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An Improved Cocaine Hydrolase: The A328Y Mutant of Human Butyrylcholinesterase is 4-fold More Efficient

WEIHUA XIE, CIBBY VARKEY ALTAMIRANO, CYNTHIA F. BARTELS, ROBERT J. SPEIRS, JOHN R. CASHMAN, and OKSANA LOCKRIDGE

Eppley Institute and Department of Biochemistry and Molecular Biology, University of Nebraska Medical Center, Omaha, Nebraska (W.X., C.V.A., O.L.) and Human BioMolecular Research Institute, Seattle, Washington (R.J.S, J.R.C.)

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ABSTRACT

Butyrylcholinesterase (BChE) has a major role in cocaine detoxication. The rate at which human BChE hydrolyzes cocaine is slow, with a k_{cat} of 3.9 min^{-1} and K_m of 14 μM. Our goal was to improve cocaine hydrolase activity by mutating residues near the active site. The mutant A328Y had a k_{cat} of 10.2 min^{-1} and K_m of 9 μM for a 4-fold improvement in catalytic efficiency (k_{cat}/K_m). Since benzoylcholine (k_{cat} 15,000 min^{-1}) and cocaine form the same acyl-enzyme intermediate but are hydrolyzed at 4000-fold different rates, it was concluded that a step leading to formation of the acyl-enzyme intermediate was rate-limiting. BChE purified from plasma of cat, horse, and chicken was tested for cocaine hydrolase activity. Compared with human BChE, horse BChE had a 2-fold higher k_{cat} but a lower binding affinity, cat BChE was similar to human, and chicken BChE had only 10% of the catalytic efficiency. Naturally occurring genetic variants of human BChE were tested for cocaine hydrolase activity. The J and K variants (E497V and A539T) had k_{cat} and K_m values similar to wild-type, but because these variants are reduced to 66 and 33% of normal levels in human blood, respectively, people with these variants may be at risk for cocaine toxicity. The atypical variant (D70G) had a 10-fold lower binding affinity for cocaine, suggesting that persons with the atypical variant of BChE may experience severe or fatal cocaine intoxication when administered a dose of cocaine that is not harmful to others.

Cocaine[1] abuse is a medical problem in the United States. About 23 million Americans have used cocaine at least once and approximately 5 million are habitual users (Das, 1993). The number of cocaine-related emergency room visits is about 100,000 annually (Schrank, 1992). Among a total of 14,843 residents of New York City who received fatal injuries from 1990 through 1992, 26.7% had cocaine or a metabolite in their urine or blood (Marzuk et al., 1995). Life-threatening symptoms due to cocaine toxicity include grand-mal seizures, cardiac arrest, stroke, and drug-induced psychosis accompanied by elevated body temperature (Rich and Singer, 1991; Das, 1993; Warner, 1993).

There is evidence that butyrylcholinesterase (BChE, EC 3.1.1.8) is the major detoxicating enzyme of cocaine. The first experiments that identified BChE as a cocaine hydrolase came from the laboratory of W. Kalow (Stewart et al., 1977, 1979; Inaba et al., 1978). They recognized that only a small percentage of cocaine appeared unchanged in the urine of humans and that the major metabolites resulted from the hydrolytic splitting of ester bonds (Fig. 1). Human plasma was known to contain only two major esterases, BChE and paraoxonase. The esterase in blood that hydrolyzes cocaine was identified as BChE by showing that diisopropyl fluorophosphate, eserine, and sodium fluoride, three characteristic inhibitors of BChE, inhibited hydrolysis of cocaine. By contrast, EDTA, an inhibitor of paraoxonase, did not inhibit hydrolysis of cocaine by plasma. Furthermore, purified BChE accounted for all the cocaine hydrolase activity seen in plasma. The metabolite produced by BChE in the test tube was ecgonine methyl ester (Fig. 1); ecgonine methyl ester was also a major metabolite found in urine, thus supporting a role for BChE in metabolism of cocaine (Inaba et al., 1978). A second metabolite found in high amounts in urine was benzoylecgonine (Fig. 1). This metabolite is produced by spontaneous hydrolysis at alkaline pH as well as by liver carboxylesterase (Brzezinski et al., 1994).

Animal studies showed that administration of purified human BChE protected mice and rats from the toxic effects of cocaine. Hoffman et al. (1996) gave mice an i.p. injection of

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[1] Cocaine possesses the "(−)" configuration unless otherwise indicated.

ABBREVIATIONS: BChE, butyrylcholinesterase enzyme.

purified human BChE and, 1 h later, an injection of cocaine. The cocaine dose of 150 mg/kg i.p. caused seizures and death in 100% of animals when animals were not pretreated with BChE. By contrast, pretreatment with BChE protected 70% of mice from death and 60% of mice from seizures. Similarly, Lynch et al. (1997) pretreated rats with BChE and found that rats were protected from the lethal effects of cocaine as well as from hypertension and arrhythmia. BChE was also effective when it was given to rats after cocaine. Rats had convulsions and died within 9 min of receiving 80 mg/kg cocaine i.p., but were protected when they were injected with BChE 3 min after receiving cocaine. It was concluded that BChE could be an effective therapy for the treatment of life-threatening cocaine-induced toxicity (Mattes et al., 1997).

Although BChE protects against cocaine toxicity by inactivating cocaine, it acts slowly. Our goal was to increase the catalytic efficiency of BChE toward (−)-cocaine by increasing its binding affinity and hydrolysis rate. Of the 24 mutants of human BChE tested, only the A328Y mutant had an improved catalytic efficiency for cocaine hydrolysis. In addition, we tested purified BChE from cat, chicken, and horse and found that the catalytic efficiency (k_{cat}/K_m) of BChE from all three animals was lower than that of wild-type human BChE, despite a 2-fold higher k_{cat} in horse BChE.

Another area we investigated was the catalytic efficiency of some of the naturally occurring genetic variants of BChE toward cocaine, including the atypical variant, the K variant, and the J variant. It is established that people with genetic variants of BChE, particularly the atypical variant (D70G), are more sensitive to the muscle relaxants succinylcholine and mivacurium and that this abnormal response is due to a decreased binding affinity for these drugs (Lockridge, 1990; Kalow and Grant, 1995). Our results suggest that people with the atypical variant of BChE have an increased risk of suffering complications from cocaine use.

Materials and Methods

Materials. Reagents for site-directed mutagenesis and expression included Pfu polymerase (Stratagene, La Jolla, CA), the expression plasmid pGS (gift from Dr. Tyler White, Scios Nova Inc., Mountain View, CA), Ultraculture without L-glutamine (BioWhittaker, Fisher Scientific Co., Fairlawn, NJ), Dulbecco's modified Eagle's medium and Ham's F12, 50/50 mix without L-glutamine (Mediatech Cellgro, Fisher Scientific Co.), CHO-KI cells (No. CCL 61; American Type Culture Collection, Rockville, MD), Qiagen plasmid purification kit and QIAquick PCR purification kit (Qiagen, Santa Clarita, CA). All oligonucleotides were synthesized by the Molecular Biology Core Facility at the University of Nebraska Medical Center. Procainamide-Sepharose was a gift from Dr. B. P. Doctor, Walter Reed Army Research Institute (Washington, DC). Other reagents were DE52 from Whatman (Maidstone, England); echothiophate iodide from Wyeth-Ayerst (Rouses Point, NY); and butyrylthiocholine iodide, benzoylcholine chloride, diisopropyl fluorophosphate, and methionine sulfoximine from Sigma Chemical Co. (St. Louis, MO). (−)-Cocaine hydrochloride was purchased from Sigma after obtaining a license from the Department of Justice, Drug Enforcement Agency (Washington, DC). (+)-Cocaine was provided by the National Institute on Drug Abuse Research Resources Drug Supply System (Rockville, MD). Cat plasma and chicken serum were from Pel-Freez Biologicals (Rogers, AK). Horse serum was from Gibco/BRL (Gaithersburg, MD). Highly purified human BChE, 280 U/ml when assayed with butyrylthiocholine, was a gift from Dr. G. Ghenbot (Centeon L.L.C., Kankakee, IL).

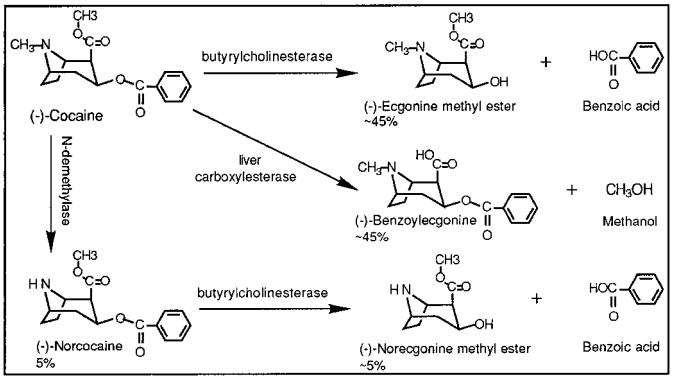

Fig. 1. Metabolism of (−)-cocaine. The naturally occurring (−)-cocaine is the pharmacologically active enantiomer.

Mutagenesis and Expression of Recombinant BChE. Mutations in human BChE were made by PCR with Pfu polymerase. Pfu polymerase was superior to *Taq* polymerase and DeepVent because it did not introduce unwanted mutations. The 1.8-kb fragment containing the mutation was cloned into the plasmid pGS and completely resequenced to make certain that only the desired mutation was present. The plasmid pGS is identical with pRc/CMV (Invitrogen, Carlsbad, CA) except that the Neo gene of pRc/CMV has been replaced by rat glutamine synthetase. Transfection of CHO-KI cells by calcium phosphate coprecipitation was followed by selection of colonies in glutamine-free, serum-free medium Ultraculture containing 50 μM methionine sulfoximine. Colonies expressing the highest levels of BChE activity were expanded. For collection of large volumes of secreted BChE, cells in 1-liter roller bottles were fed every 2 or 3 days with 100 ml of Ultraculture containing 25 μM methionine sulfoximine followed by 100 ml of Dulbecco's modified Eagle's medium/Ham's F12 without glutamine. Medium was collected from the same roller bottle for 3 to 6 months.

Purification of BChE from Culture Medium. BChE was purified from 6 to 12 liters of culture medium containing secreted BChE. Culture medium was reduced in volume to 800 ml by pouring the liquid into a 5-cm (flat diameter) dialysis bag and packing table sugar around the bag. The top of the bag was tied with a rubber band to allow refilling. The 800-ml viscous liquid was diluted with 4 volumes of 20 mM potassium phosphate 1 mM EDTA, pH 7, to reduce the salt concentration to allow the BChE to stick to the affinity gel. Particulate matter was removed by centrifugation. BChE activity was purified on procainamide-Sepharose eluted with 0.2 M procainamide, followed by ion exchange chromatography on DE52 and elution with a gradient of sodium chloride (Lockridge, 1990). The BChE was dialyzed in an Amicon Stirred cell with a PM10 membrane to concentrate the enzyme and to adjust the buffer to 0.1 M potassium phosphate, 1 mM EDTA, pH 7. Preparations used for (−)-cocaine hydrolysis had activities with 1 mM butyrylthiocholine of 20 to 150 units/ml where unit is defined as μmoles hydrolyzed per minute.

Purification of BChE from Animal Sera. BChE was purified from 3 liters of chicken serum, 1.5 liters of horse serum, and 0.35 liter of cat plasma by ammonium sulfate precipitation, ion exchange chromatography, and affinity chromatography on procainamide-Sepharose (Main et al., 1974; Ralston et al., 1983). The preparations used for (−)-cocaine hydrolysis had activities with 1 mM butyrylthiocholine of 49 U/ml (chicken BChE), 92 U/ml (horse BChE), and 9 U/ml (cat BChE).

Titration of Active Sites. The number of active sites per ml of purified BChE was titrated with echothiophate iodide and with diisopropyl fluorophosphate. The catalytic rate constant, k_{cat}, was calculated by dividing V_{max} by the concentration of active sites.

Benzoylcholine Hydrolysis. Benzoylcholine chloride concentration was verified by absorbance at 240 nm where a 0.2 mM stock solution had an absorbance of 1.874. K_m values were determined for

the range 12.5 to 50 μM benzoylcholine in 0.1 M potassium phosphate, pH 7.0, at 25°C. Hydrolysis of benzoylcholine was recorded at 240 nm. Hydrolytic activity was calculated from the difference in molar absorptivity of benzoylcholine and benzoic acid, $\Delta E = 6700$ M^{-1} cm^{-1}.

Cocaine Hydrolysis. (−)-Cocaine and (+)-cocaine stock solutions of 0.1 M were made in water and frozen. Cocaine was stable when frozen in water, but unstable in phosphate buffer at pH 7; stability was checked by high-performance liquid chromatography (HPLC). Enzyme-catalyzed hydrolysis of cocaine was recorded on a temperature-equilibrated Gilford Spectrophotometer at 240 nm where the difference in molar absorptivity between substrate and product was $\Delta E = 6{,}700$ M^{-1} cm^{-1} (Gatley, 1991). K_m values were determined in 0.1 M potassium phosphate pH 7.0 at 30°C for (−)-cocaine and at 25°C for (+)-cocaine. V_{max} and K_m values were calculated using Sigma Plot for Macintosh computer (Jandel Scientific).

Butyrylthiocholine Hydrolysis. Butyrylthiocholine stock solutions of 0.2 M were prepared in water and frozen. Hydrolysis of butyrylthiocholine was recorded at 412 nm in the presence of 0.5 mM dithiobisnitrobenzoic acid in 0.1 M potassium phosphate, pH 7.0, at 25°C. Activity was calculated from the molar extinction coefficient of 13,600 M^{-1} cm^{-1} (Ellman et al., 1961). Units of activity are expressed as micromoles substrate hydrolyzed per minute.

The competitive inhibition constant, K_i, was determined by measuring hydrolysis of butyrylthiocholine (12.5–100 μM) in the presence of (−)-cocaine (0–100 μM) and plotting 1/v versus inhibitor concentration in a Dixon plot. Each analysis was repeated three times. The butyrylthiocholine concentrations were below the range where substrate activation occurred; plots of 1/v versus 1/S were linear for the range used. For the D70G mutant the butyrylthiocholine concentrations were 105 to 630 μM and the (−)-cocaine concentrations were 117 to 702 μM.

Substrate Activation. Lineweaver-Burk plots for butyrylthiocholine hydrolysis are not linear for the range 0.01 to 40 mM butyrylthiocholine when the assays are performed in phosphate buffer. The curvature in this plot has been attributed to substrate activation at concentrations above 0.4 mM butyrylthiocholine (Radic et al., 1993; Masson et al., 1997). The equation for substrate activation (Radic et al., 1993) was used to calculate the kinetic constants for butyrylthiocholine hydrolysis with the Sigma Plot Computer Program. The K_m value describes binding at low substrate concentrations, 0.01 to 0.1 mM; the K_{ss} value describes binding at high substrate concentrations, 0.4 to 40 mM; the b value is the ratio of V_{max} at high substrate concentration divided by V_{max} at low substrate concentration. When the b value is greater than one there is substrate activation; when the b value is less than one there is substrate inhibition; when the b value is equal to one there is neither substrate activation nor substrate inhibition and double reciprocal plots are linear.

HPLC. A Waters 625 LC System with Waters 486 Tunable Absorbance Detector and Waters Delta Pak C18, 300-Å, 5-μ column were used. The elution buffer was an 80:20 mixture of 0.05 M potassium phosphate, pH 3.0, and acetonitrile. The flow rate was 0.5 ml/min. Absorbance was recorded at 220 nm. 100 μM (−)-cocaine freshly diluted in 0.1 M potassium phosphate, pH 7.0, was incubated at 37°C in the presence of 0.2 μM wild-type BChE or the A328Y mutant of BChE. 100-μl aliquots were removed for analysis of hydrolysis products by HPLC. The 100-μl aliquot was acidified to pH 3 by the addition of 1 μl of 85% phosphoric acid. It was important to acidify the samples to get a single, sharp peak for benzoic acid. Protein-containing aliquots were filtered through Millipore Ultrafree-MC 10,000 NMWL filter units by centrifugation in a microcentrifuge to remove particulates before injecting the sample on the HPLC.

Docking. Cocaine was docked into the active site of BChE with the FlexiDock program in Sybyl 6.4 on a Silicon Graphics Octane computer. The structures of (−)-cocaine and (+)-cocaine were retrieved from the Cambridge Structural Database where its code names are COCAIN10 and COCHCL. The HCl molecule was deleted from COCHCL, so that all computations were done with the base form of cocaine. Before the FlexiDock program was run, cocaine was manually aligned with butyrylcholine in the model of human BChE (Harel et al., 1992), so that the tropane ring of cocaine faced Trp-82, the carboxyl of the benzoic acid ester of cocaine was within 1.5 Å of

TABLE 1
Kinetic constants for recombinant human BChE, determined in 0.1 M potassium phosphate, pH 7.0, at 25°C for butyrylthiocholine and benzoylcholine and at 30°C for (−)-cocaine

Human BChE	Butyrylthiocholine				
	K_m	K_{ss}	b	k_{cat}	bk_{cat}
	μM	mM		min^{-1}	min^{-1}
Wild-type	23 ± 2	1.4 ± 0.2	2.5 ± 0.1	33,900 ± 1,800	84,900 ± 4,600
N68Y/Q119Y/A277W	59 ± 10	3.4 ± 0.3	4.7 ± 0.4	11,700 ± 900	55,000 ± 4,500
Q119Y	11 ± 1	1.6 ± 0.6	10.9 ± 2.7	6,300 ± 1,700	69,000 ± 18,100
Q119Y/V288F/A328Y	26 ± 8	0.5 ± 0.1	2.5 ± 0.5	19,700 ± 800	49,200 ± 2,100
E197Q	83 ± 4	21 ± 12	1.6 ± 0.2	11,500 ± 200	18,500 ± 300
V288F	9 ± 1	0.3 ± 0.1	3.2 ± 1.1	14,100 ± 4,900	45,200 ± 15,700
A328F	20 ± 1	0.7 ± 0.3	1.2 ± 0.2	32,400 ± 1,000	38,900 ± 1,100
A328Y	15 ± 1	0.2 ± 0.2	1.2 ± 0.2	38,800 ± 8,400	48,500 ± 9,900
F329A	24 ± 4	1.3 ± 0.1	3.3 ± 0.2	21,200 ± 1,300	69,900 ± 4,400
F329S	27 ± 3	2.0 ± 0.4	2.4 ± 0.1	19,000 ± 1,000	45,500 ± 2,500

	Benzoylcholine		(−)-Cocaine		
	K_m	k_{cat}	K_m	k_{cat}	k_{cat}/K_m
	μM	min^{-1}	μM	min^{-1}	M^{-1} min^{-1}
Wild-type	8 ± 1	14,500 ± 200	14 ± 1	3.9 ± 0.1	280,000
N68Y/Q119Y/A277W	20 ± 1	5,500 ± 100	60 ± 12	1.7 ± 0.2	28,000
Q119Y	31 ± 1	10,600 ± 200	56 ± 10	2.0 ± 0.2	36,000
Q119Y/V288F/A328Y	23 ± 2	6,600 ± 200	33 ± 6	2.3 ± 0.2	70,000
E197Q	29 ± 2	7,200 ± 200	17 ± 1	0.1 ± 0.01	6,000
V288F	15 ± 1	11,700 ± 300	17 ± 2	1.0 ± 0.1	60,000
A328F	8 ± 2	11,800 ± 300	24 ± 4	5.8 ± 0.4	240,000
A328Y	12 ± 1	13,300 ± 400	9 ± 1	10.2 ± 0.4	1,130,000
F329A	10 ± 1	10,100 ± 200	128 ± 20	2.7 ± 1.0	21,000
F329S	N/A	N/A	41 ± 18	1.9 ± 0.3	46,000

Ser-198, and the benzene ring of cocaine was in the acyl binding pocket of BChE. In FlexiDock the binding pocket was defined as all amino acids within 4 Å of butyrylcholine. After the binding pocket was defined, the butyrylcholine molecule was extracted. All atoms in the binding pocket, except atoms in rings and double-bonded atoms, were defined as rotatable, thus yielding 124 rotatable bonds in BChE and 7 rotatable bonds in cocaine.

Results

Choice of Mutations to Make. Amino acids in the lining of the active site gorge were selected for mutation. The goal was to allow (−)-cocaine to fit more easily into the active site. The active site gorge has several named regions including the active site Ser-198 at the bottom of the gorge, the acyl binding pocket formed by Leu-286 and Val-288 (Harel et al., 1992), the cation π site Trp-82, and the peripheral anionic site at the mouth of the gorge defined by Asp-70 (Masson et al., 1997).

TABLE 2

Binding constant of (−)-cocaine to mutants of BChE. K_i values were determined by measuring inhibition of butyrylthiocholine hydrolysis by cocaine in 50 mM potassium phosphate, pH 7.4, at 25°C

BChE	K_i
	μM
Wild-type	11 ± 1
D70G	201 ± 32
D70N	486 ± 31
G117H	440 ± 25
G117K	302 ± 33
Q119H	34 ± 35
E197D	40 ± 4
E197G	37 ± 2
L286H	24 ± 2
V288H	55 ± 4
A328F	21 ± 1
A328G	18 ± 1
A328H	27 ± 3
Y332F	22 ± 2

TABLE 3

Comparison of K_m and K_i values for (−)-cocaine. K_i values were determined by measuring inhibition of butyrylthiocholine hydrolysis by cocaine in 0.1 M potassium phosphate, pH 7.0, at 30°C

Human BChE	(−)-Cocaine	
	K_m	K_i
	μM	
Wild-type	14 ± 1	14 ± 2
Atypical (D70G)	172 ± 36	212 ± 18
A328F	24 ± 4	10 ± 2
A328Y	9 ± 2	8 ± 2
J variant (E497V)	12 ± 2	11 ± 2
J/K variant (E497V/A539T)	10 ± 2	9 ± 2
K variant (A539T)	13 ± 3	8 ± 2

(−)-Cocaine Hydrolase Activity. Each of the human BChE mutants in Table 1 was tested for activity with three substrates: butyrylthiocholine, benzoylcholine and (−)-cocaine. The maximum rate of hydrolysis of butyrylthiocholine at high substrate concentration (bk_{cat}) was higher for wild-type BChE than for the mutants. K_m values for butyrylthiocholine hydrolysis were lower for three mutants Q119Y, V288F, and A328Y. Since benzoylcholine and cocaine have benzoate as a common structural feature, it was of interest to see whether kinetic constants for benzoylcholine correlated with those for cocaine. Table 1 shows that K_m values for wild-type BChE and for all mutants were in the micromolar range for both benzoate-containing substrates. In general, the K_m values for cocaine were higher than for benzoylcholine, although we expect that this is due to the higher temperature at which cocaine hydrolysis was measured. Two mutants, E197Q and A328Y, had a lower K_m for cocaine than for benzoylcholine. Benzoylcholine was hydrolyzed at the fastest rate (highest k_{cat}) by wild-type BChE. By contrast, (−)-cocaine was hydrolyzed faster by the A328Y mutant (k_{cat} = 10.2 min^{-1}) and by the A328F mutant (k_{cat} = 5.8 min^{-1}) than by wild-type BChE (k_{cat} = 3.9 min^{-1}). The data in Table 1 show that neither benzoylcholine nor butyrylthiocholine is useful for predicting which mutant will have the best activity with (−)-cocaine.

The catalytic efficiency of an enzyme can be measured by determining k_{cat}/K_m. A comparison of k_{cat}/K_m values in Table 1 shows that the A328Y mutant was 4-fold more efficient than wild-type BChE at hydrolyzing (−)-cocaine. No other mutant outperformed wild-type BChE.

(+)-Cocaine Hydrolase Activity. (+)-Cocaine is the unnatural isomer and has no pharmacologic activity. Gatley (1991) reported that (+)-cocaine was hydrolyzed orders of magnitude more rapidly than (−)-cocaine by human wild-type BChE and that the rate of hydrolysis of (+)-cocaine was similar to that of benzoylcholine. We confirmed this result for

TABLE 4

Kinetic constants for native BChE purified from animal plasma determined in 0.1 M potassium phosphate, pH 7.0, at 25°C for butyrylthiocholine and benzoylcholine, and at 30°C for (−)-cocaine

Native BChE	Butyrylthiocholine				
	K_m	K_{ss}	b	k_{cat}	bk_{cat}
	μM	mM		min^{-1}	min^{-1}
Cat	44 ± 2	3.0 ± 0.3	2.1 ± 0.1	105,100 ± 2,500	220,600 ± 5,300
Chicken	12 ± 1	13.0 ± 2.0	2.4 ± 0.1	11,400 ± 100	27,300 ± 300
Human	23 ± 2	1.4 ± 0.2	2.5 ± 0.1	33,900 ± 1,800	84,900 ± 4,600
Horse	60 ± 8	1.0 ± 0.2	2.1 ± 0.2	66,600 ± 600	139,900 ± 12,600
	Benzoylcholine		(−)-Cocaine		
	K_m	k_{cat}	K_m	k_{cat}	k_{cat}/K_m
	μM	min^{-1}	μM	min^{-1}	$M^{-1}\ min^{-1}$
Cat	8 ± 1	12,900 ± 100	10 ± 3	2.7 ± 0.1	270,000
Chicken	8 ± 1	690 ± 9	67 ± 19	1.4 ± 0.2	21,000
Human	8 ± 1	14,500 ± 200	14 ± 1	3.9 ± 0.1	280,000
Horse	21 ± 2	11,800 ± 400	45 ± 5	7.5 ± 0.3	170,000

human wild-type BChE. Our values for (+)-cocaine were K_m = 10 μM, k_{cat} = 7500 min^{-1} and the values for benzoylcholine were K_m = 8 μM, k_{cat} = 15,000 min^{-1}. We also measured hydrolysis of (+)-cocaine by the A328Y mutant. The kinetic constants at 25°C in 0.1 M potassium phosphate, pH 7.0, were K_m = 7 μM, k_{cat} = 6,000 min^{-1}. We conclude that the A328Y mutation did not significantly alter catalytic efficiency toward (+)-cocaine.

Inhibition Constants. Because (−)-cocaine is hydrolyzed 20,000-fold more slowly than butyrylthiocholine and 4,000-fold more slowly than benzoylcholine, the amount of BChE required to measure (−)-cocaine hydrolysis is relatively high. High concentrations of mutant BChE were not available for all mutants we wanted to test. Therefore mutants in Table 2 were analyzed for cocaine binding affinity by measuring K_i values rather than K_m values. None of the mutants in Table 2 had a higher binding affinity for (−)-cocaine (that is, a lower K_i value) than wild-type BChE. The validity of K_i values as a reflection of K_m values is demonstrated in Table 3 where K_i and K_m values were found to be similar when both values were measured for the same enzyme preparation under the same conditions. Inhibition was competitive.

Animal BChE. To examine the influence of multiple amino acid differences on binding affinity and hydrolysis rate we tested purified BChE from cat, chicken, and horse. The amino acid sequence of cat BChE (Genbank accession number AF053483) is 88% identical with human BChE. Of the 70 amino acids that differ, 50 are located on the surface, 13 are buried, 4 are in the C-terminal region missing from the X-ray structure, and 3 are in the active site gorge. The three amino acid differences in the active site gorge of cat BChE are A277L and P285L at the mouth of the gorge, and F398I at the bottom of the gorge. Table 4 shows that cat BChE hydrolyzed butyrylthiocholine about 3 times faster compared with wild-type human BChE, but it hydrolyzed benzoylcholine and (−)-cocaine somewhat more slowly. Cat BChE has K_m values that are similar to those of human BChE for the three substrates in Table 4. We concluded that cat BChE was exceptionally active toward butyrylthiocholine, but no better than human BChE for hydrolysis of (−)-cocaine.

Horse BChE, like cat BChE, has three amino acid differences in the active site gorge compared with human BChE (B. P. Doctor, personal communication). They are A277V and P285L at the mouth of the gorge, and F398I at the bottom of the gorge. Horse BChE had a 2-fold higher k_{cat} for cocaine than human BChE, but its binding affinity was decreased so that its catalytic efficiency for hydrolysis of cocaine was not improved relative to human BChE.

The amino acid sequence of chicken BChE is only partially known (Jean Massoulié, personal communication). Chicken BChE has 10 amino acid differences in the active site gorge: N68L, S79T, G117S, Q119E, A277V, T284S, P285L, S287H, V288I, and A328S. Purified chicken BChE had 34% of the specific activity of human BChE with butyrylthiocholine, 5% with benzoylcholine, and 36% with cocaine (Table 4). The

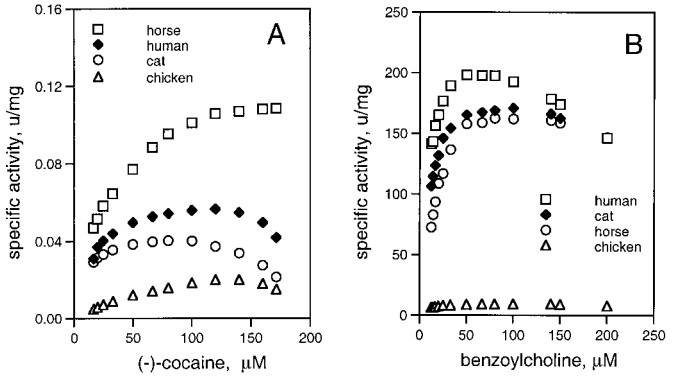

Fig. 2. Substrate inhibition. A, substrate inhibition by (−)-cocaine. B, substrate inhibition by benzoylcholine. Specific activity is expressed as micromoles hydrolyzed per minute per milligram of BChE protein. Human BChE is inhibited by (−)-cocaine concentrations greater than 120 μM and by benzoylcholine concentrations greater than 50 μM.

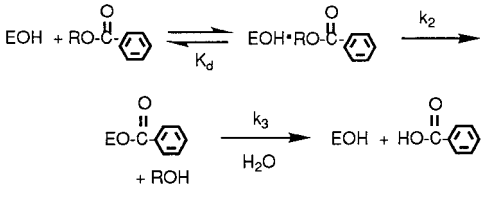

Fig. 3. Schematic diagram of the steps in substrate hydrolysis. EOH is free enzyme; R is either the choline of benzoylcholine or the ecgonine methyl group of cocaine; K_d is the dissociation constant for the Michaelis-Menten complex; k_2 is the rate constant for formation of the acyl-enzyme intermediate; k_3 is the rate constant for hydrolysis of the acyl-enzyme intermediate.

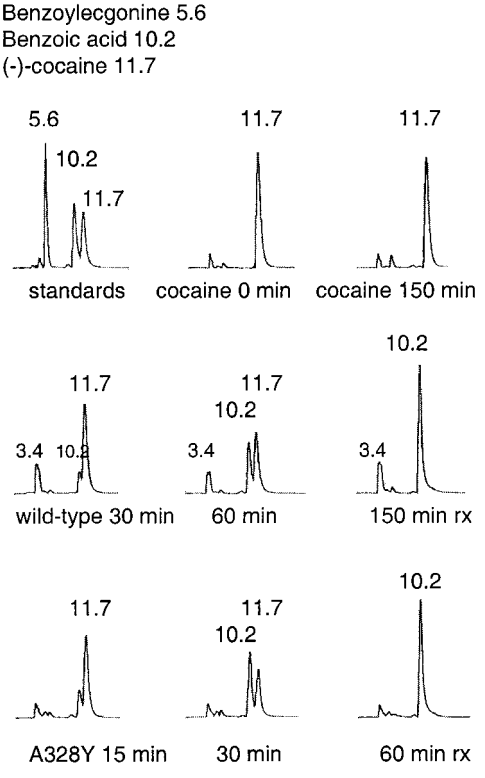

Fig. 4. HPLC analysis of the products of (−)-cocaine hydrolysis. Hydrolysis of 100 μM (−)-cocaine in 0.1 M potassium phosphate, pH 7.0, 37°C, was initiated by addition of wild-type BChE or the A328Y mutant of BChE. Aliquots of 100 μl were removed after the indicated minutes of reaction, acidified to pH 3, filtered, and injected. Absorbance was monitored at 220 nm during isocratic elution with an 80:20 mixture of 0.05 M potassium phosphate, pH 3.0, and acetonitrile. The product of cocaine hydrolysis by wild-type and A328Y was benzoic acid, eluting at 10.2 min. Neither enzyme produced benzoylecgonine. The peak at 3.4 min is a contaminant in the enzyme storage buffer. All of the (−)-cocaine was hydrolyzed within 150 min by 0.2 μM wild-type BChE and within 60 min by 0.2 μM A328Y.

second order rate constant, k_{cat}/K_m, for hydrolysis of cocaine by chicken BChE was 10% of the value for human BChE.

Substrate Inhibition. The first studies on cocaine metabolism reported an absence of cocaine hydrolases in human blood because the early assays used mM concentrations of cocaine. Stewart et al. (1977) found that the cocaine concentration had to be reduced to the μM range before hydrolysis could be observed. We examined substrate inhibition with human, cat, chicken, and horse BChE. Figure 2A shows that hydrolysis of (−)-cocaine by human BChE was inhibited when cocaine concentrations were greater than 120 μM. Cat BChE was inhibited by concentrations greater than 100 μM and chicken BChE by concentrations greater than 140 μM. Horse BChE was not inhibited by cocaine concentrations up to 170 μM. Figure 2A also shows that purified horse BChE hydrolyzed cocaine more rapidly than purified human BChE, followed by cat BChE, and last by chicken BChE.

Benzoylcholine and cocaine are the only substrates of BChE that exhibit substrate inhibition at concentrations in the μM range. In Fig. 2B we examined the pattern of substrate inhibition with benzoylcholine and found that it was similar but not identical with the pattern with (−)-cocaine. Inhibition by benzoylcholine occurred when the benzoylcholine concentration was greater than 50 μM for human, 100 μM for cat, 80 μM for horse, and 80 μM for chicken BChE. The highest specific activity with benzoylcholine was achieved by human BChE, followed by cat, horse and chicken. Chicken BChE was relatively poor at hydrolyzing both benzoylcholine and (−)-cocaine.

Rate Limiting Step for Cocaine Hydrolysis. Benzoylcholine and cocaine form the same acyl-enzyme intermediate, in which benzoic acid is esterified to the hydroxyl group of Ser-198. The fact that (−)-cocaine is hydrolyzed 4000-fold more slowly than benzoylcholine by human BChE suggests that the rate limiting step for hydrolysis of cocaine is a step leading to the formation of the acyl-enzyme intermediate, that is, k_2 in Fig. 3.

Products of (−)-Cocaine Hydrolysis. To test the possibility that the A328Y mutant might be hydrolyzing the methyl ester group of (−)-cocaine, we identified the products of hydrolysis by HPLC. Figure 4 shows that the product was benzoic acid. Both wild-type BChE and the A328Y mutant produced benzoic acid, but no benzoylecgonine. This result is consistent with the scheme in Fig. 1, where BChE hydrolyzes (−)-cocaine to ecgonine methyl ester and benzoic acid. We conclude that the methyl ester group of (−)-cocaine was not hydrolyzed by the A328Y mutant or by wild-type BChE. About 1% of the cocaine spontaneously degraded to benzoylecgonine after 150 min in 0.1 M phosphate buffer pH 7.0 at 37°C in the control incubation but not in the enzyme-containing reactions. Another result from Fig. 4 is confirmation of the faster catalytic rate of the A328Y mutant. 100 μM (−)-cocaine was consumed in 140 min by 0.2 μM wild-type BChE and in 52 min by 0.2 μM A328Y, a result consistent with a 2.6-fold higher k_{cat} value for the A328Y mutant.

Naturally Occurring Genetic Variants of Human BChE. Since BChE has a major role in detoxication of cocaine, it has been suggested that people who are unusually susceptible to the toxic effects of cocaine are those who carry a genetic variant of BChE (Kalow and Grant, 1995). We investigated the atypical variant containing the D70G mutation (McGuire et al., 1989), the J variant containing the E497V mutation (Bartels et al., 1992a), the *K* variant containing the A539T mutation (Bartels et al., 1992b) and the J/*K* variant containing the double mutation E497V/A539T. Table 5 shows that the atypical variant has a 10-fold higher K_m value for (−)-cocaine and a 10-fold lower catalytic efficiency. The J, *K* and J/*K* variants have normal binding affinities and normal k_{cat} values for (−)-cocaine.

Discussion

A328Y is a Better Cocaine Hydrolase. To explain why the A328Y mutant hydrolyzes (−)-cocaine more rapidly than wild-type BChE, we docked (−)-cocaine into the active site of BChE (Fig. 5). Cocaine was positioned with the tropane ring facing Trp-82 and the benzene ring facing the acyl binding pocket of BChE. To avoid obstructing the view all residues were deleted except 328 and 438. Figure 5A shows that in wild-type BChE the methyl ester group of (−)-cocaine is very close to His-438, overlapping the van der Waals surface of His-438, and might affect the function of His-438 in catalysis

TABLE 5
Kinetic constants for naturally occurring genetic variants of human BChE, determined in 0.1 M potassium phosphate, pH 7.0, at 25°C for butyrylthiocholine and benzoylcholine, and at 30°C for (−)-cocaine

Human BChE	Butyrylthiocholine				
	K_m	K_{ss}	b	k_{cat}	bk_{cat}
	μM	mM		min^{-1}	min^{-1}
Wild-type	23 ± 2	1.4 ± 0.2	2.5 ± 0.1	33,900 ± 1,800	84,900 ± 4,600
Atypical variant (D70G)	150 ± 30	1.2 ± 0.2	1.2 ± 0.2	32,100 ± 200	38,500 ± 300
J variant (E497V)	17 ± 2	1.0 ± 0.1	2.9 ± 0.2	32,200 ± 1,900	93,500 ± 5,500
J/K variant (E497V/A539T)	17 ± 2	1.0 ± 0.1	2.9 ± 0.1	30,500 ± 1,500	88,400 ± 4,200
K variant (A539T)	17 ± 3	0.7 ± 0.1	2.9 ± 0.2	32,500 ± 2,200	94,300 ± 6,500
	Benzoylcholine		(−)-Cocaine		
	K_m	k_{cat}	K_m	k_{cat}	k_{cat}/K_m
	μM	min^{-1}	μM	min^{-1}	$M^{-1}\ min^{-1}$
Wild-type	8 ± 1	14,500 ± 200	14 ± 1	3.9 ± 0.1	280,000
Atypical variant (D70G)	40 ± 2	15,400 ± 200	172 ± 36	4.0 ± 0.6	23,000
J variant (E497V)	11 ± 1	18,200 ± 600	12 ± 2	3.7 ± 0.2	300,000
J/K variant (E497V/A539T)	9 ± 1	13,400 ± 200	10 ± 2	3.7 ± 0.2	370,000
K variant (A539T)	8 ± 1	15,900 ± 400	13 ± 3	3.9 ± 0.2	300,000

since His-438 is part of the catalytic triad. By contrast, Tyr-328 has pushed the methyl ester away from His-438 in Fig. 5B, thus reducing the steric hindrance on His-438 and explaining the higher catalytic rate of the A328Y mutant. Our finding that the A328F mutant also has a higher k_{cat} for (−)-cocaine is consistent with this model.

Rapid Rate of Hydrolysis of (+)-Cocaine. The structural difference between (+)- and (−)-cocaine is the location of the methyl ester group on the tropane ring. (+)-Cocaine was hydrolyzed by BChE almost 2000-fold faster than (−)-cocaine, the k_{cat} values being 7500 min^{-1} and 3.9 min^{-1}, respectively. Figure 5C shows that (+)-cocaine fit easily into the active site of wild-type BChE. The absence of steric obstruction of His-438 probably explains why (+)-cocaine is rapidly hydrolyzed by BChE. The A328Y mutant hydrolyzed (+)-cocaine at the same rate as wild-type BChE, a result consistent with the model in Fig. 5C, where residue 328 is far from the methyl ester.

Distinct Binding and Catalytic Regions. Binding affinities for the two enantiomers of cocaine are nearly identical (Gatley, 1991; Berkman et al., 1997; present work), whereas rates of hydrolysis differ 2000-fold. This suggests that the binding region may not be the same as the catalytic region. A similar conclusion was reached by Berkman et al. (1997) in studies with phosphonothiolates corresponding to the transition state analogs of (−)- and (+)-cocaine hydrolysis, when they found that the position of the methyl ester group on the tropane ring was the determinant for hydrolysis but not the determinant for binding efficiency. The model by Masson et al. (1997) of three enzyme-substrate complexes preceding formation of the acyl-enzyme intermediate is consistent with the idea of distinct binding and catalytic regions.

People with Genetic Variants of BChE Are At Risk. One out of 2500 Americans and Europeans is homozygous for atypical BChE (Whittaker, 1986; Lockridge, 1990; Kalow and Grant, 1995). People with atypical BChE (D70G) are unable to breathe for 2 h after a normal dose of the muscle relaxant succinylcholine, a dose that is intended to paralyze for 3 to 5 min (Kalow and Gunn, 1957). Our results suggest that people with atypical BChE are also at risk for cocaine intoxication. The prediction is that a dose of cocaine tolerated by most people will be toxic to individuals with atypical BChE. This prediction is based on the observation that atypical BChE (D70G) has a 10-fold lower binding affinity for (−)-cocaine and a 10-fold lower k_{cat}/K_m value. This means that atypical BChE is 10-fold less efficient at hydrolyzing (−)-cocaine.

Similar predictions have been made by others based on the observation of reduced cocaine hydrolysis by plasma with low BChE activity (Jatlow et al., 1979) or by plasma with atypical BChE (Kalow and Grant, 1995; Schwartz and Johnson, 1996). The present work is the first to measure K_m and k_{cat} values for atypical BChE and (−)-cocaine. A comparison with data in the literature is listed in Table 6.

Kalow and Grant (1995) point out that people with atypical BChE are not the only ones at risk for cocaine intoxication. Any person with reduced BChE activity is at risk. "Any decrease of BChE activity that prolongs the normal half-life of cocaine would automatically favor accumulation of cocaine in blood and tissues, particularly if there is repeated cocaine

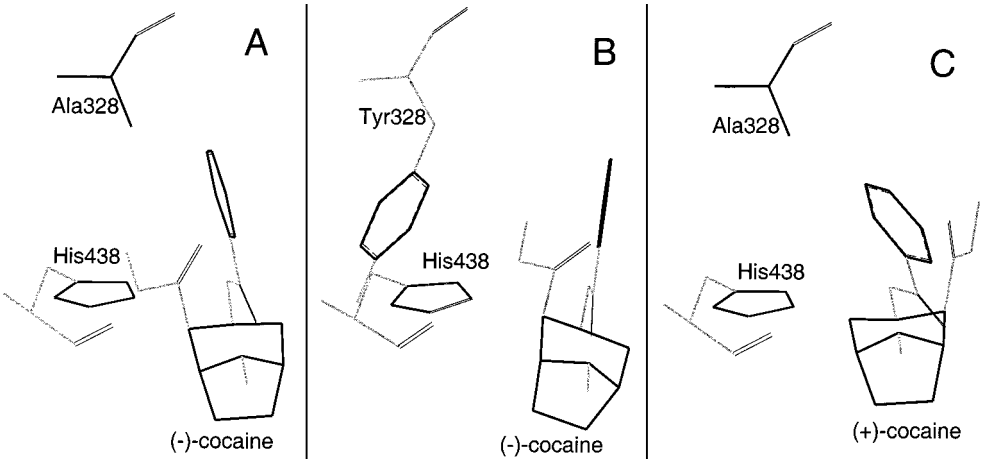

Fig. 5. Cocaine docked in the active site of BChE. A, (−)-cocaine in wild-type BChE. B, (−)-cocaine in the A328Y mutant of BChE. C, (+)-cocaine in wild-type BChE.

TABLE 6
Binding and hydrolysis constants for (−)-cocaine and human BChE[a]

BChE	K_m	K_i	k_{cat}	Reference	pH	Temp.
	μM	μM	min^{-1}			C
Wild-type	44		7	Stewart et al. (1979)	7.4	37
Wild-type		9		Gatley (1991)	7.2	30
Wild-type	12		6	Mattes et al. (1996)	7.4	37
Wild-type		37		Schwartz and Johnson (1996)	7.0	
Wild-type		8		Berkman et al. (1997)	7.4	25
Wild-type	14	14	4	Present work	7.0	30
Atypical (D70G)	172	212	4	Present work	7.0	30
Atypical (D70G)		744		Schwartz and Johnson (1996)	7.0	

[a] The k_{cat} of 7 was calculated from the V_{max} per milliliter of serum in Stewart et al. (1979) by assuming the BChE concentration in serum was 60 pmol/ml. A k_{cat} of 6 was calculated from the data of Mattes et al. (1996) who reported a k_{cat} of 24 for the tetrameric enzyme.

intake, and particularly by methods that favor its rapid absorption. Thus, all genetic and environmentally produced alterations of the activity of BChE (e.g., liver damage) are potentially able to affect the fate of cocaine" (Kalow and Grant, 1995). This conclusion is supported by clinical observations showing a correlation between life-threatening cocaine intoxication and reduced BChE activity (Devenyi, 1989; Hoffman et al., 1992; Om et al., 1993). People with silent BChE have no BChE activity in plasma (Primo-Parmo et al., 1996) and are likely to be at risk. The frequency of homozygous silent BChE is 1 in a 100,000. Since people with the K variant have a 33% decrease of BChE activity in plasma (Rubinstein et al., 1978) and people with the J variant have a 66% decrease of BChE activity in plasma (Garry et al., 1976), it is likely that both genetic variants are more susceptible to the toxic effects of cocaine, even though their binding affinities and k_{cat} values are similar to wild-type. The *K* variant is the most frequent genetic variant of BChE in American, European, and Japanese populations (Whittaker, 1986; Izumi et al., 1994) where 1 out of 69 people are homozygous for the K variant, and 1 out of 4 are carriers.

Therapy for Cocaine Overdose. Administration of wild-type human BChE has been shown to be an effective treatment for cocaine intoxication in mice and rats, preventing convulsions, arrhythmia and death (Hoffman et al., 1996; Lynch et al., 1997; Mattes et al., 1997). Administration of BChE alone to rats had no adverse effects on heart rate, blood pressure, neurologic function, cholinergic function, or behavior and it was concluded that BChE was safe (Lynch et al., 1997).

To assess the potential effectiveness of wild-type BChE for treatment of cocaine overdose one needs to know the levels of cocaine found in human blood. A safe dose is the dose used for clinical purposes. Van Dyke et al. (1976) reported 0.12 to 0.47 mg/l (0.4–1.5 μM) concentrations of cocaine in blood after topical anesthesia and vasoconstriction. Smoking and oral/nasal ingestion have yielded concentrations of cocaine up to 4.7 mg/l (15 μM) in patients who arrived in the emergency room 1 to 2 h after their last dose (Rowbotham, 1992). The range of blood cocaine concentrations in overdose fatalities is 1 to 20 mg/l (Wetli, 1987). Thus the BChE enzyme needs to bind cocaine concentrations as low as 3 μM whereas its $_{K}$m value is 14 μM. This means it will require 2 times more wild-type BChE to destroy 3 μM cocaine than to destroy 14 μM cocaine in a fixed amount of time. An improved BChE with a higher binding affinity and a higher k_{cat} could detoxify cocaine at a lower dose of BChE. The A328Y mutant of BChE is 4-fold more efficient at destroying (−)-cocaine compared with wild-type BChE.

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Send reprint requests to: Dr. Oksana Lockridge, University of Nebraska Medical Center, Eppley Institute, 600 S. 42nd St., Omaha, NE 68198-6805. E-mail: olockrid@mail.unmc.edu