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INTERACTIONS OF BAMBUTEROL WITH HUMAN SERUM CHOLINESTERASE OF THE GENOTYPES E_uE_u (NORMAL), E_aE_a (ATYPICAL) AND E_uE_a

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Abstract—Bambuterol, a carbamate ester prodrug of the bronchodilator terbutaline, was tested as inhibitor and substrate of human serum cholinesterases of the genotypes E_uE_u (the normal enzyme), E_aE_a (the atypical enzyme) and E_uE_a. The IC_{50} for the normal enzyme was 11 ± 2.2 nM (mean, SD, N = 10) and for the atypical enzyme 140 ± 6 nM (N = 13), indicating a much higher affinity of bambuterol to the normal enzyme. The heterozygotes showed a mixed behaviour; the major activity was inhibited like the normal enzyme (IC_{50} = 9.3 ± 1.9 nM, N = 9), while a residual activity (10–15%) was inhibited by bambuterol like the atypical enzyme. At a bambuterol concentration of 100 nM each of the three cholinesterase genotypes responded uniquely to bambuterol; the normal enzyme was inhibited to 2.2 ± 0.9%, the atypical enzyme to 58 ± 4.6%, and the heterozygote to 10 ± 1.2% of the basal activity. Bambuterol may therefore be added to the list of inhibitors useful in the genotyping of cholinesterases. Bambuterol was much less efficiently hydrolysed in serum containing the atypical cholinesterase than in serum containing the normal enzyme. The results of the hydrolysis experiments once again illustrate the difference in affinity of bambuterol to the genetic forms of cholinesterase, and also strengthen the evidence that cholinesterase is the major serum enzyme catalysing the hydrolysis of bambuterol.

Bambuterol is a bis-dimethylcarbamate prodrug of the bronchodilator terbutaline [1]. The biotransformation of bambuterol to yield eventually terbutaline involves hydrolytic and oxidative steps [2, 3]. The hydrolysis (Fig. 1) in blood is catalysed predominantly, if not exclusively, by plasma cholinesterase (serum cholinesterase, pseudocholinesterase, butyrylcholinesterase, acylcholine-acylhydrolase, EC 3.1.1.8) [2]. Bambuterol also is a very effective, reversible inhibitor of this enzyme [4].

Human plasma cholinesterase exists in a number of genetic variants [5]. The principal method to determine the cholinesterase genotype of a plasma or serum sample is to investigate the sensitivity of the enzyme activity towards a battery of inhibitors [5]. The aim of the present study was therefore to test bambuterol as an inhibitor for serum cholinesterases of the genotypes E_uE_u (homozygous for the usual gene), E_uE_a (heterozygous for the usual and atypical genes) and E_aE_a (homozygous for the atypical gene), and thus to explore the potential use of bambuterol as an additional tool to classify plasma cholinesterases. We also tested *in vitro* the kinetics of hydrolysis of bambuterol by serum cholinesterases of the three genotypes, since differences between genotypes in this metabolic pathway may have implications for the overall formation of terbutaline following treatment with bambuterol.

MATERIALS AND METHODS

Chemicals

Bambuterol (1-[3,5-bis(*N*,*N*-dimethylcarbamoyloxy)phenyl]-2-*t*-butylaminoethanol hydrochloride)

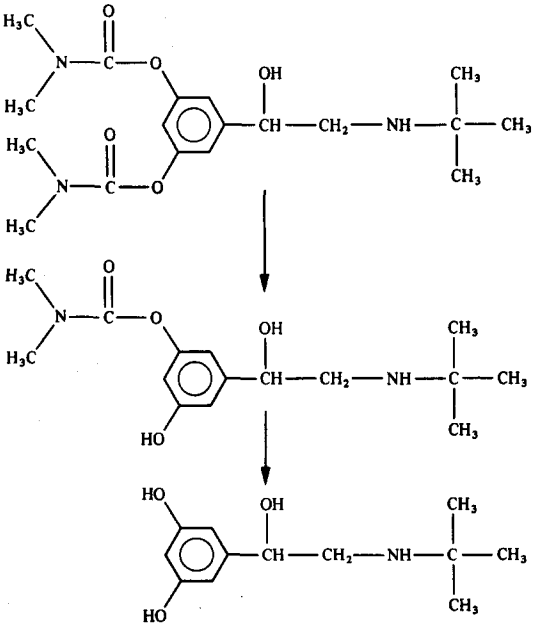

Fig. 1. Hydrolysis of bambuterol to the monocarbamate and further to terbutaline. The hydrolysis experiments described in this paper were designed in such a way that the first reaction dominated and little terbutaline was formed.

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was synthetized at the Organic Chemistry Laboratory, AB Draco (Lund, Sweden). [^{3}H]Bambuterol (1-[3,5-bis(*N,N*-dimethylcarbamoyloxy)phenyl]-2-*t*-butylamino[1-^{3}H]ethanol hydrochloride) with a specific activity of 0.85 mCi/μmol was obtained from the Radiochemical Center (Amersham, U.K.), and had a radiochemical purity of 96% when assayed on the LC-system described below. Other chemicals were of analytical grade and purchased from commercial sources. All water used was purified in a Milli-Q system (Millipore, Molsheim, France).

Human serum and cholinesterase genotyping

The serum samples were obtained from the Danish Cholinesterase Research Unit [6]. The genetic variations of the cholinesterase enzyme had been identified by the following methods: dibucaine number, fluoride number, scoline number and urea number [6]. Also, all genotypes had been verified by family studies.

Cholinesterase inhibition experiments

The efficacy of bambuterol to inhibit serum cholinesterase was determined as described in detail previously for whole blood [4], with minor modifications. The method is based on the cholinesterase assay originally described by Augustinsson *et al.* [7]. Inhibition experiments were performed with 10 serum samples containing cholinesterase of the genotype E_uE_u, with nine of the genotype E_uE_a, and with 13 of the genotype E_aE_a. The procedure was as follows.

Sample cuvette. One hundred microlitres of serum, 10-fold diluted with water, and 100 μL of an aqueous solution of bambuterol (or in controls pure water) were added to 2.75 mL of the incubation buffer (50 mM K_2HPO_4, pH 8.0, containing 100 μM 4,4-dithiodipyridine). The final bambuterol concentrations ranged between 0.1 nM and 10 μM. After preincubation for 30 min at 37° 50 μL of the substrate solution (60 mM butyrylthiocholine dissolved in 3×10^{-5}M HCl, final concentration was thus 1 mM) was added and the enzyme activity determined at 324 nm in a Varian DMS 100 double beam spectrophotometer at 37° as described previously [4]. The preincubation in the absence of the thiocholine ester is required for optimal inhibition of the cholinesterase [4].

Reference cuvette. This cuvette contained an identical mixture as the sample cuvette, but in addition 100 μM of the cholinesterase inhibitor A1397 [7], which was added dissolved in 50 μL water. This inhibitor was added after the preincubation, 2 min prior to spectrophotometric assay.

Cholinesterase activity is expressed as nmol thiocholine liberated/min/10 μL serum.

Serum-catalysed hydrolysis of [^{3}H]*bambuterol*

The hydrolysis of [^{3}H]bambuterol in serum was investigated essentially as in a previous study [2]. At the conditions chosen the monocarbamate is the dominating metabolite, while terbutaline is formed in negligible amounts. Two serum samples of each of the three genotypes were tested.

Incubations. Serum (350 μL) was equilibrated in a glass tube with a glass stopper at 37° for 2 min in a shaking water bath. Then 50 μL of an aqueous solution of [^{3}H]bambuterol was added. The final concentrations of bambuterol were 100 and 1000 nM. Each of these bambuterol concentrations was also incubated for 120 min in the absence of serum to determine the background radioactivity at the site in the LC-gradient where the monocarbamate eluted.

Metabolite determination. After incubation for 5, 10, 20, 30, 60 and 120 min, aliquots of 50 μL from the incubation mixtures were withdrawn and added to 50 μL of 5% perchloric acid. After centrifugation in a Biofuge A (Hereus), 25 μL of the supernatant was injected onto an LC-system [2], the LC-eluate collected in 0.5 min fractions, and radioactivity determined as described previously [2].

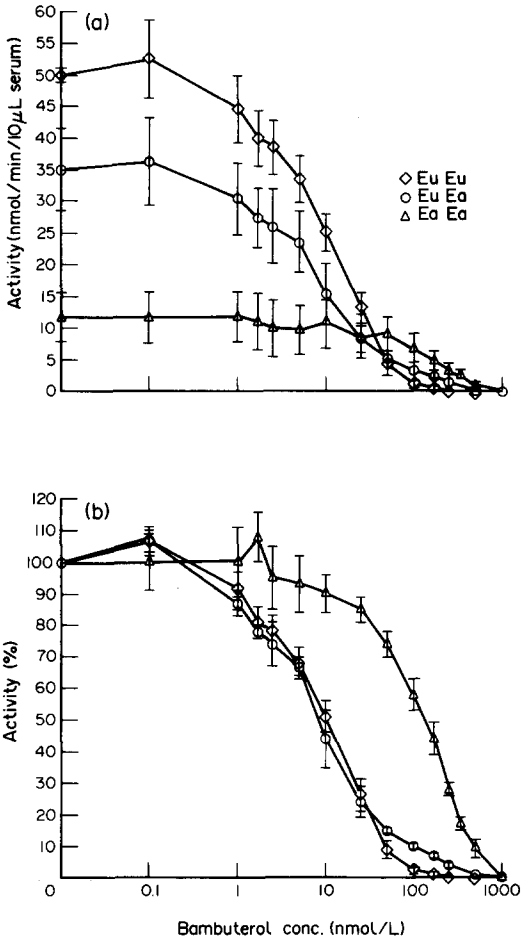

Fig. 2. Inhibition of human serum cholinesterases by bambuterol. The enzyme activities are expressed as nmol thiocholine liberated/min/10 μL serum (a), and as per cent of the activity in the absence of bambuterol (b). Means and SD are given. N = 10, 13 and 9 for E_uE_u, E_uE_a and E_aE_a, respectively.

RESULTS

Inhibition of cholinesterases of different genotypes by bambuterol

The inhibition by bambuterol of the three different genotypes of serum cholinesterase is ilustrated in Fig. 2a and b. E_uE_u was effectively inhibited with

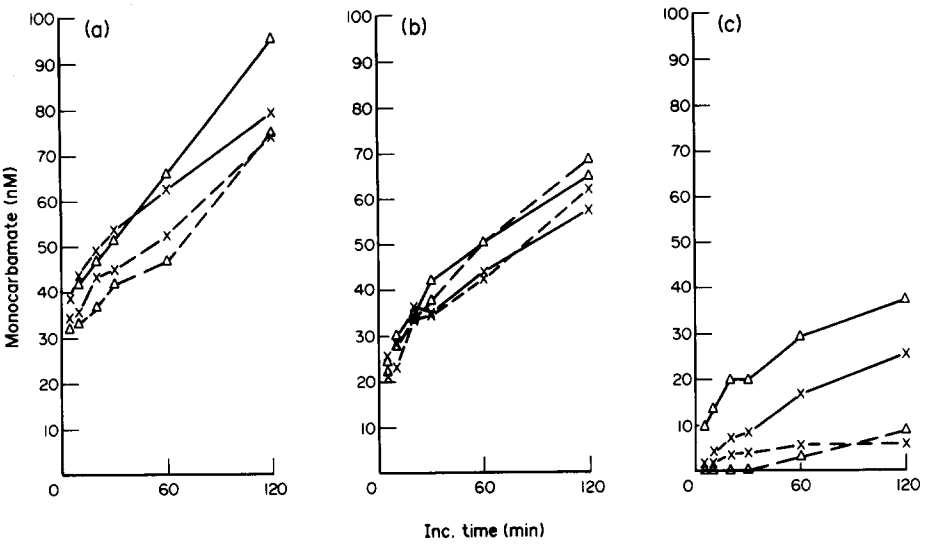

Fig. 3. Formation of [^{3}H]monocarbamate from [^{3}H]bambuterol by human serum. The serum samples contained cholinesterase of the genotypes E_uE_u (a), E_uE_a (b) and E_aE_a (c). The cholinesterase activities, measured with butyrylthiocholine as substrate, were for E_uE_u 57 (solid lines) and 41 (broken lines, for E_uE_a 38 (solid lines) and 42 (broken lines) and for E_aE_a 13 (solid lines) and 4 (broken lines). The unit for these enzyme activities are nmol thiocholine liberated/min/10 μL serum. The concentrations of [^{3}H]bambuterol were 100 nM (stars) and 1000 nM (triangles).

an IC_{50} value of 11 ± 2.2 nM (mean ± SD, N = 10), while E_aE_a was around 14-fold less sensitive, and had an IC_{50} of 140 ± 6 nM (N = 13). Serum cholinesterase of the type E_uE_a responded to bambuterol essentially as did the normal (E_uE_u) enzyme with an IC_{50} = 9.3 ± 1.9 nM (N = 9), but a residual activity (10–15% of the total activity) behaved more like the E_aE_a genotype. Thus, at a bambuterol concentration of 100 nM the three variants of cholinesterase behaved significantly differently; the remaining enzyme activity was 2.2 ±0.9% for E_uE_u, 10 ± 1.2% for E_uE_a and 58 ± 4.6% for E_aE_a.

Hydrolysis of [^{3}H]bambuterol in serum containing different genotypes of cholinesterase

The hydrolysis of [^{3}H]bambuterol was tested in six plasma samples, two of each of the genotypes E_uE_u, E_uE_a and E_aE_a. For each serum sample 100 and 1000 nM [^{3}H]bambuterol was used as substrate.

The formation of the monocarbamate (Fig. 1) from bambuterol in serum containing cholinesterase of the types E_uE_u and E_uE_a is shown in Fig. 3a and b. A rapid initial burst of monocarbamate formation was followed by a slower phase. The rate of formation of the monocarbamate did not change much as the [^{3}H]bambuterol concentration was increased 10-fold, thus the enzymes worked at nearly optimal rates at 100 nM bambuterol. Serum containing cholinesterase of the type E_aE_a behaved differently (Fig. 3c). The sample with very low cholinesterase activity hydrolysed bambuterol at a barely detectable rate. The E_aE_a-type serum sample with rather high cholinesterase activity hydrolysed bambuterol in a fashion dependent on bambuterol concentration. The initial burst of monocarbamate formation was less pronounced in this serum sample than in normals and heterozygotes. This difference was particularly clear at the low bambuterol concentration.

DISCUSSION

Bambuterol inhibited cholinesterase of the normal genotype (E_uE_u) much more effectively than the atypical enzyme (E_aE_a). Serum samples containing either of these two types of cholinesterase have approximately similar molar concentrations of the enzyme as determined by immunodiffusion and rocket immunoelectrophoresis [8]. The enzyme concentrations are in the order 40–60 nM [9, 10]. Therefore, the differences in inhibition by bambuterol are not due to variations in enzyme concentration. Instead, the inhibition characteristics for the genotypes probably reflect differences in affinity of bambuterol to the enzymes. Normal cholinesterase is a tetramer of four identical subunits [11, 12], and each subunit has one anionic and one esteratic site. The fit of substrates to both these sites is probably important for high affinity interactions between enzyme and substrate. It was recently reported [13], that the atypical enzyme has a mutation which most likely affects the anionic site of the enzyme, while the esteratic site is similar for the usual and atypical enzymes [14]. At physiological pH bambuterol, in analogy with choline esters, has a protonized amine function. This probably explains the very high affinity of bambuterol and choline esters to the normal cholinesterase, and the comparatively poor affinity to the atypical enzyme.

The E_uE_a-type of cholinesterase interestingly responded to bambuterol in a mixed manner. The major activity was inhibited like the normal enzyme, while a residual activity was more resistant, and

behaved like the atypical variant. These findings nicely illustrate the existence of at least two different forms of cholinesterase in these serum samples.

The hydrolysis of bambuterol in the serum samples containing normal and heterozygous cholinesterase studied here followed similar kinetics to those previously described for blood samples [2]. The rapid initial burst-like monocarbamate formation reflects high-affinity interaction between native enzyme and substrate, and the capacity of this phase correlated well with the butyrylthiocholine hydrolysing activity. This process involves liberation of the monocarbamate along with dimethylcarbamylation of the esteratic site. Therefore, the following slow phase of hydrolysis is dependent on regeneration of the free esteratic site. The formation of the monocarbamate by these two types of cholinesterase changed only marginally as the bambuterol concentration was increased from 100 to 1000 nM. This indicates that the enzyme was fully occupied already at 100 nM bambuterol, which is in accordance with the fact that this bambuterol concentration almost completely inhibited E_uE_u- and E_uE_a-type cholinesterase.

Serum containing the atypical cholinesterase, E_aE_a, catalysed hydrolysis of bambuterol at a much slower rate, and one of the samples had a barely detectable activity. In the serum sample containing rather high cholinesterase activity of the E_aE_a-type, the rate of hydrolysis of bambuterol, and the capacity of the rapid initial phase, depended on the bambuterol concentration. This illustrates, like the inhibition data, that higher bambuterol levels are required to occupy this enzyme.

The results presented in this report demonstrate that relatively simple inhibition experiments with bambuterol discriminate between three of the commonest genetic variants of human serum cholinesterase. Bambuterol, a very non-toxic compound, could therefore be included in the set of inhibitors used for genotyping this enzyme. Serum containing the atypical form of cholinesterase hydrolysed bambuterol at a much slower rate than serum with normal enzyme. This strengthens the evidence that cholinesterase is the major serum enzyme catalysing this hydrolysis, but the quantitative importance of this metabolic route in humans *in vivo* is not yet established. Pharmacokinetic studies in human volunteers are in progress to investigate if individuals with atypical cholinesterase have impaired capacity of forming terbutaline from bambuterol.

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