

Characterization of Species Differences in Tissue Diltiazem Deacetylation Identifies *Ces2a* as a Rat-Specific Diltiazem Deacetylase[§]

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ABSTRACT

Diltiazem, a calcium channel blocker, is mainly metabolized via demethylation or deacetylation in humans. Diltiazem demethylation is catalyzed by cytochrome P450 2D6 and 3A4. Although it was previously reported that the area under the curve ratio of deacetyldiltiazem to diltiazem after oral dosing with diltiazem in rats was sevenfold higher than in humans, the molecular mechanisms underlying this species difference remain to be clarified. In the present study, we compared the diltiazem deacetylase activity in liver, intestinal, renal, and pulmonary microsome preparations of human and experimental animal tissues to identify the specific deacetylase enzyme(s) involved in deacetylation. Diltiazem deacetylase activity was detected in rat liver and small intestine microsome preparations, but not in those from human, monkey, dog, and mouse tissues. Further purification of rat liver microsome

(RLM) proteins identified four carboxylesterase (Ces) enzymes (*Ces1d*, *Ces1e*, *Ces1f*, and *Ces2a*) as potential candidate deacetylases. On the basis of their tissue distribution, the *Ces2a* enzyme was considered to be the enzyme that was responsible for diltiazem deacetylation. Furthermore, recombinant rat *Ces2a* expressed in Sf21 cells displayed efficient diltiazem deacetylase activity with similar *K_m* values as RLM. In addition, the inhibitory characteristics of various chemical inhibitors were similar between recombinant rat *Ces2a* and RLM. In conclusion, we determined that only rat tissues were able to catalyze diltiazem deacetylation. The characterization of Ces enzymes in animal species, as undertaken in this study, will prove useful to predict the species-specific pharmacokinetics differences between the *in vivo* models used for drug development.

Introduction

The calcium channel blocker diltiazem, which is widely used for treating hypertension and angina pectoris, is metabolized via *N*-demethylation, deacetylation, and *O*-demethylation (Fig. 1) (Yeung et al., 1990; Molden et al., 2002). *N*-demethylation and deacetylation are the major metabolic pathways, and the area under the curve (AUC) ratio of *O*-demethyldiltiazem, deacetyldiltiazem, and *N*-demethyldiltiazem reported in humans was 1:3.4:28, respectively (Molden et al., 2002). *N*-Demethyldiltiazem and deacetyldiltiazem have coronary vasodilation activity, with approximately one-fifth and one-half the potency of diltiazem, respectively (Yabana et al., 1985). In humans, diltiazem *N*- and *O*-demethylation are catalyzed by cytochrome P450 (CYP) 3A4 and 2D6, respectively, whereas the esterase(s) responsible for diltiazem deacetylation has not been identified.

Esterases are expressed in various organs, including the liver, small intestine, kidneys, and lungs, and contribute to the hydrolysis of a number of clinically used drugs containing ester, amide, or thioester bonds (Fukami and Yokoi, 2012). Carboxylesterases (CES) are well-known to catalyze the hydrolysis of various drugs and xenobiotics

(Laizure et al., 2013) and lipid metabolism (Zhao et al., 2005). In mammals, CES enzymes are divided into five families. The CES1 and CES2 families are primarily involved in drug hydrolysis. In humans and dogs, both the CES1 and CES2 families consist of a single isoform. In the cynomolgus monkey, although the CES1 family consists of a single isoform, the CES2 family appears to consist of two isoforms (Williams et al., 2010, 2011). In contrast, a number of CES isoforms exist in rodents. Eight *Ces1* isoforms (*Ces1a*–*Ces1h*) and eight *Ces2* isoforms (*Ces2a*–*Ces2h*) are found in mice. In rats, five *Ces1* isoforms (*Ces1a*–*Ces1f*) and seven *Ces2* isoforms (*Ces2a*–*Ces2j*) exist (Holmes et al., 2010). According to a previous report (Imai, 2006), in primates, CES1 is mainly expressed in the liver and CES2 is highly expressed in the small intestine, kidneys, and liver. In dogs, both CES1 and CES2 are expressed in the liver, but neither is expressed in the small intestine. In mice, most *Ces1* isoforms are expressed in the liver, and only a portion, such as *Ces1e*, *Ces1f*, and *Ces1g*, is expressed in the small intestine, whereas *Ces2* isoforms are mostly expressed in the small intestine (Jones et al., 2013). The expression profile of *Ces* isoforms in rats remains to be comprehensively determined.

In humans, the substrate specificity of the CES1 and CES2 enzymes can partly be explained by the relative size of the acyl and alcohol groups found within the compound (Fukami and Yokoi, 2012). Indeed, CES1 prefers compounds with a large acyl group and a small alcohol group, whereas CES2 prefers compounds with a small acyl group and a large alcohol group. Recently, Ozaki et al. (2013) measured

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ABBREVIATIONS: AADAC, arylacetamide deacetylase; AUC, area under the curve; BNPP, bis(4-nitrophenyl) phosphate; CES, carboxylesterase; CYP, cytochrome P450; DFP, diisopropyl fluorophosphate; DMSO, dimethylsulfoxide; HPLC, high-performance liquid chromatography; NaF, sodium fluoride; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride; RLM, rat liver microsome; RT-PCR, reverse transcription-polymerase chain reaction; sRLM, solubilized RLM.

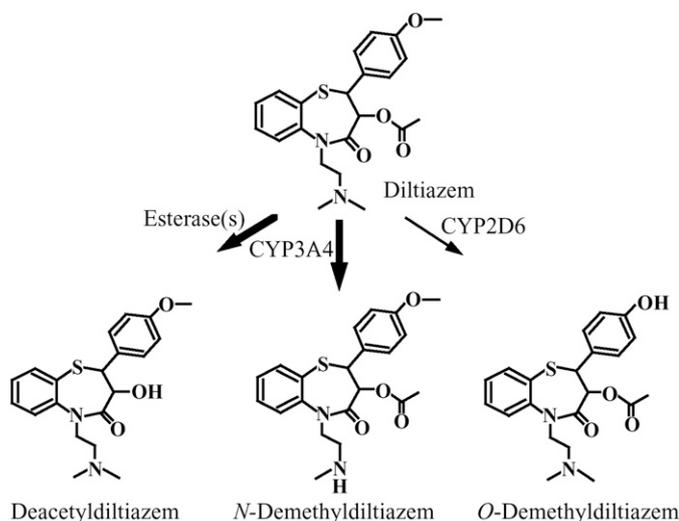


Fig. 1. Diltiazem metabolic pathways in humans.

the hydrolase activity of several rat Ces enzymes toward paraben derivatives and found that the substrate specificity of the Ces1 and Ces2 families does not necessarily correspond with that in humans. In addition, it has been shown that most substrates were hydrolyzed by multiple isoforms in rats (Robbi and Beaufay, 1994; Sanghani et al., 2002). Because the substrate specificity of each Ces isoforms in rats remains to be characterized, it is very difficult to predict the rat isoforms that are involved on the basis of their chemical structures.

Yeung et al. (1990) reported that the deacetyldiltiazem to diltiazem AUC ratio after a single oral dose of diltiazem was higher in rats (0.82) than in humans (0.12), dogs (0.58), and rabbits (0.15), implying the existence of species differences in diltiazem deacetylase activity. These previous observations prompted us to clarify the molecular mechanisms behind this particular species difference between humans and experimental animals.

Materials and Methods

Chemicals and Reagents. Diltiazem hydrochloride, deacetyldiltiazem, phenylmethylsulfonyl fluoride (PMSF), diisopropyl fluorophosphate (DFP), and eserine sulfate were purchased from Wako Pure Chemical Industries (Osaka, Japan). Bis(4-nitrophenyl) phosphate (BNPP) sodium salt, sodium fluoride (NaF), and ethopropazine hydrochloride were obtained from Sigma-Aldrich (St. Louis, MO). All primers were commercially synthesized at Hokkaido System Sciences (Sapporo, Japan). Other chemicals were of the highest commercially available grade.

Tissue Microsomes. Human liver (pooled, $n = 50$) and intestinal microsomes (pooled, $n = 7$) were purchased from Corning (Corning, NY). Human renal (pooled, $n = 6$) and pulmonary microsomes (individual) were purchased from KAC (Kyoto, Japan). Monkey liver (pooled, $n = 10$, males) and intestinal microsomes (pooled, $n = 10$, males) and dog liver (pooled, $n = 8$, male) and intestinal microsomes (pooled, $n = 3$, males) were obtained from Xenotech (Lenexa, KS). Protease inhibitors, such as phenylsulfonyl fluoride, leupeptine, aprotinin, and bestatin, were not included in the suspension buffer.

Pooled liver, jejunum, renal, and pulmonary microsomes were prepared from three rats (7-week-old Sprague-Dawley male rats, 210–230 g) and three mice (6-week-old C57BL/6J male mice, 20–25 g) purchased from SLC Japan (Hamamatsu, Japan), according to the method described in our previous study (Kobayashi et al., 2012). For the purification of diltiazem deacetylating enzyme(s), rat liver microsomes (RLM) were prepared from five pooled samples from 7-week-old Sprague-Dawley female rats (140–160 g). The protein concentrations were determined according to the Bradford method (1976) using γ -globulin as a standard.

Human Plasma. Human plasma samples (from five healthy Japanese volunteers: 22- to 30-year-old males) were obtained according to our previous report (Hioki et al., 2011). The use of human plasma samples was approved by the Ethics Committees of Kanazawa University (Kanazawa, Japan; No. 216).

Diltiazem Deacetylase Activity. Diltiazem deacetylase activities were determined as follows: a typical incubation mixture (final volume of 0.2 ml) contained 100 mM potassium phosphate buffer (pH 7.4) and enzyme sources (tissue microsomes and rat liver cytosol, 0.2 mg/ml; solubilized RLM [sRLM], 0.5 mg/mL; 50–70% ammonium sulfate precipitated fraction, 20 μ L; DEAE Sephacel fraction, 20 μ L; Superdex 200 fraction, 10 μ L; hydroxyapatite chromatography fraction, 20 μ L; Sf21 cell homogenates expressing esterases, 0.1 mg/ml; plasma, 2.5 μ L). In a preliminary study, we confirmed that the rates of deacetyldiltiazem formation were linear with respect to the protein concentration (<1.4 mg/ml) and incubation time (<50 minutes). Diltiazem was dissolved in distilled water. The reactions were initiated by the addition of diltiazem (at a final concentration of 200 μ M) after a 2-minute preincubation period at 37°C. After the 30-minute incubation, the reactions were terminated by the addition of 200 μ l ice-cold methanol. The supernatant obtained by centrifugation at 12,000g for 5 minutes was diluted 10-fold with mobile phase, and a 40- μ L aliquot of the diluted supernatant was subjected to high-performance liquid chromatography (HPLC). The HPLC analysis was performed using an L-2130 pump (Hitachi, Tokyo, Japan), an L-2200 autosampler (Hitachi), an L-2400 UV detector (Hitachi), and a D-2500 Chromato-Integrator (Hitachi) equipped with a Wakopak eco-ODS column (5 μ m particle size, 4.6 mm i.d. \times 150 mm; Wako Pure Chemical Industries). The eluent was monitored at 237 nm with a noise-base clean Uni-3 (Union, Gunma, Japan), which can reduce the noise by integrating the output and increasing the signal by threefold after differentiating the output and by an additional fivefold after amplification with an internal amplifier, resulting in the maximal 15-fold amplification of the signal. The mobile phase was composed of a 35% methanol/27% acetonitrile solution containing 5 mM ammonium acetate and 0.02% triethylamine. The flow rate was set at 1.0 ml/min. The column temperature was set at 35°C. The quantification of deacetyldiltiazem was obtained by comparing the HPLC peak height with that of an authentic standard. Because diltiazem is partially deacetylated in a nonenzymatic manner, the total amount of deacetyldiltiazem in the control mixture, incubated without the enzyme sources, was subtracted from the amount of deacetyldiltiazem obtained in the presence of the enzyme sources. The activities were measured in triplicate, and the mean value was calculated.

The kinetic analyses of diltiazem deacetylation were performed between 50 and 1000 μ M. The parameters were estimated from fitted curves using software (KaleidaGraph, Synergy Software, Reading, PA) designed for nonlinear regression analyses.

Purification of the Diltiazem Deacetylating Enzymes. All of the procedures were carried out at 4°C. The RLM were solubilized in 100 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol, 1 mM EDTA, 0.1% tertigol type-NP9, and 0.3% sodium cholate for 8 hours and were then centrifuged at 105,000g for 1 hour. The collected supernatant represented the sRLM. Proteins in the sRLM precipitated between 50 and 70% saturated ammonium sulfate were dissolved in 20 mM Tris-HCl buffer (pH 7.4) containing 20% glycerol, 1 mM EDTA, and 0.1% tertigol type-NP9 and then were dialyzed against the same buffer to remove the ammonium sulfate. The dialyzed fraction was applied to a DEAE Sephacel anion exchange column (2.5 \times 4.0 cm; GE Healthcare, Buckinghamshire, UK) equilibrated with 20 mM Tris-HCl buffer (pH 7.4) containing 20% glycerol, 1 mM EDTA, and 0.1% tertigol type-NP9. The proteins were eluted using the gradient method in addition to the equilibration buffer containing 1 M KCl at a rate of 0.5 mL/min. The eluate fractions were collected continuously as 5-mL aliquots, and their protein concentrations were determined according to the Bradford method (Bradford, 1976). The diltiazem deacetylation activity of the fractions was evaluated, as described above. Fractions showing activity (fractions 20–23) were pooled and centrifuged at 4000–5000g using a Centricon YM-30 (Millipore, Billerica, MA) to concentrate the proteins and exchange the buffer. Concentrated proteins were diluted in 50 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol, 150 mM NaCl, and 0.1% tertigol type-NP9. The diluted proteins were then applied to a Superdex 200 10/300 GL gel filtration column (GE Healthcare) equilibrated with 50 mM potassium phosphate

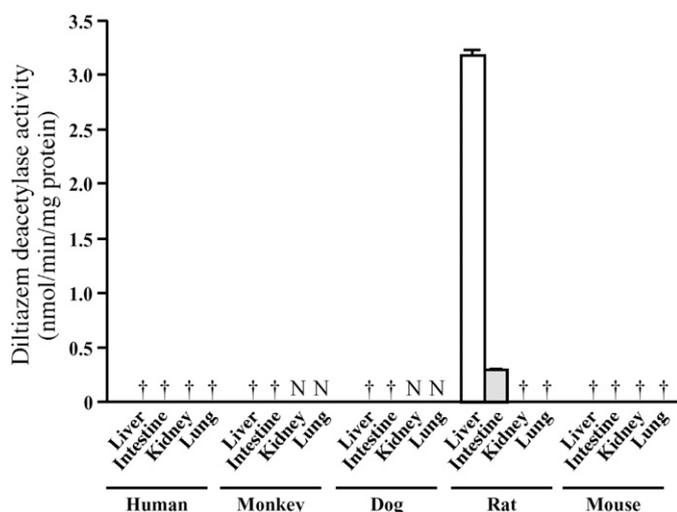


Fig. 2. Diltiazem deacetylase activity in liver, small intestine, kidney, and lung microsomes from human, monkey, dog, rat, and mouse. Tissue microsomes (0.2 mg/mL) were incubated with diltiazem (200 μ M) for 30 minutes. Kidney and lung microsomes from monkey and dog were not available commercially. Each column represents the mean \pm S.D. of triplicate determinations. †, Not detected; N, not available.

buffer (pH 7.4) containing 20% glycerol, 150 mM NaCl, and 0.1% tertigol type-NP9. The proteins were eluted using the same buffer at a rate of 0.25 mL/min, and the diltiazem deacetylase activity of each 0.5-mL fraction was evaluated. Fractions with enzyme activity (fractions 30–32) were pooled and applied to a hydroxyapatite column (1.5 \times 3.0 cm; Bio-Rad, Hercules, CA) equilibrated with 100 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol, 1 mM EDTA, and 0.1% tertigol type-NP9. The proteins were then eluted using the gradient method with the addition of an equilibration buffer at 300 mM. The eluted fractions (1 mL) were then subjected to SDS-PAGE, followed by silver staining. The diltiazem deacetylase activity of the fractions containing proteins weighing approximately 60 and 70 kDa on silver staining was measured, as described above. The final fractions showing enzyme activity (fraction 56–67) were pooled and concentrated using a Centricon YM-30.

Gel Electrophoresis and Protein Identification. SDS-PAGE was performed according to the method described previously, with slight modifications (Tabata et al., 2004). Briefly, proteins were separated on a 7.5% polyacrylamide gel. After electrophoresis, the gels were stained with a 0.05% Coomassie Brilliant Blue solution. The protein band was excised using a clean scalpel, and the amino acid sequence was analyzed at Wako Pure Chemical Industries.

RNA Preparation from Rat Tissues and Reverse Transcription-Polymerase Chain Reaction Analyses. The abdominal cavities of rats were opened, and several tissues were excised. Total RNA samples from the rat liver, jejunum (enterocytes), kidneys, and lungs were freshly isolated using RNAiso. Real-time reverse transcription-polymerase chain reaction (RT-PCR) was performed for the quantitative determination of Ces mRNA using a MX3000P real-time polymerase chain reaction (PCR) system (Stratagene, La Jolla, CA).

The sequences and position of the primers are shown in Supplemental Table 1. A 1- μ L portion of the reverse-transcribed mixture was added to a PCR mixture containing 0.4 μ M each primer and 12.5 μ L SYBR Premix Ex Taq solution in a final volume of 25 μ L. After an initial denaturation step at 95°C for 30 seconds, amplification was performed with 40 cycles of denaturation, annealing, and extension. The PCR conditions for each Ces enzyme are shown in Supplemental Table 2. The amplified products were monitored by measuring the increase in the intensity of the SYBR Green (Takara Bio, Shiga, Japan) dye that binds to the amplified double-stranded DNA during the PCR. The target mRNA copy number in the samples was defined based on a standard curve using the PCR products generated using real-time RT-PCR primer pairs. The specificity of all of the primer pairs was confirmed by PCR product digestion with the appropriate restriction enzymes and sequence analysis. The Ces mRNA expression levels were normalized with glyceraldehyde-3-phosphate dehydrogenase mRNA levels.

Construction of an Expression System for Rat Ces2a in Sf21 Cells. An expression system for rat Ces2a was constructed using the Bac-to-Bac Baculovirus Expression System (Invitrogen, Carlsbad, CA), according to the manufacturer's protocol. The rat Ces2a cDNA was obtained by RT-PCR from a rat liver RNA sample using the Ces2a SalI and the Ces2a XhoI primers (Supplemental Table 1). The nucleotide sequence (referred to as accession no. NM_144743) was confirmed by DNA sequence analysis performed at FASMAG (Kanagawa, Japan). The PCR product was transferred into the pFastBac1 vector using the appropriate restriction enzymes. The pFastBac1 vector containing the Ces2a cDNA was transformed into DH10Bac-competent cells, followed by transposition of the inserts into bacmid DNA. Nonrecombinant bacmid DNA (mock) was also prepared using the same procedure. The steps following the infection of *Spodoptera frugiperda* Sf21 cells (Invitrogen) with bacmid DNA were performed according to the method described previously, with slight modifications (Iwamura et al., 2012).

Inhibition Studies on Diltiazem Deacetylase Activity. To confirm the deacetylase activity of rat Ces2a for diltiazem in rat livers, we performed inhibition studies using recombinant rat Ces2a and RLM with typical esterase inhibitors. Organophosphates, such as BNPP and DFP, are general Ces inhibitors (Heymann and Krisch, 1967; Yamaori et al., 2006). PMSF is a general serine esterase inhibitor (Johnson and Moore, 2000). Eserine, NaF, and ethopropazine are cholinesterase inhibitors (Preuss and Svensson, 1996; Yamaori et al., 2006; Takahashi et al., 2009). Eserine is also an inhibitor of human CES2 (Takahashi et al., 2009). The final inhibitor concentration was 100 μ M for BNPP, DFP, and PMSF; 1 mM for eserine and NaF; and 10 μ M for ethopropazine. DFP, PMSF, and ethopropazine were dissolved in dimethylsulfoxide (DMSO). The final concentration of DMSO in the incubation mixture was 1%. The other inhibitors were dissolved in distilled water. The experimental procedure and conditions were the same as described above. It was confirmed that a 1% DMSO concentration did not inhibit diltiazem deacetylase activity, and the activity in the control samples was also determined in the presence of 1% DMSO.

Results

Diltiazem Deacetylase Activity in the Microsomes from Human, Monkey, Dog, Rat, and Mouse Tissues. Diltiazem deacetylase activity in the liver, small intestine, kidney, and lung microsomes isolated from human, monkey, dog, rat, and mouse tissues was

TABLE 1
Purification of the enzyme(s) involved in diltiazem deacetylation in RLM

Step	Total Protein	Total Activity	Specific Activity	Purification	Recovered Activity
	mg	nmol/min	nmol/min/mg	Fold	%
RLM	969.2	538.3	0.6	1.0	100.0
sRLM	937.9	381.3	0.4	0.7	70.8
50–70% (NH ₄) ₂ SO ₄	212.2	478.2	2.3	4.1	88.8
DEAE Sephacel	175.8	629.8	3.6	6.5	117.0
Superdex 200	1.4	475.7	338.6	609.6	88.4
Hydroxyapatite	0.1	150.0	125.0	225.0	27.9

measured at 200 μM substrate concentration (Fig. 2). These organs were selected because they are the main organs involved in drug metabolism and express several esterases. Diltiazem deacetylase activity was only detected in rats. The activity in the male rat liver and intestinal microsomes was 3.18 ± 0.05 and 0.29 ± 0.01 nmol/min/mg protein, respectively. The activity in the female rat liver was 4.23 ± 0.13 nmol/min/mg protein, which is 1.3-fold greater than the activity measured in male rats (data not shown). In contrast to the microsomes, the rat liver cytosol samples showed no activity (data not shown).

Purification and Identification of the Enzyme(s) Responsible for Diltiazem Deacetylation. To identify the enzyme responsible for diltiazem deacetylation, we sought to purify the enzyme(s) from female rat liver microsomes. The efficiency of protein recovery for each step of the purification procedure is shown in Table 1. The protein staining profile and diltiazem deacetylase activity are shown in Fig. 3. Rat liver microsomes were solubilized in a solution containing tergitol type-NP9 and sodium cholate, and we confirmed that the diltiazem deacetylase activity of sRLM was retained (70.8%). Upon

ammonium sulfate precipitation, the 50%–70% ammonium sulfate-saturated fraction exhibited the highest deacetylase activity and was purified on a DEAE Sephacel anion exchange column. By monitoring the protein concentration and diltiazem deacetylase activity of the eluate, we observed that the candidate protein was mainly found in fractions 20–23. The pooled proteins (fractions 20–23) were concentrated using a Centricon YM-30 and subsequently loaded on a Superdex 200 gel filtration column. Again, the active fractions (fractions 30–32) were pooled and were subsequently subjected to hydroxyapatite chromatography purification. Fractions 53–71 displayed some activity, but the highest activity was found in fractions 62–64 (10.5–10.8 nmol/min/ μL fraction) (Fig. 3B). In these fractions, two bands of approximately 60 and 70 kDa were detected by SDS-PAGE and silver staining (Fig. 3A). The intensity of the 60-kDa protein band was correlated with the diltiazem deacetylase activity found in fractions 58–68. The intensity of the 70-kDa protein band was highest in fraction 50, which showed lower diltiazem deacetylase activity (0.6 nmol/min/ μL fraction) than fractions 62–64 (data not shown). Overall, compared with the initial RLM fraction, we achieved a 225-fold purification of enzymatically active proteins with a 0.12% yield. However, the specific activity in the final step was lower than in the Superdex 200-purified fractions (Table 1). This decrease may have been caused by the deterioration of the hydroxyapatite after multiple uses. Finally, based on the amino acid sequence analysis of the excised 60-kDa protein band, *Ces1d* (accession no. NP_579829), *Ces1e* (NP_113753), *Ces1f* (NP_001096829), and *Ces2a* (NP_653344) emerged as candidate proteins (Table 2).

mRNA Expression Levels of Rat Ces Enzymes. To determine which of the *Ces* isoforms are responsible for diltiazem deacetylation among the four candidates, their tissue distribution at the mRNA expression level was evaluated in comparison with the activities of the rat tissue microsomes, as shown in Fig. 2. In addition, the mRNA levels of the other *Ces* isoforms were also evaluated. All of the mRNA levels were determined as the copy numbers normalized to glyceraldehyde-3-phosphate dehydrogenase levels (Fig. 4). In the *Ces1* family, *Ces1a* was not detected in the liver, small intestine, kidneys, or lungs. The expression of *Ces1c* was limited to the liver, whereas *Ces1d*, *Ces1e*, and *Ces1f* were expressed in various organs. In the *Ces2* family, *Ces2a* was expressed in the liver and at a lower level in the small intestine. *Ces2c*, *Ces2g*, and *Ces2h* were expressed in the liver, small intestine, and kidneys. These *Ces2* isoforms were also detected in the lungs, but only in female rats. *Ces2e* was expressed in the liver and barely expressed in the small intestine of female rats. The expression of *Ces2i* and *Ces2j* was limited to the liver and small intestine, respectively. The only isoform for which the expression profile corresponded to the diltiazem deacetylase activity profile was *Ces2a*.

Kinetic Analyses of the Diltiazem Deacetylase Activity of Recombinant Rat *Ces2a* and RLM. To examine whether rat *Ces2a* might be responsible for diltiazem deacetylation, we performed a kinetic analysis of the diltiazem deacetylase activity of a recombinant *Ces2a* protein (Fig. 5; Table 3). The activities of both recombinant *Ces2a* and RLM followed the Michaelis-Menten kinetics model. The K_m , V_{max} , and CL_{int} values of recombinant *Ces2a* were $116.8 \pm 5.3 \mu\text{M}$,

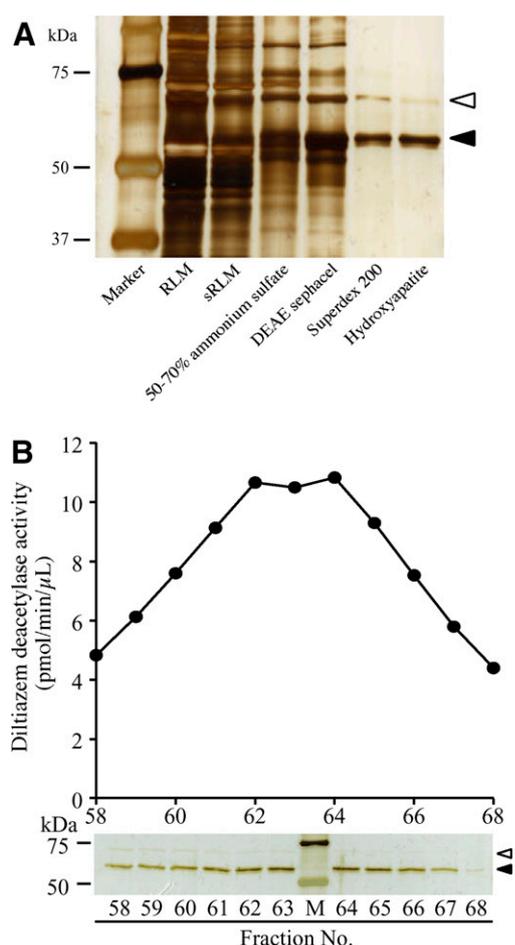


Fig. 3. Purification of the enzyme(s) responsible for diltiazem deacetylation from RLM. (A) Protein profiles of sRLM and RLM fractionated by ammonium sulfate and column chromatography. The proteins were separated by SDS-PAGE and were visualized by silver staining: lane 1, molecular weight standard; lane 2, 30 μg proteins; lane 3, 10 μg proteins; lane 4, 6 μg proteins; lane 5, 8 μg proteins; lane 6, 0.6 μg proteins; lane 7, 0.1 μg proteins. The open and closed arrowheads indicate the 70- and 60-kDa protein bands, respectively. (B) Diltiazem deacetylase activity in the fractions eluted from hydroxyapatite chromatography and approximately 60- and 70-kDa protein bands of each fraction. The 60- and 70-kDa protein bands were visualized by silver staining. For the diltiazem deacetylase activity, 20 μL fractions were incubated with 200 μM diltiazem for 30 minutes.

TABLE 2
Candidate proteins involved in diltiazem deacetylation in rat

Protein	Gene	Molecular Weight	pI	Accession No.
<i>Ces1d</i>	<i>Ces1d</i>	62,147	9.41	NP_579829
<i>Ces1e</i>	<i>Ces1e</i>	61,669	5.71	NP_113753
<i>Ces1f</i>	<i>Ces1f</i>	62,308	6.29	NP_001096829
<i>Ces2a</i>	<i>Ces2a</i>	61,802	6.19	NP_653344

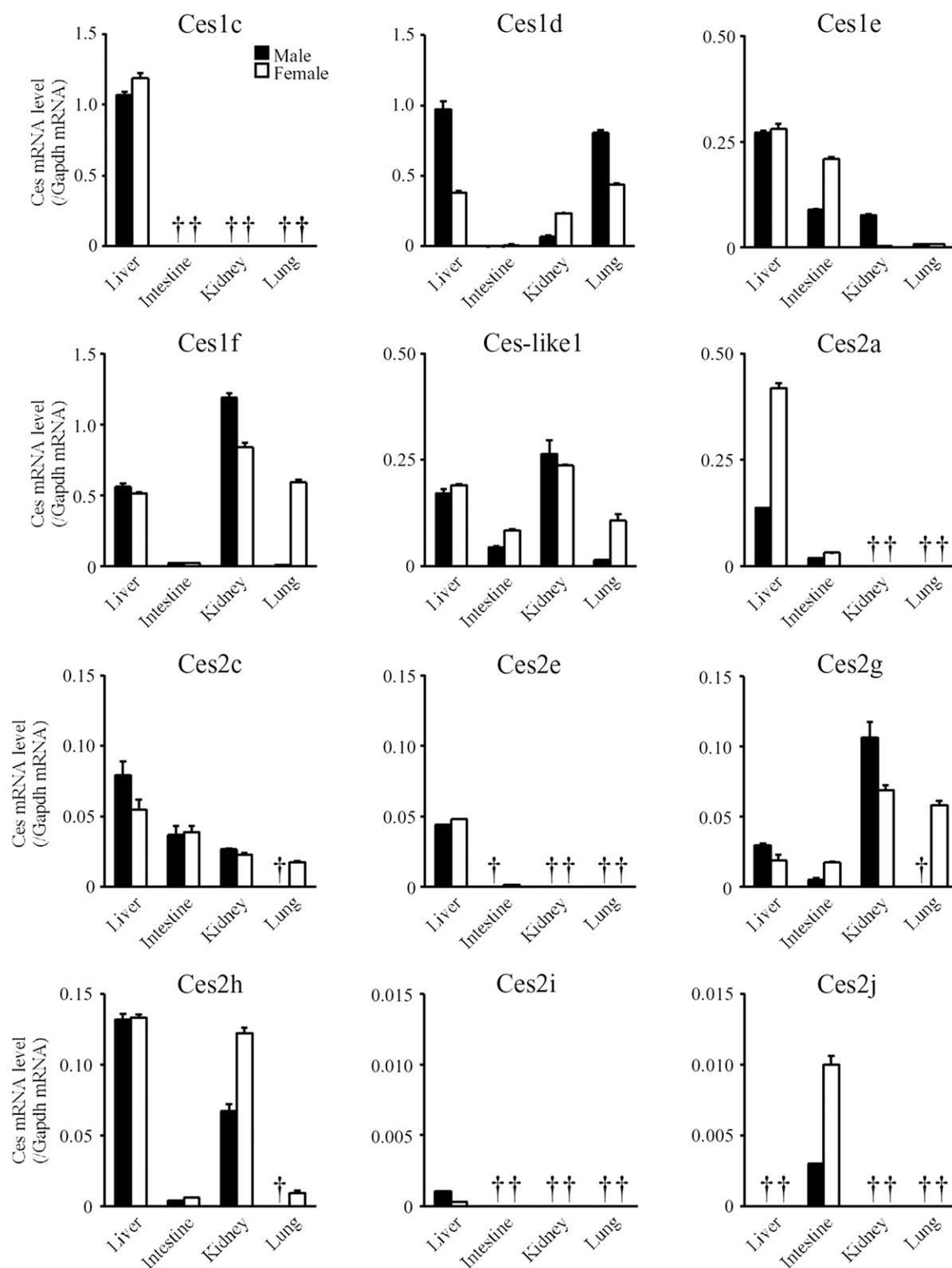


Fig. 4. The mRNA expression profiles of rat Ces isoforms in liver, small intestine, kidney, and lung from male and female rats. Total RNA samples were freshly isolated from three rats, and pooled samples were used. The copy numbers of Ces mRNA were determined by real-time RT-PCR analyses and were normalized with glyceraldehyde-3-phosphate dehydrogenase levels. A standard curve for the determination of copy number was constructed using PCR products with each primer pair used for real-time RT-PCR. Each column represents the mean \pm S.D. of triplicate determinations. †, Not detected.

18.3 ± 0.5 nmol/min/mg protein, and 156.8 ± 3.1 μ L/min/mg protein, respectively (Table 3). Those values for the RLM were 359.4 ± 9.7 μ M, 11.6 ± 0.4 nmol/min/mg protein, and 32.2 ± 0.3 μ L/min/mg

protein, respectively. Thus, recombinant Ces2a showed a similar K_m value as the RLM, suggesting that rat Ces2a is the main enzyme responsible for diltiazem deacetylation in the rat liver.

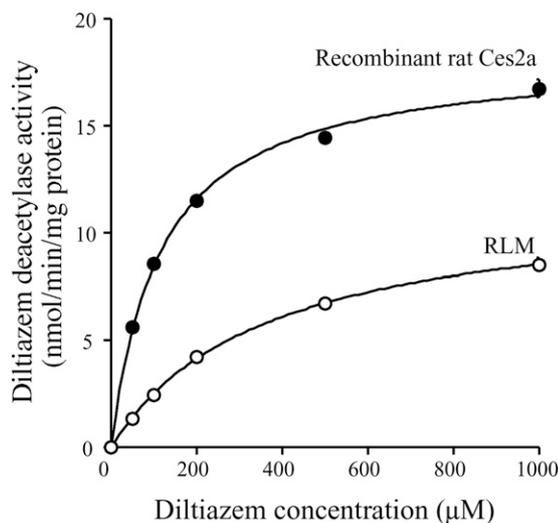


Fig. 5. Kinetic analyses of diltiazem deacetylation by recombinant rat Ces2a and RLM. Recombinant rat Ces2a (0.1 mg/mL) or RLM (0.2 mg/mL) were incubated with diltiazem for 30 minutes. The diltiazem deacetylase activity was measured by HPLC. Each point represents the mean \pm S.D. of triplicate determinations.

The Effects of Chemical Inhibitors on Diltiazem Deacetylation by Recombinant Ces2a and RLM. To investigate whether Ces2a is the principal enzyme for diltiazem deacetylation in the rat liver, we compared the effects of various esterase inhibitors on the diltiazem deacetylase activity of recombinant Ces2a and RLM (Fig. 6). The activity of both recombinant Ces2a and RLM was efficiently inhibited by BNPP, DFP, PMSF, and eserine, but not by NaF and ethopropazine. Such similarity in the inhibition profiles between recombinant Ces2a and RLM supported the hypothesis that Ces2a is the enzyme that is responsible for diltiazem deacetylation in rats.

Discussion

In humans, diltiazem is primarily metabolized to *N*-demethyldiltiazem, *O*-demethyldiltiazem, and deacetyldiltiazem by CYP3A4, CYP2D6, and esterase(s), respectively (Molden et al., 2002; Williams et al., 2002); however, the esterase(s) responsible for the deacetylation remained to be identified. Yeung et al. (1990) reported that the AUC ratio of deacetyldiltiazem to diltiazem after an oral dose of diltiazem was much higher in rats (0.82) than in humans (0.12), suggesting the existence of species differences in the enzyme activity and/or substrate specificity of the esterase(s) involved in diltiazem deacetylation. In this study, we sought to clarify the molecular underpinnings of this species difference in diltiazem deacetylase activity between humans and experimental animals via *in vitro* experiments.

First, we measured the diltiazem deacetylase activity of tissue microsomes isolated from humans, monkeys, dogs, rats, and mice (Fig. 2). Among the tested samples, diltiazem deacetylase activity was detected only in the liver and small intestine microsomes of rats. In addition, we confirmed that rat plasma had no diltiazem deacetylase

activity (data not shown). Our results support a previous report by LeBoeuf and Grech-Belanger (1987) showing that diltiazem was deacetylated in the rat liver and small intestine, but not in the kidneys or lungs. Although deacetyldiltiazem was detected in human plasma after an oral dose of diltiazem (Yeung et al., 1990), no diltiazem deacetylase activity was detected in the microsomes of human livers, the small intestine, the kidneys, and the lungs (Fig. 2). In addition, we confirmed that human plasma did not show diltiazem deacetylase activity in an examination of plasma samples from five healthy Japanese volunteers (data not shown). This apparent contradiction may be partly accounted for by diltiazem deacetylation that occurs in other organs that were not examined. In addition, during a 30-minute incubation of 200 μ M diltiazem at 37°C, 7.8% and 0.25% of the added diltiazem were enzymatically (with 0.2 mg/mL RLM) and nonenzymatically deacetylated, respectively. Therefore, nonenzymatic deacetylation may be another reason for the detection of deacetyldiltiazem in human plasma. Initially, it was suggested that the enzyme(s) catalyzing diltiazem deacetylation in the main drug metabolism organs, such as the liver and small intestine, was expressed only in rats.

To identify the enzyme(s) responsible for diltiazem deacetylation in rats, we purified enzymatically active proteins from rat liver microsomes. Through several purification steps (Table 1; Fig. 3A), we finally restricted the candidate diltiazem deacetylating enzymes to proteins weighing approximately 60 kDa (Fig. 3). The amino acid sequences of these 60-kDa proteins were analyzed, and four Ces enzymes, Ces1d, Ces1e, Ces1f, and Ces2a (Table 2), were identified as candidates. Using purified Ces enzymes, Hosokawa et al. (1990) reported that Ces1f preferentially hydrolyzes malathion and palmitoyl-CoA, that Ces1d selectively hydrolyzes butanilicaine, and that Ces1d and Ces1e hydrolyze isocarboxazid. Additionally, Robbi and Beaufay (1994) have reported that recombinant Ces1e hydrolyzes acetanilide. Among those chemicals, by acyl group size, the descending order is palmitoyl-CoA, isocarboxazid, butanilicaine, malathion, and acetanilide. In addition, Ozaki et al. (2013) investigated the substrate specificity of rat Ces isoforms using parabens with various alkyl chain lengths, although the substrate specificities of Ces1d, Ces1e, and Ces1f could not be clearly determined from the size of the alcohol or acyl moieties. In the same report, although information regarding the substrate specificity of rat Ces2 was very limited, it was revealed that rat Ces2a appears to prefer compounds with a large alcohol moiety, which is a characteristic of diltiazem. Because we considered that it would be difficult to determine which Ces isoform is responsible for diltiazem deacetylation on the basis of the chemical structure of their substrates, we observed the expression levels of Ces isoforms in the rat liver, small intestine, kidneys, and lungs (Fig. 4). Because antibodies specific for each Ces isoform are not available, we determined the mRNA expression levels in this study.

We found that the Ces1 isoforms were highly expressed in the liver, which is similar to humans (Sato et al., 2002). It is noteworthy that Ces1e and Ces-like1, which are probably isoforms in the Ces1 family, were expressed in the rat small intestine. It has been reported that Ces1e was highly expressed in the rat intestine (Mentlein et al., 1987), although CES1 was not expressed in the human small intestine at protein levels (Taketani et al., 2007). The Ces2 isoforms, except for Ces2j, were commonly expressed in the liver, whereas some isoforms were moderately expressed in the small intestine. Our results are supported by previous papers (Mello et al., 2008; Ohura et al., 2014) that reported Ces isoform mRNA expression in the rat liver, jejunum, or ileum. Compared with these previous studies, an advantage of the present study was the determination of the expression profiles of all of the Ces isoforms in rats. Among the candidate isoforms (Ces1d,

TABLE 3

Kinetic parameters of diltiazem deacetylation in RLM and recombinant Ces2a

Enzyme Source	K_m	V_{max}	CL_{int}
	μ M	nmol/min/mg	μ L/min/mg
RLM	359.4 \pm 9.7	11.6 \pm 0.4	32.2 \pm 0.3
Recombinant Ces2a	116.8 \pm 5.3	18.3 \pm 0.5	156.8 \pm 3.1

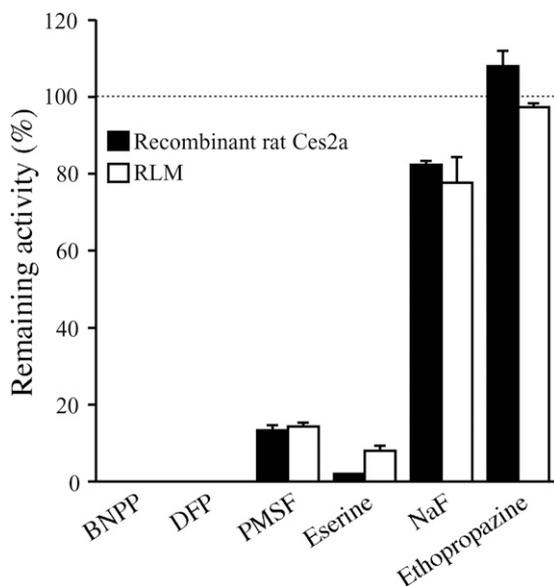


Fig. 6. Inhibitory profile of various chemical inhibitors against diltiazem deacetylase activity. Recombinant rat Ces2a (0.1 mg/mL) or RLM (0.2 mg/mL) were incubated with 200 μM diltiazem for 30 minutes. The controlled activity values by recombinant rat Ces2a and RLM were 9.55 ± 0.14 and 4.23 ± 0.13 nmol/min/mg protein, respectively. Each column represents the mean ± S.D. of triplicate determinations.

Ces1e, Ces1f, and Ces2a), only Ces2a showed an expression profile that corresponded to the profile of diltiazem deacetylase activity. Furthermore, the sex difference in the diltiazem hydrolase activity (higher in females) was consistent with the difference in the Ces2a expression level in the liver (Fig. 4). Consequently, Ces2a was suggested to be the enzyme responsible for diltiazem deacetylation.

Previously, it was reported that the rat Ces1f isoform, the trivial name of which is carboxylesterase pI 6.2, catalyzes diltiazem deacetylation (Vmax: 3.0 nmol/min/mg protein of purified Ces1f) (Luan et al., 1997). However, it was unclear whether Ces1f was the sole enzyme exhibiting diltiazem hydrolysis because the activity was evaluated only by recombinant rat Ces1d and Ces1f. In the present study, we found that Ces1f was expressed in rat kidneys and lungs (Fig. 4). In

addition, it has been reported that Ces1f protein is expressed in the rat kidney (Yan et al., 1994). Because the rat kidney and lung microsomes did not show any activity (Fig. 2), the contribution of Ces1f to diltiazem deacetylation would be quite low. There are no reports regarding diltiazem deacetylation by recombinant Ces1e. It was demonstrated that the rat Ces1e protein is highly expressed in the liver and intestine and is expressed in the kidneys at relatively low levels (Mentlein et al., 1987), which is almost consistent with the mRNA expression results of this study. Diltiazem deacetylation was not detected in rat kidney microsomes. These observations reminded us of the major contribution of Ces2a to diltiazem deacetylation in rats.

To confirm the role of rat Ces2a in diltiazem deacetylation, we measured the diltiazem deacetylase activity of recombinant Ces2a and compared its Km value with that in RLM (Table 3; Fig. 5). The similarity of Km values between them suggested that Ces2a is responsible for diltiazem deacetylation in the rat liver. Furthermore, this idea was further supported by the similarity of the inhibition profiles of RLM and recombinant Ces2a, as determined with various inhibitors (Fig. 6). Because information regarding potent inhibitors against rat Ces2a is lacking, we used general inhibitors that are used for human CES enzymes. Eserine, which is known to be a human CES2 inhibitor (Takahashi et al., 2009), also inhibited rat Ces2a activity. Although it is known that eserine potently inhibits human arylacetamide deacetylase (AADAC) activity (Watanabe et al., 2010), we confirmed that recombinant rat AADAC did not show any diltiazem deacetylation (data not shown). In addition, we evaluated the effects of calcium chloride (1 mM) on diltiazem deacetylation in RLM, because paraoxonase is known to be activated by calcium chloride (Hioki et al., 2011). However, the activation was not observed (94.7 ± 0.7% of control, data not shown). Based on these results, we concluded that rat Ces2a is the enzyme responsible for diltiazem deacetylation.

Why isn't diltiazem deacetylated in the human liver and small intestine? When we measured the diltiazem deacetylase activity using recombinant human CES1, CES2, and AADAC, which are representative esterases involved in tissue drug hydrolysis, none of them displayed any activity (data not shown). The amino acid sequence homology between human CES2 and rat Ces2a is approximately 67% (Fig. 7). The amino acids within the catalytic triad Ser, Glu, and His

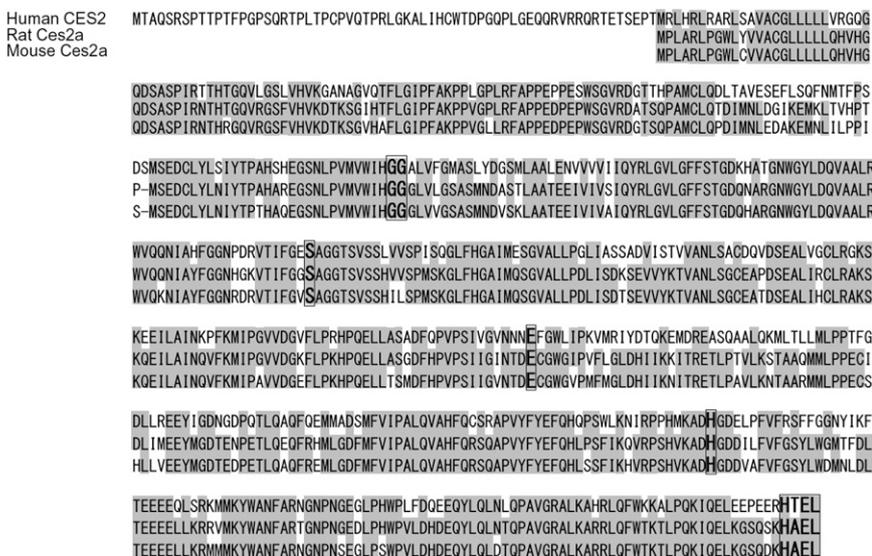


Fig. 7. Alignment of amino acid sequences of human CES2 (NP_003860), rat Ces2a (NP_653344), and mouse Ces2a (NP_598721). Amino acids identical to rat Ces2a are shown in gray boxes. Bold letters in boxes indicate the conserved amino acids, which are part of an oxyanion hole (GG), catalytic triad (S, E, and H), and retention sequence (HXEL).

are conserved, and the sequences around the serine residue in the active site are also highly similar between species. Although the mouse Ces2a, which is highly expressed in the duodenum, jejunum, and ileum (Jones et al., 2013), shows a high level of homology with the rat Ces2a at the amino acid level (88%), no diltiazem deacetylase activity was detected in mouse tissue microsomes (Fig. 2). Crystal structure analysis would be useful to obtain the answer to these questions, but one can imagine that subtle differences in amino acid residues in Ces2 between the rat and other species would determine the specificity of substrate recognition.

In conclusion, we found that diltiazem deacetylase activity was detected only in the rat liver and small intestine and that Ces2a is the enzyme responsible for this activity. In addition, our study clarified the expression profile of all of the rat Ces isoforms in several tissues. The present study provides useful information regarding rat Ces enzymes and will help us to understand the species-specific differences in drug pharmacokinetics between humans and rats.

Authorship Contributions

Participated in research design: Kurokawa, Fukami, Nakajima.

Conducted experiments: Kurokawa, Fukami.

Contributed new reagents or analytic tools: Kurokawa, Fukami.

Performed data analysis: Kurokawa, Fukami.

Wrote or contributed to the writing of the manuscript: Kurokawa, Fukami, Nakajima.

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