( $v_1$ ) of test compounds in the mesenteric vein was calculated by  $v_1 = Q_b \times C_b$ . The degradation rate ( $v_2$ ) of test compounds and the appearance rate ( $v_3$ ) of their metabolites in the intestinal lumen were calculated by  $v_2 = Q_l \times (C_{in} - C_{out})$  and  $v_3 = Q_l \times C_{M,out}$ , respectively, where  $Q_b$  and  $Q_l$  are the flow rates of vascular and intestinal perfusion, respectively,  $C_b$  is the concentration of test compound in the mesenteric vein,  $C_{in}$  and  $C_{out}$  are the concentrations of test compounds at the entrance and exit of the jejunal segment, respectively, and  $C_{M,out}$  is the concentration of their metabolites at the exit of the jejunal segment.  $C_{out}$  and  $C_{M,out}$  were corrected by the concentration of FD-4.

The apparent absorption clearance into the mesenteric vein ( $CL_{app}$ ) and the degradation clearance of isovaleryl-PL in the jejunal mucosa ( $CL_{deg}$ ) were calculated as follows:  $CL_{app} = AUC_{P,b} / AUC_{P,l} \times Q_b = \text{absorbed amount} / AUC_{P,l}$  and  $CL_{deg} = AUC_{M,b} / AUC_{P,l} \times Q_b + AUC_{M,l} / AUC_{P,l} \times Q_l = \text{degraded amount} / AUC_{P,l}$ , respectively, where  $AUC_{P,l}$  and  $AUC_{M,l}$  are the areas under the curve of parent compound and metabolite in the intestinal lumen at steady-state, respectively. It was assumed that the concentration of parent compound in the intestinal loop decreased according to first-order kinetics when calculating the AUC in the intestinal lumen.  $AUC_{P,b}$  and  $AUC_{M,b}$  are areas under the curve of parent compound and metabolite, respectively, in the mesenteric vein at steady-state.

For comparison of the absorption parameters with the reported values, the permeability rate constant ( $P_{eff}$ ) was calculated as:  $P_{eff} \text{ (cm/min)} = Q_l \times (1 - C_{out} / C_{in}) / 2\pi RL$ , where R,

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the radius of the segment, was assumed to be 0.178 cm (Yamashita et al., 1997) and L is the length of the segment (i.e., 10 cm).

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## RESULTS

### Determination of Treatment Conditions for Bis-*p*-nitrophenyl Phosphate (BNPP)

BNPP was perfused in the jejunal lumen at an initial concentration of 400  $\mu\text{M}$ , it was extremely slowly absorbed in the mesenteric vein and its appearance rate ( $v_{1, \text{BNPP}}$ ) was about 0.7 nmol/min at steady-state (Fig. 1). The disappearance of BNPP in luminal fluid was less than 3%. The appearance rate of BNPP in the mesenteric vein showed a large inter-individual variability, but steady-state was achieved in each jejunum loop 40 min after starting perfusion. The large inter-individual variability is thought to be due to the low absorbability of BNPP, due to its hydrophilicity, and the fact that the BNPP molecule taken up into the enterocyte is covalently bound to CES. Although morphological changes were not observed after 40 min treatment with BNPP at this concentration (data not shown), when the BNPP concentration in perfusate was increased to more than 800  $\mu\text{M}$ , histological damage was induced in the rat jejunum. Therefore, it was decided to pre-perfuse the jejunum loop with BNPP at 400  $\mu\text{M}$  for 40 min.

### Enzyme Activity of Jejunum Mucosal S9 Prepared after Perfusion with BNPP

BNPP has been reported to almost completely inhibit hydrolase activity of purified CES isozymes (Brandt et al., 1980). In order to confirm that treatment with BNPP at 400  $\mu\text{M}$  for 40 min has no effect on other hydrolases except CES, the remaining activity of esterase and aminopeptidase was measured. The BNPP-perfused jejunum was washed well to remove free BNPP and then its mucosa was stripped to prepare mucosal homogenate 9000g supernatant (S9). *p*-Nitrophenyl acetate (PNPA) and L-leucyl-*p*-nitronilide (Leu-*p*-NA) were selected as substrates for esterase and aminopeptidase, respectively. PNPA is hydrolyzed by several esterases, not only CES.

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Therefore, it is possible that PNPA might be hydrolyzed even in the mucosal S9 maximally inhibited by BNPP. The esterase activity for PNPA in jejunal S9 after BNPP treatment was significantly decreased to  $0.45 \pm 0.23$   $\mu\text{mol}/\text{min}/\text{mg}$  protein compared with a control activity of  $1.78 \pm 0.10$   $\mu\text{mol}/\text{min}/\text{mg}$  protein ( $p < 0.05$ ). The inhibition percent was about 75%. However, external addition of BNPP at 1 mM in the control jejunal S9 induced about 85% inhibition of PNPA hydrolysis. These data indicated that the present treatment condition with BNPP could inhibit about 90% of CES activity without any modulation of other esterases. In addition, hydrolysis of Leu-*p*-NA in the jejunal S9 was not affected by treatment with BNPP ( $39.0 \pm 1.89$  nmol/min/mg protein after BNPP treatment compared with a control value of  $38.7 \pm 5.02$  nmol/min/mg protein). This result indicated that the treatment of BNPP in *in situ* single-pass perfusion could specifically inhibit CES without any modulation of other esterase and aminopeptidase.

### **Effect of BNPP on Absorption of Leu-*p*-NA in Rat Jejunal Single-pass Perfusion**

In order to confirm the effect of BNPP on intestinal absorption, Leu-*p*-NA (500  $\mu\text{M}$ ), a non-ester compound, was perfused in the jejunal lumen with or without BNPP treatment (400  $\mu\text{M}$  for 40 min). When Leu-*p*-NA is perfused in the jejunum, Leu-*p*-NA is transported into the epithelial cell and then hydrolyzed to *p*-nitroaniline by aminopeptidase, expressed both in BBM and inside the cell. The *p*-nitroaniline formed in the epithelial cell is then transported into the mesenteric vein and jejunal lumen. In this experiment, *p*-nitroaniline was measured to evaluate the effect of BNPP on the aminopeptidase activity and transport characteristics of Leu-*p*-NA and *p*-nitroaniline. As shown in Fig. 2, the appearance rates of *p*-nitroaniline into the mesenteric vein and jejunal lumen after BNPP perfusion were not significantly different from those of a control in

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which only MES buffer was perfused (i.e., without BNPP). These results show that BNPP affects neither aminopeptidase activity nor the membrane transport of Leu-*p*-NA and/or *p*-nitroaniline. *p*-Nitroaniline converted from Leu-*p*-NA in the enterocyte was transported at a comparable rate in the jejunal lumen and the mesenteric vein. Since *p*-nitroaniline (pKa 1.0) in both sites is mainly present in the unionized form, *p*-nitroaniline in the mucosal cells may be transported to both luminal and venous sides at the same rate by passive diffusion. Thus, it was confirmed that treatment with BNPP (400  $\mu$ M) for 40 min could be used to evaluate the contribution of CES to hydrolysis during the absorption process in rat jejunum single-pass perfusion.

#### **Absorption of Isovaleryl-PL in Rat Jejunal Single-pass Perfusion after BNPP Treatment**

When isovaleryl-PL was perfused at 300  $\mu$ M in rat jejunum, with or without pre-treatment with BNPP (400  $\mu$ M for 40 min), steady-state was achieved after perfusion for 30 min in both cases. Absorption clearance ( $CL_{app}$ ) and degradation clearance ( $CL_{deg}$ ) of isovaleryl-PL were determined at steady-state. As shown in Table 2,  $CL_{app}$  of isovaleryl-PL after BNPP treatment was three-fold greater than that of control. The elevation of intracellular concentration of isovaleryl-PL resulted in the increase of  $CL_{app}$  could probably be due to the substrate escaped from being hydrolyzed by CES. The  $CL_{deg}$  of isovaleryl-PL after BNPP treatment decreased to 23% of control, indicating the inhibition of 77% of the intestinal hydrolysis of isovaleryl-PL. Since the pre-treatment condition with BNPP could inhibit about 90% of CES activity, these data suggest that CES contributes about 85% of the intestinal hydrolysis of isovaleryl-PL during absorption. The permeability rate constant ( $P_{eff}$ ) of isovaleryl-PL after BNPP treatment



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was decreased to half of control values.

### **Inhibition Study of CES in Rat Jejunal and Ileal S9**

An inhibition study using BNPP was performed to determine the contribution of CES in the hydrolysis of valeryl-PL and isovaleryl-PL in jejunal and ileal S9. BNPP inhibited the hydrolysis of both substrates in dose-dependent manner. Both jejunal and ileal S9 showed a similar inhibition curve (Fig. 3). The  $IC_{50}$  for valeryl- and isovaleryl-PL was about 0.1  $\mu$ M. Furthermore, the residual hydrolytic activity for valeryl- and isovaleryl-PL was  $9.00 \pm 1.40\%$  and  $16.4 \pm 2.97\%$ , respectively, at 100  $\mu$ M of BNPP. These data suggest that CES was mainly responsible for the hydrolysis of PL ester compounds in rat intestine, with other esterases expressed in the S9 fraction contributing about 10–20%.

### ***In Vitro* Hydrolase Activity in Rat Jejunal and Ileal S9**

It has been reported that the expression level and activity of P-450 isozymes are decreasing along with the length of the small intestine from the duodenum to the ileum (De Waziers et al., 1990; Liu et al., 2006). However, the distribution of CES isozyme has not been determined yet. Therefore, hydrolase activity was measured in jejunal and ileal S9. Table 3 lists the hydrolase activity for each enantiomer of the racemic PL ester derivatives (butyryl-, valeryl-, isobutyryl- and isovaleryl-PL) in jejunal and ileal S9. There was no significant enantio-selectivity in hydrolase activity for the various racemic PL derivatives. The hydrolysis of butyryl- and valeryl-PL in jejunal S9 was significantly faster than in ileal S9. The hydrolase activity for isobutyryl- and isovaleryl-PL in the jejunum was higher than the ileum, although significant difference was not observed due to low activity with relatively large inter-individual variation.

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Enzyme kinetic parameters for hydrolysis of racemic valeryl-, isovaleryl-PL and PNPA were calculated by fitting the data to the Michaelis–Menten equation by nonlinear least-squares analysis, using the MULTI program (Table 4). The  $V_{\max}$  values for valeryl-PL, isovaleryl-PL and PNPA were 1.7- 2.5-fold higher in the jejunum than in the ileum, although their  $K_m$  values were nearly same in jejunal and ileal S9. These data suggest that the higher hydrolase activity of the jejunum might be due to the higher expression level of esterases. Furthermore, PL derivatives had a much smaller  $K_m$  value than PNPA, suggesting that valeryl- and isovaleryl-PL have a higher affinity for esterase than PNPA.

#### **Expression Level of CES Isozyme mRNA in the Jejunum and Ileum**

Figure 4a shows the result of RT-PCR for two major CES2 isozymes, AB010635 and AY034877, in the jejunum and ileum. The relative expression levels of AB010635 and AY034877, shown in Fig. 4b, were about 1.5-fold and 1.6-fold higher, respectively, in the jejunum than in the ileum. Hydrolase B/C, which belongs to the CES1 group, was also detectable in the rat intestine under annealing conditions of 56°C, 30 cycles, using twice the amount of initial total RNA that was used in the measurement of AB010635. The expression level of hydrolase B/C was much lower than CES2 isozymes in rat intestine (data not shown). Although hydrolase B/C does not affect intestinal hydrolase activity, it was observed that the mRNA level of hydrolase B/C was about 10-fold higher in the jejunum than in the ileum.

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## DISCUSSION

Previously, we evaluated intestinal first-pass hydrolysis using isovaleryl-PL as a model ester compound (Masaki et al., 2006; Yoshigae et al., 1998). The capacity for intestinal hydrolysis was remarkable and the degradation clearance was limited only by the rate of isovaleryl-PL uptake in the mucosal cells. In the present study, we have identified the major hydrolytic enzyme responsible for the extensive intestinal first-pass hydrolysis.

CES is the preferential candidate as the major intestinal esterase. Therefore we performed the *in situ* perfusion experiment under CES inhibition. Recently, Quinney et al. (2005) reported that loperamide, an opioid compound, competitively inhibited CES, especially human CES2 isozyme, hCE2, with a  $K_i$  of 1.5  $\mu\text{M}$ . However, the effect of loperamide on the activity of other hydrolases is not mentioned in detail, and reduction of gastro-intestinal motility by loperamide might affect drug absorption in other ways (Callreus et al., 1999). On the other hand, bis-*p*-nitrophenyl phosphate (BNPP) is well known as a potent and specific inhibitor of CES isozymes (Mentlein et al., 1988; Block & Arndt, 1978; Heymann & Krisch, 1967). We first determined that BNPP noncompetitively inhibited PNPA hydrolysis in the rat jejunal S9 with a  $K_i$  value of  $44.9 \pm 4.95$  nM. Since BNPP inhibits CES with a low  $K_i$  value by covalent binding, we surmised that a CES-inhibited condition might be obtained by a short pre-perfusion with BNPP at low concentrations. In fact, pre-perfusion with 400  $\mu\text{M}$  BNPP for 40 min inhibited around 90% of CES activity and this inhibition continued after washout of BNPP. BNPP specifically inhibited CES without any inhibition of aminopeptidase activity or transport of L-leucyl-*p*-nitroanilide (Leu-*p*-NA) and *p*-nitroaniline in our *in situ* experiment. When we further examined the effect of BNPP on Caco-2 cell membrane transport, it was observed that BNPP affected neither active transport, e.g. P-glycoprotein and peptide

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transporters, nor passive diffusion (data not shown).

When isovaleryl-PL was perfused into the jejunum lumen without BNPP treatment (Fig. 5a; control condition), isovaleryl-PL was extensively hydrolyzed to PL and isovaleric acid (IVA) in the mucosal tissue at a rate nearly the same as its uptake rate into cells (Masaki et al., 2006). Therefore, the intracellular concentration of isovaleryl-PL was low (97.7 nmol/g tissue), resulted in the low efflux of isovaleryl-PL into both direction of absorption (vascular side) and secretion (luminal side). Consequently, the apparent absorption clearance of isovaleryl-PL ( $CL_{app}$ ) was remarkably low and its  $P_{eff}$  ( $16.6 \times 10^{-3}$  cm/min) was relatively large. Furthermore the hydrolysates, PL and IVA, were transported by passive diffusion according to pH-partitioning theory into vascular and luminal compartments, as described in a previous report (Masaki et al., 2006). Under pre-treatment condition with BNPP (Fig. 5b), the  $CL_{deg}$  was reduced to 32.5  $\mu$ L/min due to inhibition of mucosal hydrolysis, following the increase of intracellular concentrations of isovaleryl-PL. Since isovaleryl-PL is passively transported through the epithelial membrane in the same way as PL, the increased intracellular concentration resulted in the higher apparent absorption clearance of isovaleryl-PL ( $CL_{app}$ ). Furthermore isovaleryl-PL could be secreted also into luminal compartment following pH-partitioning theory. Finally,  $P_{eff}$  ( $7.84 \times 10^{-3}$  cm/min) was decreased to half value of control.

The membrane permeability of PL is great enough to achieve complete absorption (Walter et al., 1996). Therefore, it might be predicted that the  $P_{eff}$  of isovaleryl-PL under non-hydrolyzing conditions is identical to that of PL in PL perfusion. However, the  $P_{eff}$  of isovaleryl-PL after BNPP pre-treatment was still 3.5-fold larger than the reported  $P_{eff}$  of PL ( $2.20 \times 10^{-3}$  cm/min; Masaki et al. 2006). The enzymes showing residual hydrolyzing activity could form PL that is effluxed from the cell. The intestinal hydrolysis of prodrug

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intrinsically increased its membrane permeability due to the presence of two molecules, prodrug and parent drug, in the mucosal cell.

A comparison of  $CL_{deg}$  between the control and BNPP pre-treated conditions, suggested that CES accounted for about 85% of hydrolysis of isovaleryl-PL in the rat jejunal single-pass perfusion experiment (Table 2). Interestingly, this value is identical to that of the inhibition of isovaleryl-PL hydrolysis by BNPP in jejunal S9 (84%). These results suggest that CES-mediated *in vivo* hydrolysis of test ester compounds could be estimated by *in vitro* BNPP inhibition in the S9 fraction. Since human CES isozymes are involved in 97–99% of the hydrolysis of isovaleryl- and valeryl-PL in human jejunal and ileal S9 (preliminary data), human CES may be responsible for more than 95% of hydrolysis of isovaleryl-PL during *in vivo* absorption. The identity of the major hydrolyzing enzyme in the human intestine will be confirmed in an inhibition study of numerous ester compounds in human small intestinal S9.

Satoh & Hosokawa (2006) have reported on the intestinal expression of CES2 isozymes in humans and experimental animals. In human intestine, the mRNA expression level of the CES2 isozyme, hCE2, is slightly higher in the jejunum than in the ileum according to northern blot analysis (Quinney et al., 2005), while Van Gelder et al. (2000) reported slightly higher hydrolase activity in rat jejunum only. In the present study, we demonstrated a proximal-to-distal decrease of CES isozymes based on enzyme kinetic analysis and mRNA expression level. In a recent report, three isozymes of the rat CES2 family, D50580, AY034877 and AB010635, have been cloned from rat liver (Sone & Wang, 1997; Sanghani et al., 2002). However, D50580 could hardly be detected using RT-PCR (data not shown), which agrees with previous results for northern blot analysis (Sanghani et al., 2002). AB010635, encoding for RL4, was the most abundant of the three

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CES2 isozymes expressed in Wistar rat intestine. This suggests that AB010635 and AY034877 are the most important isozymes for hydrolysis of isovaleryl-PL in rat intestine.

In the present study, the rat intestine showed non-enantioselective hydrolysis of PL ester derivatives (Table 3). This finding was different from *S*- and *R*-preferential hydrolysis of rat liver and plasma, respectively (Yoshigae et al., 1997). Human intestinal microsomes and purified hCE2 also showed non-enantioselective hydrolysis (Imai et al., 2006). Furthermore, the substrate specificity of rat intestine, i.e. fast hydrolysis for straight acyl PL-derivatives, moderate hydrolysis for isobutyryl-PL, and markedly low hydrolysis for isovaleryl-PL, was quite similar to that of purified hCE2, despite the expression of two major isozymes in rat intestine. hCE2 was 70% and 67% identical to amino acid sequences encoded by AB010635 and AY034877, respectively, while AB010635 and AY034877 were 74% identical. Although these CES2 isozymes commonly have an endoplasmic reticulum retention signal at the C-terminus and catalytic triad residues, they differ in their glycosylation sites (there are two glycosylated Asn residues in hCE2 compared with none and one in AB010635 and AY034877, respectively). In spite of these differences, rat and human intestinal CESs have many functional similarities. Thus, the hydrolysis of ester compounds in the human intestine may be predicted using the *in situ* rat intestinal perfusion model, although it will be necessary to clarify the similarity of the hydrolyzing characteristics of human and rat intestines.

CES2 isozymes were shown to be the major intestinal esterases in first-pass hydrolysis and with higher expression levels in the jejunum than in the ileum. Interestingly, it may be possible to estimate the contribution of CES to intestinal hydrolysis during absorption

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from *in vitro* hydrolysis in the S9 fraction. Furthermore, it was proposed that mucosal hydrolysis increased the intestinal permeation of ester-containing drugs due to lowering of its intracellular concentration. Therefore, substrate specificity for CES2 isozymes will be helpful in the development of oral prodrugs.

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#### Footnote

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**Fig. 1.** Absorption of BNPP in the rat jejunal single-pass perfusion experiment. Data represents the appearance rate of BNPP in the mesenteric vein. Rat jejunal loop (10 cm) was perfused with 400  $\mu$ M BNPP at flow rate of 3.0 and 0.3 mL/min in the mesenteric vein and jejunal lumen, respectively. Each point represents mean  $\pm$  S.D. ( $n = 3$ ).

**Fig. 2.** Absorption and secretion of *p*-nitroaniline derived from 500  $\mu$ M Leu-*p*-NA in rat jejunal single-pass perfusion experiment, with or without BNPP treatment. a) and b) represent the appearance rate of *p*-nitroaniline in the mesenteric vein and in the jejunal lumen, respectively. Closed and open circles represent the appearance rate of *p*-nitroaniline for the control and CES-inhibited condition, respectively. The BNPP treatment was performed the perfusion with 400  $\mu$ M BNPP for 40 min before the perfusion of Leu-*p*-NA. The perfusion flow rate was 3.0 and 0.3 mL/min for the mesenteric vein and jejunal lumen, respectively. Each point represents mean  $\pm$  S.D. ( $n = 3$ ).

**Fig. 3.** Inhibition of the hydrolase activity for valeryl- and isovaleryl-PL by BNPP in rat jejunal and ileal S9. Closed circles and squares represent the hydrolase activity for valeryl- and isovaleryl-PL remaining in the jejunal S9, respectively. Open circles represents the activity for valeryl-PL remaining in the ileal S9. Both jejunal and ileal S9 were diluted with pH 7.4 HEPES buffer (50 mM) to 20–25  $\mu$ g/mL. Substrate concentration was 100  $\mu$ M for valeryl- and isovaleryl-PL. The remaining hydrolase activity was plotted against the logarithm of BNPP concentration. Each point represents mean  $\pm$  S.D. ( $n = 3$ ).

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**Fig. 4.** RT-PCR of carboxylesterase isozymes in rat jejunum and ileum. RT-PCR was performed on the total RNA of both the jejunum and ileum. a) mRNA expression of CES isozymes in the jejunum and ileum. Amplified PCR products of GAPDH, AB010635 and AY034877 were detected by staining with ethidium bromide. b) mRNA expression ratio of CES isozymes in the jejunum compared with the ileum. Data represent the mRNA expression level of each CES isozyme in the jejunum normalized by that in the ileum ( $n = 4$ ). The mRNA level for both CES2 isozymes in the jejunum is significantly higher than in the ileum ( $p < 0.05$ ).

**Fig. 5.** Scheme of intestinal absorption of isovaleryl-PL

**TABLE 1**

The conditions for RT-PCR and the sequences of the forward and reverse primers

	Sequence of primer	Position	Denaturation	Annealing	Extension	Cycles
GAPDH <sup>a</sup>	5'-ACCACAGTCCATGCCATCAC (forward)	1369–1388	95°C, 15 s	59°C, 30 s	72°C, 30 s	26 cycles
	5'-TCCACCACCCTGTTGCTGTA (reverse)	1820–1801				
AB010635 <sup>b</sup>	5'-ACGGTCTCCACTACAGTGGC (forward)	811–830	95°C, 15 s	59°C, 30 s	72°C, 32 s	27 cycles
	5'-AATAGCTGGGTGCATGTTGG (reverse)	1339–1320				
AY034877 <sup>b</sup>	5'-AATCTGAGGTGGTCTACAAG (forward)	794–813	95°C, 15 s	58°C, 30 s	72°C, 33 s	32 cycles
	5'-TGCTTGATGAAGCTGGGCAG (reverse)	1337–1318				
Hydrolase B/C <sup>c</sup>	5'-CCAAAGACCCAAGGATGTAG (forward)	1382–1401	95°C, 15 s	56°C, 30 s	72°C, 20 s	30 cycles
	5'-TGAGGTTGTCTCTTAGCCAG (reverse)	1678–1659				

<sup>a</sup> glyceraldehyde-3-phosphate dehydrogenase (GAPDH: Gene Bank accession number; NM017008)<sup>b</sup> AB010635 and AY034877 were Gene Bank accession numbers.<sup>c</sup> Hydrolase B / Hydrolase C (Gene Bank accession numbers: X81825 for hydrolase B, U10698 for hydrolase C)



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**TABLE 2**

Kinetic parameters for absorption of isovaleryl-PL in rat single-pass perfusion experiment, with or without BNPP treatment.

	Control condition	CES-inhibited condition <sup>a</sup>
CL <sub>app</sub> (μL/min)	2.42 ± 0.79	7.60 ± 0.74**
CL <sub>deg</sub> (μL/min)	141 ± 20.5	32.5 ± 5.4**
P <sub>eff</sub> (×10 <sup>-3</sup> cm/min)	16.6 ± 1.26	7.84 ± 2.10**

<sup>a</sup> In the CES-inhibition condition, the jejunum was perfused with 400 μM BNPP for 40 min before perfusion with isovaleryl-PL.

\*\* Statistical significance refers to the difference between kinetic parameters in the CES-inhibited condition and that in the control condition ( $p < 0.01$ ).

Values represent mean ± S.D. ( $n = 3$ ).

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**TABLE 3**

Hydrolase activity in rat jejunal and ileal S9.

	Jejunum		Ileum	
<b>Hydrolysis of PL derivatives<sup>a</sup></b>	nmol/min/mg protein		nmol/min/mg protein	
Substrate	<i>R</i>	<i>S</i>	<i>R</i>	<i>S</i>
Butyryl-PL	101 ± 18.2*	81.5 ± 24.7*	51.7 ± 6.84	45.5 ± 11.2
Valeryl-PL	108 ± 12.0*	121 ± 13.6*	73.5 ± 18.0	79.8 ± 7.63
Isobutyryl-PL	57.0 ± 16.1*	49.4 ± 14.6	46.8 ± 16.7	45.8 ± 19.0
Isovaleryl-PL	7.21 ± 1.85	9.26 ± 2.39	4.10 ± 1.20	6.19 ± 4.69

<sup>a</sup> The jejunal and ileal S9 were diluted with pH 7.4 HEPES (50 mM) at 30 µg/mL and 55 µg/mL, respectively. The concentration of PL ester derivatives was 100 µM.

\* Statistical significance refers to the difference between activity in the jejunum compared with that in the ileum. Hydrolase activity in the jejunum is significantly higher than in the ileum ( $p < 0.05$ ).

Each point represents mean ± S.D. ( $n = 3$ ).

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**TABLE 4**

Kinetic parameters for hydrolysis of valeryl-PL, isovaleryl-PL and PNPA in rat intestinal S9.

	$K_m$ ( $\mu\text{M}$ )	$V_{\max}$ (nmol/min/mg protein)	$V_{\max}/K_m$ (mL/min/mg protein)
Valeryl-PL <sup>a</sup>			
Jejunum	11.2 $\pm$ 3.40	256 $\pm$ 43.1*	23.8 $\pm$ 4.20
Ileum	9.09 $\pm$ 2.28	149 $\pm$ 39.8	16.9 $\pm$ 4.88
Isovaleryl-PL <sup>a</sup>			
Jejunum	13.7 $\pm$ 1.71	29.1 $\pm$ 3.81*	2.12 $\pm$ 0.27
Ileum	8.60 $\pm$ 2.08	11.8 $\pm$ 3.40	1.39 $\pm$ 0.30
PNPA <sup>b</sup>	( $\mu\text{M}$ )	( $\mu\text{mol/min/mg protein}$ )	(mL/min/mg protein)
Jejunum	338 $\pm$ 67.4	2.65 $\pm$ 0.31*	7.93 $\pm$ 0.70**
Ileum	414 $\pm$ 54.0	1.45 $\pm$ 0.40	3.57 $\pm$ 1.09

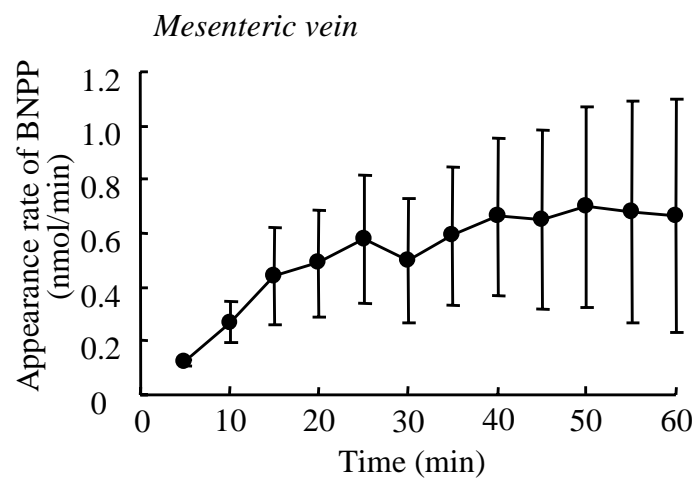
<sup>a</sup> The intestinal S9 were diluted with pH 7.4 HEPES buffer (50 mM) at 5 and 25  $\mu\text{g/mL}$ . Valeryl- and isovaleryl-PL concentrations were 2  $\mu\text{M}$  to 200  $\mu\text{M}$ .

<sup>b</sup> The intestinal S9 were diluted with pH 7.4 HEPES buffer (50 mM) at 25–45  $\mu\text{g/mL}$ . PNPA concentrations were 25  $\mu\text{M}$  to 500  $\mu\text{M}$ .

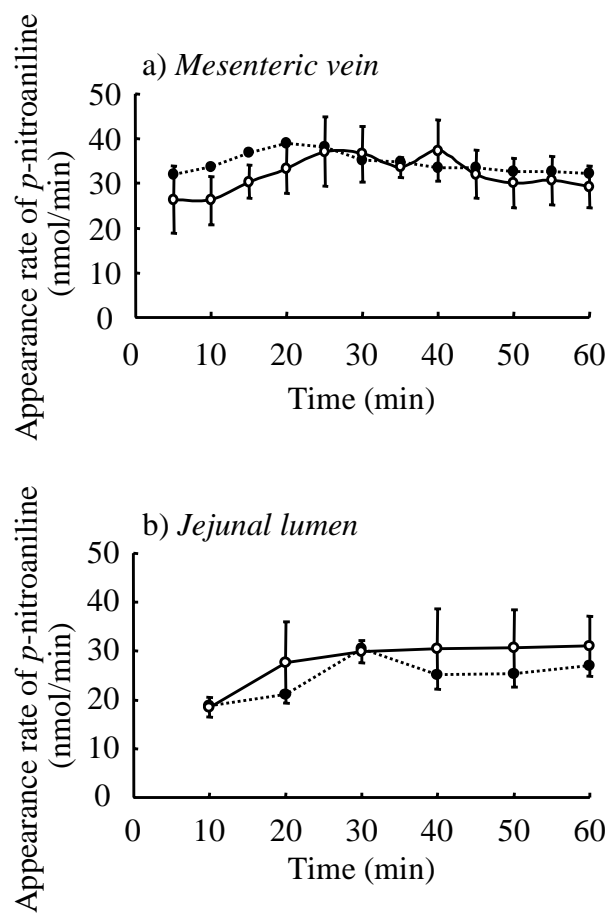
\* Statistical difference of activity in the jejunum against to that in the ileum represents. Hydrolase activity in the jejunum is significantly higher than in the ileum (\*;  $p < 0.05$ , \*\*;  $p < 0.01$ ).

$K_m$  and  $V_{\max}$  were calculated by fitting the data to the Michaelis–Menten equation by nonlinear least-squares analysis, using the MULTI program. Values represent mean  $\pm$  S.D. ( $n = 3$ ).

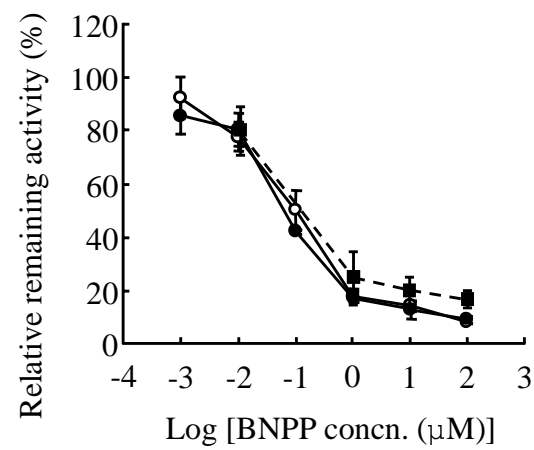
**Fig. 1.**



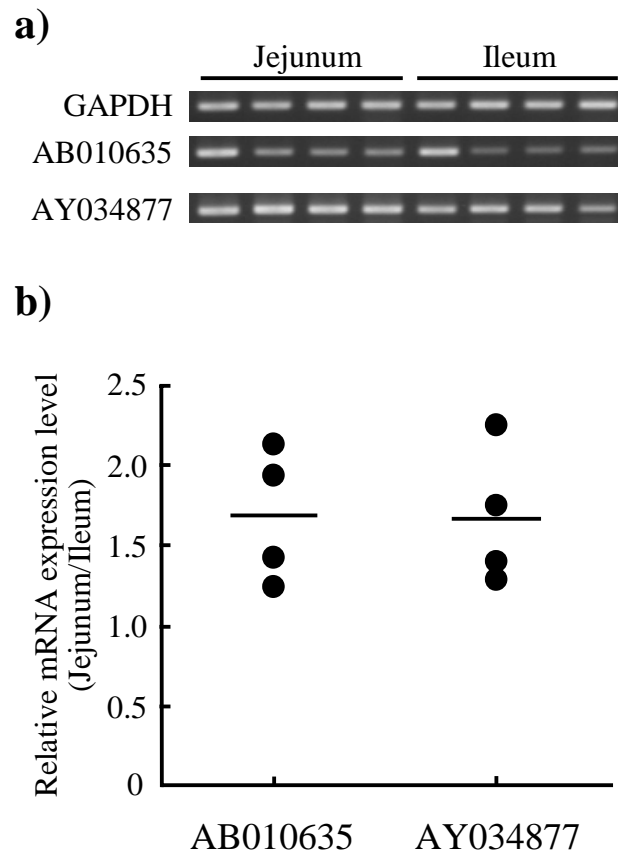
**Fig. 2.**



**Fig. 3.**



**Fig. 4.**



**Fig. 5.**

