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Interaction of Human Butyrylcholinesterase Variants with Bambuterol and Terbutaline

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Bambuterol, a dimethylcarbamate, carbamoylates butyrylcholinesterase (BChE; EC 3.1.1.8). The carbamoylated enzyme is not very stable and the final product of the two-step hydrolysis is a bronchodilator drug, terbutaline (1-(3,5-dihydroxyphenyl)-2-*t*-butylamino-ethanol sulphate). Both bambuterol and terbutaline inhibit BChE, but their affinities differ in human serum BChE variants (U, A, F, K and S) due to their positive charge. Bambuterol inhibition rate constants for the homozygous usual (UU), Kalow (KK), fluoride-resistant (FF) or atypical (AA) variant ranged from 4.4 to 0.085 $min^{-1} \mu M^{-1}$. Terbutaline showed competitive reversible inhibition for all BChE variants. The dissociation constants for UU, FF and AA homozygotes were 0.18, 0.31 and 3.3 mM, respectively. The inhibition rate or dissociation constants for heterozygotes were distributed between the respective constants for the corresponding homozygotes. A 50-fold difference in inhibition between the UU and AA enzyme might affect terbutaline release in humans. The affinity of all studied BChE variants for terbutaline was low, which suggests that terbutaline originating from bambuterol hydrolysis should not affect the hydrolysis of bambuterol by BChE.

Keywords: Bambuterol; Human butyrylcholinesterase variants; Inhibition; Terbutaline

INTRODUCTION

The activity of human plasma butyrylcholinesterase (BChE; EC 3.1.1.8) is of clinical importance in several therapeutic fields as well as in the inactivation of naturally occurring toxins and synthetic poisons and drugs.[1,2] Plasma BChE is also involved in the controlled-release of terbutaline from bambuterol, a bis-carbamoyl ester, which has been approved for the treatment of asthma due to its bronchodilating property.[3–5] The high therapeutic index of bambuterol is the result of a remarkable selective inhibition of BChE with respect to the related enzyme acetylcholinesterase (EC 3.1.1.7); the ratio of the inhibition rate constants k_{BChE}/k_{AChE} is 16000.[6]

At least 20 different human BChE variants have been characterized so far. Their origin is in nucleotide substitution, deletion or insertion, which alter BChE protein and its catalytic properties, or even lead to a partial or complete absence of BChE protein due to premature termination of protein synthesis.[7] The qualitative variants which include atypical (A) and the fluoride-resistant (F) BChE can be distinguished from the usual enzyme (U) by a lower degree of inhibition by dibucaine, sodium fluoride and the dimethylcarbamate, Ro 02-0683.[8–10] The Kalow variant (K) as the quantitative variant of BChE is present in concentrations of ~70% of the usual variant level in plasma because of variation in its expression or stability.[11] The discovery of healthy individuals whose BChE is totally lacking activity, as well as of individuals exhibiting extremely reduced levels of BChE activity (only 2%–3% of the usual level) led to the recognition of the silent variant (S).[7]

Bambuterol is transformed into active terbutaline in a two-step hydrolysis by BChE and by an oxidative process (Figure 1).[12] The carbamoyl-BChE conjugate spontaneously decarbamoylates at a rate ensuring slow terbutaline release. In spite of the clinical importance of bambuterol and terbutaline the kinetics of their interactions with human BChE variants has not been extensively

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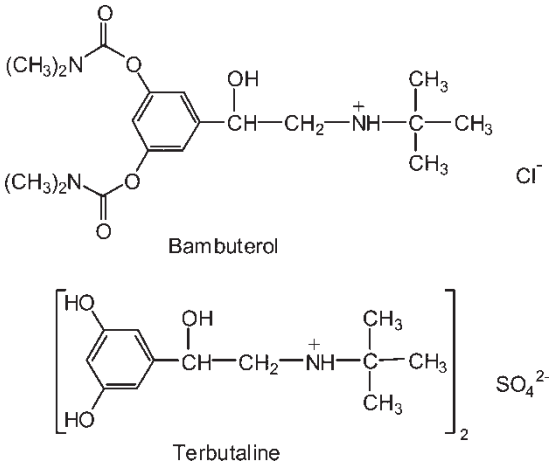

FIGURE 1 Chemical structures of bambuterol and terbutaline.

studied until now. This paper presents the kinetics of bambuterol inhibition of human BChE variants. Due to possible inhibition by terbutaline, dissociation constants for the reversible complex of BChE variants with terbutaline were also determined.

MATERIALS AND METHODS

Materials

Propionylthiocholine iodide (PTCh), benzoylcholine chloride (BzCh), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) and dibucaine hydrochloride were purchased from Sigma Chemical Co., USA. Bambuterol (5-[-(*tert*-butylamino)-1-hydroxyethyl]-*m*-phenylene-bis(dimethylcarbamate) hydrochloride) and terbutaline (1-(3,5-dihydroxyphenyl)-2-*t*-butylaminoethanol sulphate) were supplied by Astra Draco AB, Sweden (Figure 1). Ro 02-0683 (dimethylcarbamate of (2-hydroxy-5-phenylbenzyl)trimethylammonium bromide) was supplied by Hoffman La Roche, USA. Sodium fluoride was purchased from Kemika, Croatia.

The serum specimens were collected at the Institute for Medical Research and Occupational Health, Zagreb, and at the Cholinesterase Investigation Unit at St. James's University Hospital, Leeds, UK, during the Cholinesterase Proficiency Programme organized by Dr. R. T. Evans in 1994–98.[13] One atypical serum specimen was provided by Dr. O. Lockridge, University of Nebraska Medical Center, Eppley Institute, Omaha, USA.

All spectrophotometric measurements were performed on a Cary 300 spectrophotometer (Varian Inc., Australia).

Determination of BChE Phenotypes

Human serum BChE specimens were phenotyped at 25°C in 66.7 mM phosphate buffer (pH = 7.4) by determining the percentage of inhibition (*i.e.* inhibitor numbers) of BzCh hydrolysis (50 μM) in the presence of dibucaine (50 μM), sodium fluoride (50 μM) or Ro 02-0683 (10 nM).[10] The final dilution of serum was 200-times. The inhibition by dibucaine and sodium fluoride was measured immediately after the addition of inhibitor and substrate to the reaction mixture, whereas the procedure for Ro 02-0683 inhibition required a 2 h pre-incubation of the enzyme with inhibitor prior to the addition of substrate. The hydrolysis of benzoylcholine resulted in a decrease in absorbance at 240 nm.

BChE Activity Measurements

Enzyme activities with PTCh were determined at 25°C in 100 mM phosphate buffer (pH = 7.4) and 0.3 mM DTNB using the Ellman spectrophotometric method.[14] The final dilution of serum was 150- or 300-times. The increase in absorbance was recorded at 412 nm ($\varepsilon_M = 14220\ M^{-1}\ cm^{-1}$).[15]

Progressive Inhibition

Enzyme samples were incubated with bambuterol in the absence of PTCh for various intervals up to 30 min. The inhibition reaction was stopped by the addition of PTCh (4.0 mM final concentration), and the extent of inhibition was determined by measuring the residual activity. At least three bambuterol concentrations ranging from 10^{-8} to 10^{-5} M were used for each enzyme preparation. The first-order rate constants (k_{obs}) were calculated by linear regression analysis at any given inhibitor concentration (i):

$$\ln \frac{v_0}{v_i} = k_{obs} \cdot t \qquad (1)$$

where v_0 and v_i stand for the enzyme activity in the absence and in the presence of inhibitor at time t. The second-order inhibition rate constant (k_i) was calculated from:

$$k_i = k_{obs}/i \qquad (2)$$

Reversible Inhibition

Reversible inhibition by terbutaline (1.0–20 mM) was measured with PTCh (s; 0.05–1.0 mM) in the absence (v_0) and in the presence (v_i) of terbutaline concentration (i). For each substrate concentration, the apparent dissociation constant (K_{app}) was calculated by linear regression analysis using the Hunter-Downs equation.[16]

$$K_{app} = \frac{v_i \cdot i}{v_0 - v_i} = K_i + \frac{K_i}{K_{(s)}} \cdot s \qquad (3)$$

TABLE I Activities (±SD) of human BChE variants with benzoylcholine (BzCh), and propionylthiocholine (PTCh), and inhibitor numbers (±SD) with dibucain (DN), fluoride (FN) and Ro 02-0683 (RoN). The number of BChE specimens is given in brackets; SD denotes standard deviation between specimens

		Activity/$\mu mol^{-1}\,min^{-1}\,ml^{-1}$		Inhibitor numbers		
BChE		BzCh	PTCh	DN	FN	RoN
UU	(6)	1.1 ± 0.3	5.3 ± 1.3	81.2 ± 1.4	60.5 ± 1.4	96.4 ± 1.1
US	(2)	0.60 ± 0.4	3.4 ± 1.8	77.3 ± 2.3	55 ± 2.6	95.7 ± 1.2
UK	(3)	0.61 ± 0.20	3.7 ± 0.3	81.3 ± 1.2	61.4 ± 0.5	96.4 ± 1.0
KK	(1)	0.13	0.76	75	48	90
UA	(3)	0.64 ± 0.03	2.7 ± 0.3	63.8 ± 3.0	50.6 ± 1.5	74.0 ± 4.7
AK	(2)	0.53 ± 0.03	1.9 ± 0.2	52.3 ± 4.7	42.3 ± 2.7	60.8 ± 0.8
UF	(3)	0.60 ± 0.08	2.6 ± 0.5	70.3 ± 4.0	46.3 ± 1.5	96.0 ± 1.0
FF	(2)	0.47 ± 0.09	2.0 ± 0.3	69.2 ± 6.0	33.7 ± 4.6	92.7 ± 3.1
AF	(3)	0.59 ± 0.11	2.8 ± 1.2	46.5 ± 1.2	31.1 ± 1.3	55.6 ± 2.5
AA	(3)	0.58 ± 0.09	1.4 ± 0.3	24.3 ± 0.6	26.0 ± 4.6	10.0 ± 3.5

In this equation, K_i is the enzyme-ligand dissociation constant (K_i), while $K_{(s)}$ is the enzyme-substrate dissociation constant corresponding either to the Michaelis constant (K_m) or to the substrate inhibition constant (K_{ss}).

RESULTS AND DISCUSSION

Enzyme activities and dibucaine, sodium fluoride and Ro 02-0683 inhibitor numbers for 28 specimens were determined in order to establish phenotypes conclusively before we continued with the study (Table I). Phenotypes were classified according to the reference values of activities and inhibitor numbers.[10,13] For specimens with quantitative variants US, UK, and KK, DNA level analysis was performed earlier, since these phenotypes are indistinguishable from the usual phenotype using inhibition measurements.[13]

Progressive inhibition by bambuterol followed first-order kinetics at any given inhibitor concentration (Figure 2A), and the first-order rate constant (k_{obs}) was a linear function of bambuterol concentration (Figure 2B). The resulting bimolecular rate constants (k_i) for bambuterol inhibition (Table II) express the first step of bambuterol hydrolysis in the production of terbutaline. Bambuterol is a potent inhibitor of BChE, and the quantitative variants, KK, US and UK, are affected by it to the same extent as the usual variant. Similar rates of inhibition between U and K variants are not surprising, because mutation in the K variant (alanine 539 to threonine) results in a lower level of expression, and consequently in a lower BChE concentration in serum.[11] The inhibition rate constant for atypical BChE was about 50 times lower than that for the usual enzyme. Neutralization of the charge obtained by the point mutation of aspartate 70 to glycine found in the atypical variant resulted in a decreased reactivity to positively charged bambuterol (Table II), as well as to dibucaine or Ro 02-0683 (cf. Table I).[8,17,18] The inhibition potency of bambuterol in fluoride homozygous BChE was slightly lower than in the usual enzyme because the mutations found in the fluoride variant, threonine 243 into methionine or glycine 390 into valine, are not very close to the active serine of BChE.[9,19]

When the inhibition of the heterozygotes UA, AK, AF, and UF was allowed to proceed for a longer time at low concentrations of bambuterol, we observed a deviation from the kinetics described by Equation (1). This might be due to the existence of two BChE variants having different inhibition rates and not contributing equally to the measured activity.[17] It is theoretically possible that the deviation is the result of decarbamoylation of the catalytic serine, but this seems unlikely since it is known that usual and atypical variants decarbamoylate spontaneously at similar and very slow rates, of about 0.003 min^{-1}.[18] Re-inhibition of free BChE by the monocarbamate produced after the first step of bambuterol hydrolysis

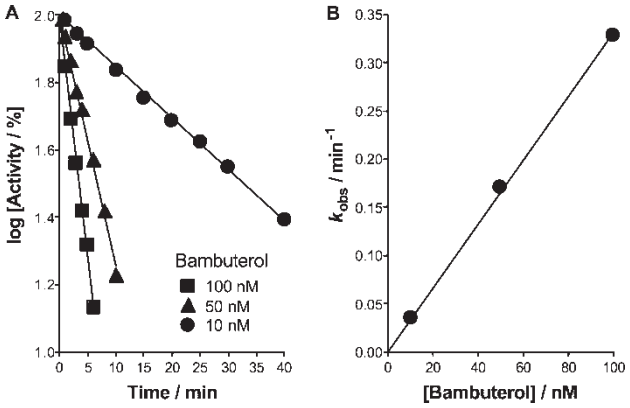

FIGURE 2 Inhibition of human BChE (UK phenotype) by bambuterol. Points on plot (A) indicate logarithm of residual activity *vs.* time of inhibition. The slopes of the lines on plot (A), k_{obs}, were plotted as a function of bambuterol concentrations on plot (B). The overall inhibition rate constant is the slope of the line on plot (B) (see Equations 1 and 2).

TABLE II Second-order rate constants, k_i, for the inhibition of human BChE variants by bambuterol. Equation (2) was applied to calculate k_i (±SE) from k_{obs} (8–13 values) obtained in at least 3 experiments. The number of BChE specimens is given in brackets

BChE		Bambuterol/nM	$k_i/10^6\,M^{-1}\,min^{-1}$
UU	(5)	10–100	4.4 ± 0.2
US*	(1)	10, 30	6.7 ± 0.3
UK	(3)	10–100	3.7 ± 0.2
KK*	(1)	10–500	3.8 ± 0.1
UA	(3)	10–100	2.6 ± 0.0
AK	(2)	10–1000	2.4 ± 0.1
UF	(2)	10–500	1.5 ± 0.1
FSFF	(2)	10–1000	0.95 ± 0.08
AF	(3)	100–1000	0.74 ± 0.04
AA	(3)	500–10000	0.085 ± 0.002

*Calculated from only 3 values of k_{obs}

is also improbable due to a great excess of nonhydrolysed bambuterol. Tunek and colleagues showed that bambuterol is about 10-times more potent as an inhibitor of usual and atypical BChE than monocarbamate.[3] The double decarbamoylation which must occur prior to the generation of terbutaline, may account for prolonged production of the active drug terbutaline, allowing bambuterol administration in humans to a single dose a day.[5,20–22]

The dissociation constants of the enzyme-terbutaline complex (K_i) (Table III) were determined from the effect of substrate upon the degree of inhibition (Figure 3). Terbutaline reversibly inhibited all investigated BChE variants and the inhibition was competitive with PTCh. $K_{(s)}$ constants were lower than the corresponding K_i, which shows that BChE has lower affinity for terbutaline binding (affinity equals $1/K_i$) than for the substrate. The calculated $K_{(s)}$ value for the usual variant was in good agreement with the K_m constant published earlier.[23] The competition of terbutaline with substrate concentrations close to the K_m value indicates that terbutaline binds to the catalytic site.[16] The obtained K_i constants for homozygotes UU, FF and AA differentiate well between the variants, but to a smaller extent than does bambuterol (cf. Table II). The usual variant

TABLE III Dissociation constants (±SD) derived from Equation (3) for the reversible inhibition of human BChE variants by terbutaline; n is the number of K_{app} constants obtained from the listed range of PTCh concentrations. The number of BChE specimens is given in brackets

BChE		PTCh/mM	n	K_i/mM	K_s/mM
UU	(3)	0.05–0.35	13	0.18 ± 0.15	0.017 ± 0.014
US*	(2)	0.05–0.3	6	0.11 ± 0.05	0.011 ± 0.005
KK*	(1)	0.1–0.5	6	0.56 ± 0.40	0.11 ± 0.08
AK	(1)	0.05–0.3	18	0.64 ± 0.17	0.053 ± 0.014
UA	(3)	0.05–0.3	18	0.67 ± 0.16	0.065 ± 0.021
UF	(2)	0.05–0.5	18	0.96 ± 0.12	0.059 ± 0.007
FF	(2)	0.05–0.7	16	0.30 ± 0.32	0.016 ± 0.017
AF	(2)	0.05–1.0	25	2.4 ± 1.1	0.17 ± 0.08
AA	(3)	0.05–1.0	35	3.3 ± 0.7	0.11 ± 0.02

*Calculated from one experiment only.

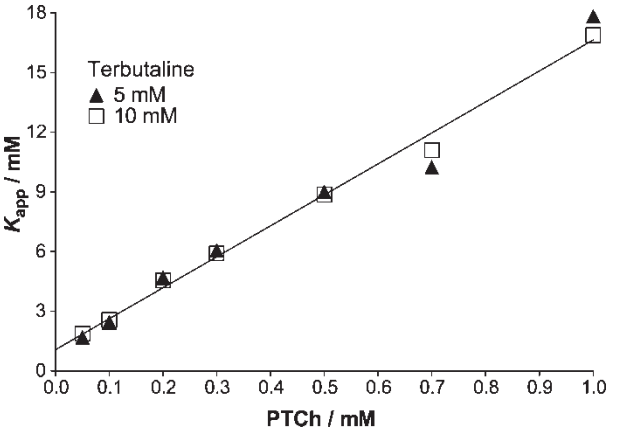

FIGURE 3 Reversible inhibition of human BChE (UF phenotype) by terbutaline. Points indicate calculated K_{app} from measured activities with PTCh in presence of given terbutaline concentrations using Equation (3).

has the highest affinity for terbutaline. Fluoride FF variant has about 2-fold, while atypical 20-fold lower affinity than the usual variant. Terbutaline inhibition constants for heterozygotes with U, F or A variants are distributed between the respective constants of the corresponding homozygotes. The one exception is the UF variant that has an even lower affinity than FF.

Our findings provide new information on the kinetics of bambuterol hydrolysis by BChE and comprise the inhibition of human BChE by both the "substrate" (bambuterol) and the "product" (terbutaline) of the reaction. Both compounds are positively charged and their affinities are higher for the usual BChE than for atypical BChE, but the affinity of terbutaline for any BChE variant is too low to interfere with bambuterol inhibition. A 50-fold difference in bambuterol inhibition between the usual and atypical variants might affect the rate of terbutaline release in humans. However, the second hydrolytic step (hydrolysis of the monocarbamate to terbutaline) coupled with the oxidation of monocarbamate probably limits the rate of terbutaline release and compensates for the slower inhibition of the atypical BChE variant by bambuterol. This is substantiated by the findings that subjects with atypical BChE were able to produce terbutaline as efficiently as subjects with the usual BChE.[12]

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