0026-895X/02/6202-220–224$7.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 62:220–224, 2002

Vol. 62, No. 2
1697/997370
Printed in U.S.A.

Re-engineering Butyrylcholinesterase as a Cocaine Hydrolase

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Received February 26, 2002; accepted April 19, 2002

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

To address the problem of acute cocaine overdose, we undertook molecular engineering of butyrylcholinesterase (BChE) as a cocaine hydrolase so that modest doses could be used to accelerate metabolic clearance of this drug. Molecular modeling of BChE complexed with cocaine suggested that the inefficient hydrolysis (k_{cat} = 4 min^{-1}) involves a rotation toward the catalytic triad, hindered by Tyr332. To eliminate rotational hindrance and retain substrate affinity, we introduced two amino acid substitutions (Ala328Trp/Tyr332Ala). The resulting mutant BChE reduced cocaine burden in tissues, accelerated plasma clearance by 20-fold, and prevented cocaine-induced hyperactivity in mice. The enzyme's kinetic properties (k_{cat} = 154 min^{-1}, K_M = 18 μM) satisfy criteria suggested previously for treating cocaine overdose (k_{cat} >120 min^{-1}, K_M < 30 μM). This success demonstrates that computationally guided mutagenesis can generate functionally novel enzymes with clinical potential.

Cocaine overdose remains a serious problem with no broadly effective remedies (Wetli, 1987; Schrank, 1992; Hollander, 1995; Marzuk et al., 1995). An alternative to conventional therapy with receptor antagonists is to enhance metabolic inactivation (Landry et al., 1993; Gorelick, 1997). Cocaine metabolism is partly driven by carboxylesterase. For detoxication, however, the more relevant enzyme is butyrylcholinesterase (BChE), which converts cocaine to the less active derivatives ecgonine methyl ester and benzoic acid (Inaba et al., 1978). Pretreating rats with human BChE reduces cocaine-induced cardiac effects (Mattes et al., 1996) and can prevent death (Lynch et al., 1997), but BChE has such low efficiency that huge amounts could be needed to detoxify a human patient. Clearly, practical treatment requires a cocaine hydrolase with improved kinetic properties.

Because human BChE hydrolyzes unnatural (+)-cocaine 2000-fold faster than naturally occurring (−)-cocaine, we recently used molecular modeling to investigate BChE complexes with each of these stereoisomers (Sun et al., 2001). Our model predicted that both forms of cocaine bind similarly within the active site of BChE but not oriented for immediate catalysis. We hypothesized that the differing catalytic efficiencies with these two substrates reflect differences in a key step: rotation of cocaine's benzoic ester group toward the catalytic triad. Inspection of the model suggested that (+)-cocaine could easily rotate about the axis formed by a cation-π interaction between its ammonium group and Trp82 of BChE. In contrast, (−)-cocaine would rotate less readily because of steric hindrance and a relatively strong π-π interaction with Tyr332.

Accordingly, we developed a rational strategy to create an effective cocaine hydrolase from BChE in two steps: 1) replacing Tyr332 with Ala, to reduce the steric hindrance and the π-π interaction that impede rotation and 2) replacing Ala328 with Trp to provide a cation-π interaction (Gallivan and Dougherty, 2000) to restore substrate affinity lost in disabling the π-π interaction. Herein, we report the properties and some applications of this re-engineered BChE, along with further molecular modeling studies aimed at explaining its improved catalytic efficiency with cocaine.

Experimental Procedures

Materials. Reagents for producing recombinant BChE included a site-directed mutagenesis kit (Stratagene, La Jolla, CA), an expression plasmid pRc/CMV incorporating human BChE (Xie et al., 1999), Dulbecco's modified Eagle's medium (DMEM; Fisher Scientific, Fairlawn, NJ), human embryonic kidney 293 cells (American Type Culture Collection, Manassas VA), and a plasmid purification kit (QIAGEN, Valencia, CA). Oligonucleotides were synthesized by Mayo's Molecular Biology Core Facility. Echothiophate iodide was from Wyeth-Ayerst (Rouses Point, NY). Synthetic (+)-cocaine was provided by the National Institute on Drug Abuse Research Resources Drug Supply System (Rockville, MD), and (−)-[^{3}H]cocaine was purchased from New England Nuclear (Boston, MA). Other materials, all from Sigma (St. Louis, MO) were: natural (−)-cocaine (purchased

This work was supported by the Mayo Foundation for Medical Education and Research and National Institute on Drug Abuse grant DA011707 (to O.L.).

ABBREVIATIONS: BchE, butyrylcholinesterase; DMB, A328W/Y332A butyrylcholinesterase; DMEM, Dulbecco's modified Eagle's medium; 3D, three-dimensional.

under an institutional license); butyrylthiocholine iodide; benzoylcholine chloride; and human plasma.

Molecular Modeling. The 3D structure of A328W/Y332A was generated from the computationally generated 3D model of wild-type BChE by changing residues A328 and Y332 to tryptophan and alanine, respectively, with the LINK, EDIT, PARM, and SANDER modules of the AMBER 5.0 program (Pearlman et al., 1995). Cocaine was docked to the catalytic gorge of A328W/Y332A by the EUDOC program (*ceramic version*) as described previously (Pang et al., 2001). A box of 5.5 × 4.0 × 10.0 Å was defined in the binding pocket of A328W/Y332A to confine ligand translation. Translational and rotational increments were set at 1.0 Å and 10° of arc, respectively. The most energetically stable, EUDOC-generated, Michaelis-Menten complex of cocaine and enzyme was then refined by a molecular dynamic simulation. Complexes were simulated for 1.0 ns in a TIP3P water box with a periodic boundary condition at constant temperature (298°K) and pressure (1 atm). A time-average of 1000 instantaneous structures of the complex at 1-ps intervals was generated with the CARNAL module of AMBER 5.0.

Mutagenesis of BChE. Mutations were generated from wild-type human BChE in a pRc/CMV expression plasmid. Using plasmid DNA as template and primers with specific base-pair alterations, mutations were made by polymerase chain reaction with *Pfu* polymerase, for replication fidelity. Modified plasmid DNA was transformed into *Escherichia coli,* amplified, and re-sequenced to ensure correctness. Purified plasmids were then transfected into human embryonic kidney 293 cells by calcium phosphate precipitation, and recombinant BChE was expressed in serum-free DMEM.

Expression and Purification of BChE Tetramers. Stable lines expressing human BChE were made in CHO-K1 cells (American Type Culture Collection). As described previously (Xie et al., 1999), a 1.8-kilobase DNA sequence encoding the signal peptide and 574 amino acids of the enzyme was cloned into pGS. To promote BChE assembly into a stable tetrameric form, this plasmid was cotransfected with a pRc/RSV plasmid (Invitrogen, Carlsbad, CA) that expressed the signal peptide and 45 N-terminal amino acids of the rat *COLQ* gene (Krejci et al., 1997; Altamirano and Lockridge, 1999). Colonies resistant to 50 μM methionine sulfoximine and 0.8 mg/ml G418 were expanded as 1-liter roller cultures (Ultraculture medium plus methionine sulfoximine and G418 alternated with glutamine-deficient DMEM/Ham's F-12). The secreted BChE was purified by affinity chromatography on procainamide-Sepharose followed by anion exchange chromatography on DE52 (Arpagaus et al., 1990). Purified BChE was dialyzed in phosphate-buffered saline, concentrated to 1 mg/ml, filter sterilized, and stored at 4°C. Active sites in the preparation were titrated by overnight incubation at 25°C with echothiophate (Radic et al., 1991).

Enzyme Assays and Kinetics. To measure hydrolysis of efficient substrates butyrylcholine and synthetic (+)-cocaine, classical spectrophotometric methods were used (Ellman et al., 1961; Gatley, 1991). To measure the slower hydrolysis of natural cocaine we devised a sensitive method based on liberation of [^{3}H]benzoic acid from (−)-[^{3}H]cocaine. The radiolabeled substrate was pre-extracted with toluene to eliminate any free benzoic acid, then mixed with unlabeled cocaine. Substrate mixtures (50 nCi, 50 μl) were incubated for 60 min in scintillation vials with 50 μl of enzyme in 0.1 M sodium phosphate, pH 7.4. Reactions were stopped by addition of 300 μl of 0.02 M HCl to neutralize liberated benzoic acid. After partitioning into 4 ml of toluene-based fluor (or, with high protein samples, after extraction into pure toluene), product was measured by scintillation counting. In some cases residual substrate was measured identically with one exception: before extraction, samples were alkalinized with 300 μl of 1 M Na_2CO_3, so neutral [^{3}H]cocaine (but not ionized benzoic acid) would partition into the organic phase. To obtain a signal 2-fold above background in the radiometric assay, only 0.001 unit of wild-type BChE was required, about 10,000 less than typically used in spectrophotometric assays for cocaine hydrolysis. However, the two methods yielded similar estimates of K_M and V_{max}.

Tissue and Plasma Samples. To simulate cocaine clearance in vitro, 1-ml samples at pH 7.6 were prepared with 0.85 ml of human plasma plus 1) phosphate-buffered saline, 2) wild-type or mutant BChE (4.2 μg/ml), or 3) BChE and inhibitor (echothiophate, 0.1 mM). For in vivo studies, male Sprague-Dawley rats (250~350 g) were anesthetized with urethane (1.45 mg/kg, i.p.). Catheters were placed in the tail vein and carotid artery of each rat, and A328W/Y332A was administered (1 or 3 mg/kg, i.v.), followed 10 min later by (−)-[^{3}H]cocaine (6.8 mg/kg, 30 μCi, total). Fifteen minutes later, 1 ml of arterial blood was drawn into a heparinized tube. The rats were then euthanized with sodium pentobarbital (200 mg/kg, i.v.) and perfused with 60 ml of NaCl solution containing inhibitors of BChE and carboxylesterase (echothiophate, 10^{-5}M; tetraisopropyl pyrophosphoramide, 10^{-5}M; and saturated sodium fluoride, 25 μl/ml). Brain, heart, kidney, liver, and spleen were collected on dry ice, then homogenized in cold 10 mM sodium phosphate, pH 7.4, with 0.5% Tween 100 and enzyme inhibitors. After centrifugation (8000*g*, 10 min, 4°C), the supernatants were immediately assayed for cocaine.

Locomotor Activity. Adult male 129Sv mice (52–94 days old, 24–35 g), kept in cages without bedding that could spuriously trigger a beam counter, were acclimated for 1 h in a dimly lit, sound-proof room. Mice then received either cocaine alone (25 mg/kg, i.p.), saline alone, or BChE (2.8 IU/g) followed 1 h later by cocaine. Each mouse received cocaine only once. Motion was detected as beam interruptions with an opposed light-emitting diode and a photodiode detector connected to a microprocessor for quantitation.

Statistical Analysis. Treatment effects were subjected to analysis of variance using StatView 4.5 (Abacus Concepts, Berkeley, CA); $p < 0.05$ was considered statistically significant.

Results

Improving Cocaine Hydrolase Activity. Modified BChE with double mutations predicted to enhance cocaine hydrolase activity (A328W/Y332A) was stably expressed and purified in milligram quantities. Compared with wild-type BChE, the two mutations synergistically caused a 40-fold increase in catalytic power (k_{cat}) versus (−)-cocaine and an 11-fold rise in catalytic efficiency (k_{cat}/K_M), with little apparent decline in affinity (Table 1). By contrast, k_{cat}/K_M with butyrylthiocholine was nearly the same in the mutant (580 ± 12 $min^{-1}\mu M^{-1}$) as in wild-type BChE (595 ± 2.6 $min^{-1}\mu M^{-1}$), whereas k_{cat}/K_M for (+)-cocaine actually fell almost 2-fold (from 760 ± 3 to 460 ± 16 $min^{-1}\mu M^{-1}$). Hence, the selected mutations selectively enhanced hydrolysis of clinically relevant (−)-cocaine. It was also striking that k_{cat} with *both* cocaine stereoisomers was pH-dependent in A328W/Y332A, whereas that of wild-type BChE was pH-dependent only with the unnatural isomer (Fig. 1).

To explain the enhanced catalytic properties, molecular modeling was used to investigate 3D-complexes of cocaine with A328W/Y332A and wild-type BChE. In the predicted A328W/Y332A complex, the ammonium nitrogen atom of (−)-cocaine engaged in cation-π interactions with Trp82 and

TABLE 1
Kinetic constants of BCHE and mutants with (−)-cocaine
Hydrolysis of (−)-cocaine was determined radiometrically. Data were fitted to the Michaelis-Menten equation by nonlinear regression; V_{max} was divided by titrated enzyme concentrations to calculate k_{cat}.

Enzyme	(−)-Cocaine	
	K_M	k_{cat}
	μM	*min^{-1}*
Wild-type	4.5 ± 0.3	4.1 ± 0.4
Tyr332Ala	245 ± 64	109.5 ± 0.7
Ala328Trp	3.1 ± 0.1	50 ± 0.6
Ala328Trp/Tyr332Ala	18 ± 2	154 ± 14

Trp328, whereas the cocaine phenyl ring interacted with Trp328 only (Fig. 2). In key respects, this (−)-cocaine-A328W/Y332A complex resembles the (+)-cocaine–BChE complex rather than the (−)-cocaine–BChE complex, even though A328W/Y332A and BChE differ by only two residues at the active site. In fact, the two esters of (−)-cocaine in A328W/Y332A overlaid perfectly with those of (+)-cocaine in BChE. This overlay is consistent with the pH-dependence data indicating that the mutant enzyme acts similarly upon both cocaine stereoisomers.

The catalytic properties of BChE with single mutations clarified the contributions of the two mutations in A328W/Y332A (Table 1). In Tyr332Ala, for example, k_{cat} was 26-fold higher than in wild-type BChE. However, the accompanying 50-fold increase in K_M was equivalent to a 2.4 kcal/mol reduction in affinity, attributable to the lost π-π interaction. In two other singly mutated enzymes, Ala328Tyr and Ala328Trp, k_{cat} values were 4- and 13-fold above wild-type but K_M values were reduced from 4.5 μM to about 3.0 μM. These properties suggest that the other A328W/Y332A mutation restores (−)-cocaine binding affinity via cation-π interaction, without impairing catalytic function.

Enhancing Cocaine Metabolism. A rationale to use hydrolases for cocaine detoxication is that circulating enzymes should draw drug from target tissues by reversing concentration gradients. With its 40-fold increase in k_{cat} for (−)-cocaine and its K_M in the concentration range typical of fatal overdose (Wetli, 1987), A328W/Y332A should be more clinically effective than wild-type BChE. This prediction was tested first on cocaine removal from human plasma in vitro (Fig. 3). Cocaine half-life in normal plasma was 154 min. Adding wild-type BChE (4.2 μg/ml) reduced half-life to 86 min, but the same amount of A328W/Y332A reduced half-life to 5 min and converted all cocaine to benzoic acid within 30 min. A328W/Y332A was also highly effective in vivo. When injected into rats before cocaine challenge, it caused dose-dependent reduction of cocaine levels in all sampled tissues, including heart and brain (Fig. 4). Enzyme inactivated by echothiophate did not alter cocaine removal (not shown).

To obtain further evidence for therapeutic potential, we measured locomotor activity in mice. In control animals, a moderate dose of cocaine (25 mg/kg, i.p.) regularly induces a dramatic hyperactivity that can be quantitated as interruptions of a light beam (see *Experimental Procedures*). Remarkably, however, mice pretreated with A328W/Y332A showed no more locomotor activity after injection of cocaine than after injection of saline solution (Fig. 5). In other words, the mutant BChE virtually abolished overt behavioral signs of response to cocaine.

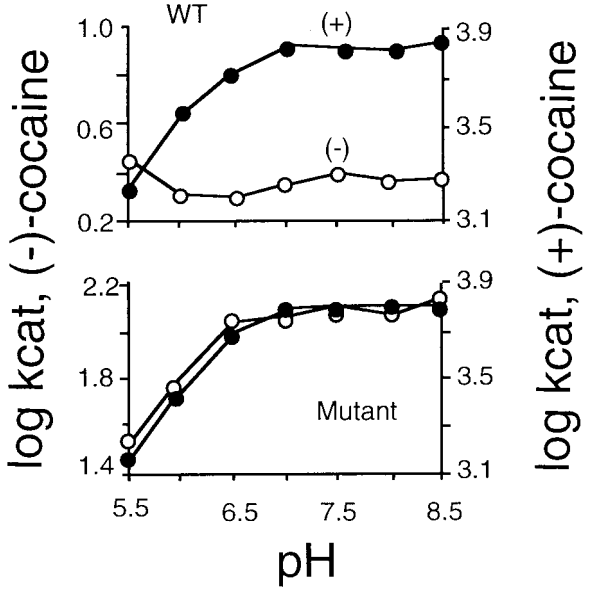

Fig. 1. Cocaine hydrolysis and pH-dependence. Reactions were carried out in triplicate in 0.1 M sodium phosphate buffers at 25°C for (+)-cocaine (●) and 37°C for (−)-cocaine (○). Apparent k_{cat} values (min^{-1}) were determined after fitting kinetic data directly to the Michaelis-Menten equation.

Discussion

Why A328W/Y332A Mutations Improve Cocaine Hydrolysis. Although cocaine's two isomers differ more than 1000-fold in rates of hydrolysis, wild-type BChE binds them nearly equally well (Gatley, 1991; Berkman et al., 1997; Xie et al., 1999; Sun et al., 2001). We argue that BChE's low efficiency with (−)-cocaine is caused not by weak binding or poor catalysis

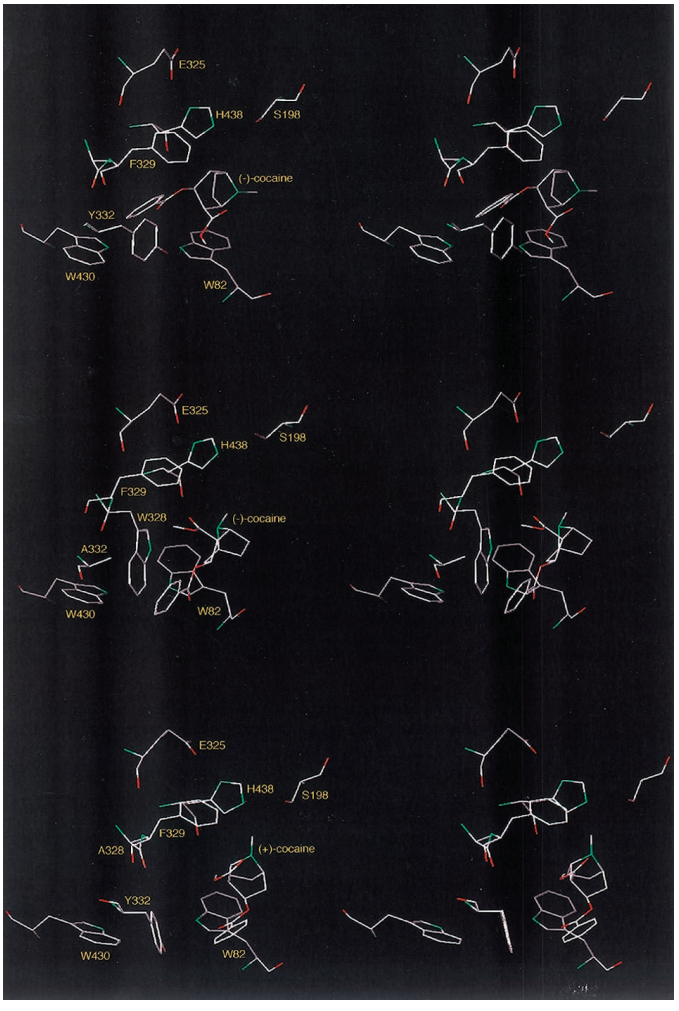

Fig. 2. Cocaine complexes with A328W/Y332A and wild-type BChE. Molecular modeling was performed according to protocols described previously (Sun et al., 2001). Close-up views (looking down into active site) of time-averaged complexes from 1.0 ns (1.0-fs time step) molecular dynamics simulations. Top, (−)-cocaine-BChE [protein data bank (PDB) code: 1EHO); middle, (−)-cocaine-A328W/Y332A (PDB code: 1KCJ); bottom, (+)-cocaine-BChE (PDB code: 1EHQ).

per se but by difficulty in moving substrate from binding site to active site. The assembled data on pH-dependence support the view that substrate movement is important for cocaine hydrolysis by BChE. Our initial studies with native BChE had shown a pH-dependent k_{cat} for hydrolysis of (+)-cocaine, but a pH-independent k_{cat} for hydrolysis of the poor substrate, (−)-cocaine (Sun et al., 2001). The implication was that formation/decay of the acyl-enzyme intermediate is the rate-limiting step with (+)-cocaine, whereas the rate-limiting step with (−)-cocaine occurs earlier. More than one interpretation can be considered, but we lean toward the view that, after initial binding, (−)-cocaine is selectively hindered in rotating into close contact with residues of the catalytic triad. Because the differential effects of pH are lost in the double mutant enzyme, we suggest that this hindrance has been reduced or eliminated in A328W/Y332A.

That suggestion fits the structural picture. Our original structural models identified intermolecular interactions, not available to (+)-cocaine, that stabilize (−)-cocaine in its initial binding orientation on BChE and may sterically hinder the benzoic ester in approaching S198 for hydrolysis. For example, with (−)-cocaine, an aromatic core of Phe329, Tyr332, and Trp430 in wild-type BChE traps cocaine's phenyl ring and can be expected to impede rotation (Sun et al., 2001). We propose that the mutation Y332A alleviates the problem by breaking up this core of aromatic residues. The Trp 328 mutation may also help prevent ligand entrapment in A328W/Y332A because it introduces a pyrrole ring whose location forces (−)-cocaine to rotate 180° about its ammonium group, relative to the orientation in BChE. Consequently, the drug can enter cation-π interaction with Trp328 in the mutant enzyme and position its phenyl ring as does (+)-cocaine in BChE, free to rotate toward Ser198 (Fig. 2). Overall it seems that the catalytic efficiency of A328W/Y332A for (−)-cocaine was achieved by eliminating rotational hindrance without compromising substrate binding. Basically, a π-π interaction of the phenyl ring (which hinders rotation) has been exchanged for a cation-π interaction of the ammonium group (which improves binding affinity and favors rotation).

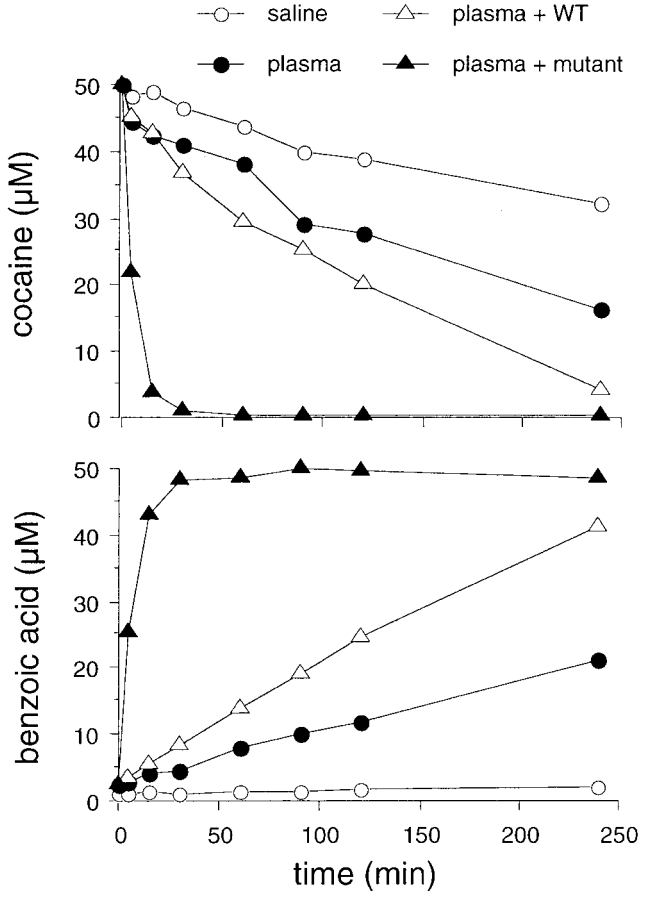

Fig. 3. Accelerated cocaine breakdown in plasma. Cocaine breakdown was measured in vitro in human plasma plus wild-type BChE or A328W/Y332A (4.2 μg/ml) in a final volume of 1 ml (pH 7.4–7.7). After addition of [^{3}H]cocaine, separate aliquots were sampled periodically to assay residual [^{3}H]cocaine (top) and liberated [^{3}H]benzoic acid (bottom).

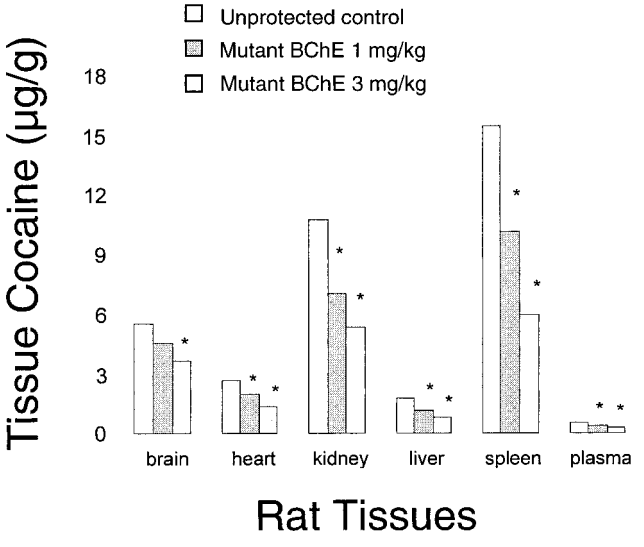

Fig. 4. A328W/Y332A pretreatment and tissue cocaine levels. A328W/Y332A was injected 10 min before [^{3}H]cocaine, and cocaine levels were assayed in tissue samples collected 15 min later. Data are means ± S.E.M. in micrograms per gram, wet weight (n = 5). ⋆, significantly different from control samples by analysis of variance and post hoc Scheffé's F test ($p < 0.05$). Regression analysis demonstrated dose-dependent, A328W/Y332A-induced reduction of cocaine levels in all tissues including brain ($p < 0.05$).

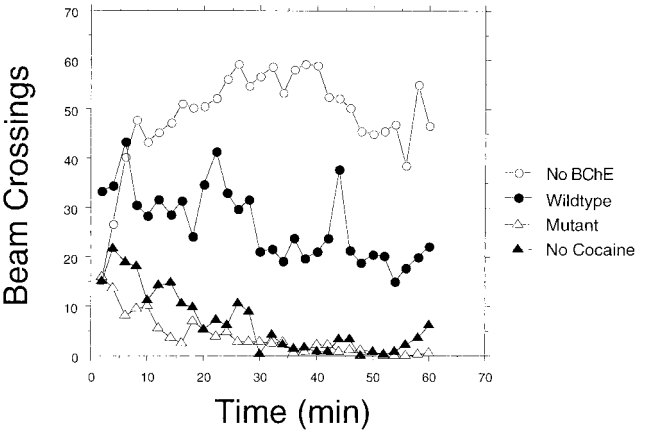

Fig. 5. Locomotor activity in mice as a measure of cocaine toxicity. Mice were pretreated with saline or BChE (wild-type or mutant, 2.8 units/g, i.p.) 1 h before receiving cocaine (25 mg/kg, i.p.). Beam breaks were summed every 2 min for 60 min after injection of cocaine (mean values are shown, n = 10). The loss of cocaine-induced hyperactivity in mice treated with A328W/Y332A BChE was highly significant ($p < 0.001$).

Clinical Potential of A328W/Y332A. It has been estimated that a cocaine hydrolase with $k_{cat} > 120\ min^{-1}$ and $K_M < 30\ \mu M$ would be effective against overdose (Landry et al., 1993). A328W/Y332A meets these criteria. In fact, the enzymatic properties of A328W/Y332A, despite its modestly elevated K_M, give this enzyme a considerable advantage in eliminating cocaine from the blood circulation, especially when plasma levels are high. Patients intoxicated with cocaine can have drug levels up to 60 μM (Wetli, 1987; Benowitz, 1993), a concentration that just reaches the saturation point for A328W/Y332A.

One sign of therapeutic potential in A328W/Y332A is the ability to accelerate cocaine removal from isolated plasma and to cause dose-related reduction of tissue cocaine levels in vivo. Because A328W/Y332A reduced the cocaine buildup in heart, it might combat the cardiovascular arrhythmias that are prime factors in lethal cocaine overdose (Foltin et al., 1995). A328W/Y332A effects in brain were weaker than in heart, possibly because cocaine was retained in lipid-rich white matter or bound tightly to membrane transporters (Jones et al., 1995). Nonetheless, pretreatment with A328W/Y332A did cause dose-dependent reductions of cocaine levels in nervous tissue.

The data on locomotor activity suggest that these reductions had a large behavioral impact. Hyperactivity is one of the striking effects of cocaine in doses that generate moderate plasma levels, on the order of 2 μM (Taylor and Ho, 1977; Benowitz, 1993). Blockade of this effect by a modified BChE is consistent with other evidence that sequestration or metabolic conversion of circulating cocaine lessens behavioral effects in rodents (Baird et al., 2000; Mets et al., 1998). In the long run, there is reason for optimism that sustained delivery of a cocaine hydrolase, possibly by gene transfer, would be a useful approach to therapy of cocaine addiction.

Further studies of toxicology, physiology, and behavior are required to determine whether A328W/Y332A has real clinical potential. It is particularly important to evaluate this agent in a paradigm that realistically reflects the classic overdose emergency. The question is whether a re-engineered BChE will be able to reverse cocaine toxicity that has been already been established. This is a subject of ongoing investigation.

Although a bacterial cocaine hydrolase of even higher catalytic efficiency has recently been reported (Larsen et al., 2002), the modified BChE is worth exploring because, as a nearly natural human protein, it is unlikely to provoke immunological reaction or adverse effects in patients. We have docked (−)-cocaine into the active site of the bacterial enzyme. The predicted structure of the complex (Y. P. Pang, unpublished observations) suggests that the higher catalytic efficiency of the bacterial enzyme is conferred by the location of the catalytic triad that permits an immediate hydrolysis without a rotation of cocaine after binding. Therefore, if the structural advantages of the bacterial enzyme and DMB could be combined, these first-generation cocaine hydrolases might lead to new enzymes with even greater clinical promise.

Possibly the most significant aspect of our work is the demonstration that functionally new enzymes can be created readily when mutagenesis is guided by molecular modeling. We are especially encouraged to find that the kinetic properties (k_{cat} and K_M) and the stereochemical requirement of a substrate in the chiral environment of an enzyme can be altered at will. Rational enzyme mutagenesis may therefore open doors toward a variety of conceptually novel and effective medicines.

Acknowledgments

We thank Drs. Larry Schopfer and Terrone Rosenberry for suggestions and discussion concerning studies of pH dependence.

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