

Dibucaine Inhibition of Serum Cholinesterase

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The dibucaine number (DN) was determined for serum cholinesterase (EC 3.1.1.8, SChE) in plasma samples. The ones with a DN of 79-82 were used, because they had the "usual" SChE variant. The enzyme was assayed colorimetrically by the reaction of 5,5'-dithiobis-[2-nitrobenzoic acid] (DTNB) with the free sulfhydryl groups of thiocholine that were produced by the enzyme reaction with butrylthiocholine (BuTch) or acetylthiocholine (AcTch) substrates, and measured at 412 nm. Dibucaine, a quaternary ammonium compound, inhibited SChE to a minimum within 2 min in a reversible manner. The inhibition was very potent. It had an IC_{50} of 5.3 μ M with BuTch or 3.8 μ M with AcTch. The inhibition was competitive with respect to BuTch with a K_i of 1.3 μ M and a linear-mixed type (competitive/noncompetitive) with respect to AcTch with inhibition constants, K_i and K_i' of 0.66 and 2.5 μ M, respectively. Dibucaine possesses a butoxy side chain that is similar to the butryl group of BuTch and longer by an ethylene group from AcTch. This may account for the difference in inhibition behavior. It may also suggest the existence of an additional binding site, other than the anionic binding site, and of a hydrophobic nature.

Keywords: Dibucaine, Dibucaine binding site, Dibucaine number, Inhibition kinetics, Pseudocholinesterase

Introduction

Serum cholinesterase (EC 3.1.1.8, SChE), also known as pseudocholinesterase or butrylcholinesterase, is an esterase of an unknown physiological role (Masson *et al.*, 1998). It was previously suggested to hydrolyze the choline esters that may arise *in vivo*, and which would inhibit acetylcholinesterase (EC 3.1.1.7, AChE) (Lehman and Silk, 1953). However, SChE was found useful in terminating the neuromuscular

block of the muscle relaxants suxamethonium and mivacurium. It hydrolyses these compounds which are sometimes used in clinical anesthesia to facilitate intubation or other medical procedures (Goedde *et al.*, 1968; Hersh *et al.*, 1974; Cook *et al.*, 1989; Ostergard *et al.*, 1992; Fleming *et al.*, 1996). However, some patients were suxamethonium-sensitive and quantitatively low in SChE activity, a genetically-determined property (Kalow and Genest, 1957; Thompson and Wittaker, 1966; Hobbiger and Peck, 1969; Viby-Mogensen, 1981; Cattozzo *et al.*, 1993; Mitchell and Harrop-Griffiths, 1994; Sockalingam and Green, 1995). Thus, a pretest must be used to determine the SChE phenotype prior to suxamethonium or mivacurium infusion, otherwise, there is the risk of prolonged muscular relaxation and apnea. Kalow and Davis (1958), using dibucaine as an inhibitor and benzoylcholine as a substrate under standard conditions, were able to differentiate between the different genetic variants of this polymorphic enzyme. They found that most of the population (97%) had the "usual" phenotype and were dibucaine sensitive with around an 80% inhibition, dubbed the dibucaine number (DN) of 80. About 3% of the population had an "intermediate" phenotype with a DN around 62, and an "atypical" phenotype of 1 in 4000 with a DN around 20. The DN appeared to be a constant characteristic for any one individual. Although the dibucaine number has been extensively used by employing different techniques and procedures (Garry and Routh, 1965; Doutin and Brodeur, 1970; Silk *et al.*, 1979; Arnold, 1981; Gottardis *et al.*, 1990; Palleschi *et al.*, 1990; Holownia *et al.*, 1995), still little is known about the mode of inhibition of this enzyme by dibucaine. Cholinesterases have an affinity for cationic substrates and inhibitors, particularly those that have a positively-charged nitrogen atom that is linked to methyl or ethyl groups. They also combine with the enzyme at two different sites as follows; an anionic site that binds the positive charge on the substrate or inhibitor, and an esteratic site that binds the carboxyl group of the ester linkage that is to be broken. SChE has three specific substrates-benzoylcholine, butrylcholine, butrylthiocholine, and many nonspecific substrates including acetylthiocholine (Silk *et al.*, 1979). In the present study, we investigated the kinetics of dibucaine

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inhibition of SChE in the presence of a specific substrate, BuTch, and a nonspecific substrate, AcTch, so that we could clarify some aspects of the dibucaine inhibition of SChE and gain some insight of the enzyme-binding site.

Materials and Methods

Butrylthiocholine (BuTch) and benzoylcholine were purchased from Fluka Chemie AG (Buchs, Switzerland). Acetylthiocholine (AcTch) was from BDH Chemicals Ltd. (Poole, England). Quinidine sulfate was from Winlab Limited (Berkshire, United Kingdom). Dibucaine and 5,5'-dithiobis-[2-nitrobenzoic acid] (DTNB) were purchased from the Sigma Chemical Co. (St. Louis, USA). All of the other chemicals were of analytical grade. Plasma from healthy individuals was obtained fresh from donors to the blood bank of the King Khalid University Hospital, Riyadh.

Dibucaine number determinations DN was determined as described by Kalow and Genest (1957). The assay mixture consisted of a 66.7 mM phosphate buffer (pH 7.4), 50 μ M benzoylcholine, and 20 μ l plasma. The total volume was 4 ml. The reaction was conducted for 3 min at 37°C. The reaction was terminated by the addition of quinidine sulfate. The absorbance with or without the addition of 10 μ M dibucaine, was read at 240 nm. The percent inhibition was then calculated, which was equivalent to the DN.

Enzyme assay SChE activities were measured spectrophotometrically according to the method of Garry and Routh (1965). Initial velocities were determined at 37°C by using 20 μ l plasma samples as the enzyme source that was added to a 4.5 ml Tris-saline buffer (pH 7.4) that contained 50 mM Tris, 11.4 mM NaCl, and 0.252 mM DTNB. Different concentrations of BuTch or AcTch were added to the assay mixture as required. The resulting color read at 412 nm. Determinations were carried out in duplicate, and the activities were measured after 5 min. The reaction was linear during this period. Blanks that contained all of the compounds except the enzyme were used to eliminate any spontaneous hydrolysis of the substrate. A unit of enzyme activity was defined as the amount of enzyme that liberates 1 μ mole-SH groups per minute per ml plasma.

Kinetic studies Enzyme-kinetic measurements were carried out to elucidate the action of dibucaine on the SChE activity and BuTch- or AcTch-SChE interactions. Enzyme assay experiments were performed in the absence or presence of various concentrations of dibucaine (0-10 μ M) as a function of different substrate (BuTch or AcTch) concentrations (10-200 μ M). The nature of inhibition that was produced by each set of experiments was determined by analysis of the double-reciprocal plots of the initial velocity versus BuTch or AcTch concentrations. The primary or secondary replots were obtained using the computer program Grafit (Leathebarrow, 1992).

Results

In the present study the plasma samples that were used had

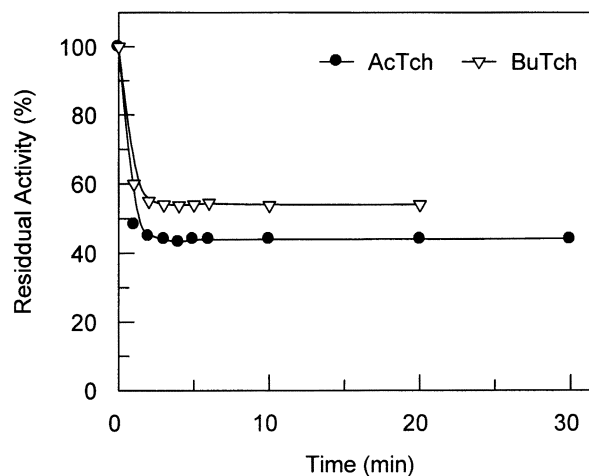


Fig. 1. Time dependence of the inhibition of human SChE-catalyzed hydrolysis of BuTch (∇) or AcTch (\bullet) at 200 μ M each in the presence of 5 μ M dibucaine. Each point represents the average of 4 determinations with an experimental error of 3%.

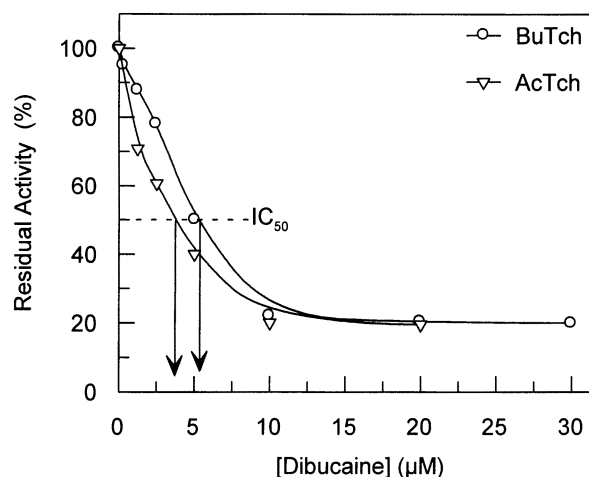


Fig. 2. Inhibition of human SChE activity by varying concentrations of dibucaine (1.25-30 μ M) in the presence of 200 μ M of either BuTch (\circ) or AcTch (∇). Each point is an average of 3-5 measurements with an experimental error of 3%.

the "usual" SChE type with a DN between 78-82%. The inhibitory effects of any one dibucaine concentration on SChE in the presence of either the specific substrate BuTch or the non-specific substrate AcTch, were found to reach maximum within 2 min of incubation and then remain constant (Fig. 1). Dibucaine (1.25-20 μ M) inhibits SChE activity in the presence of BuTch by 12-78%, or AcTch by 30-80%, in a concentration-dependent manner with an IC_{50} of 5.3 μ M and 3.8 μ M, respectively (Fig. 2).

Lineweaver-Burk analysis of dibucaine effects It appears from the Lineweaver-Burk plot of SChE activity in the presence of varying concentrations of dibucaine (0-10 μ M),

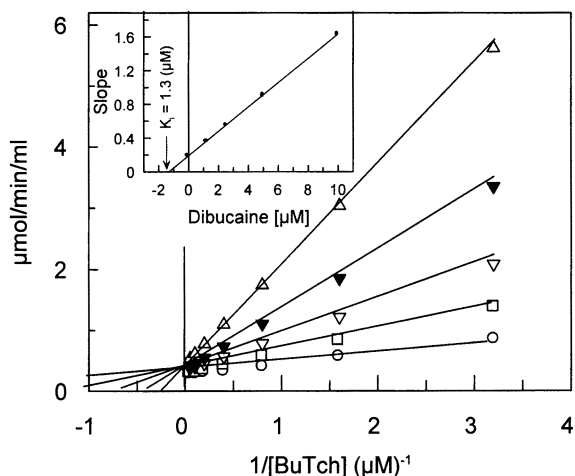


Fig. 3. Kinetic analysis of dibucaine inhibition of the human SChE-catalyzed hydrolysis of BuTch. The data are represented as a double-reciprocal plot of the initial rate of SH production as a function of the BuTch concentration (3.13-200 μM) in the absence (\circ) and presence of 1.25 μM (\square); 2.5 μM (∇); 5 μM (\blacktriangledown) and 10 μM (\triangle) dibucaine. Each point is an average of 3-5 measurements with an experimental error of 3%. Inset shows a secondary replot of the slope values that were derived from the primary double-reciprocal plot vs. dibucaine concentration.

and BuTch as the substrate, that dibucaine increases K_m (220-1000%) with no effect on V_{max} in a competitive type of inhibition (Fig. 3). The inset of the same figure shows a replot of the slopes of the Lineweaver-Burk plots against the dibucaine concentrations that produce an inhibition constant (K_i) of 1.3 μM . However, these results were obtained with similar concentrations of dibucaine, and AcTch showed an increase in K_m (200-3850%) and a decrease in V_{max} (35-78%) in a mixed-type inhibition, competitive/noncompetitive (Fig. 4 A). The replots of the slopes and intercepts of the lines against the dibucaine concentrations (Fig. 4 A, inset and Fig. 4 B, respectively) gave straight lines with a K_i of 0.66 μM for the competitive and a K_i of 2.5 μM for the noncompetitive inhibitions.

Discussion

The K_m value with the specific substrate BuTch was 4.55 μM , which is comparable to that of the other specific substrate benzoylcholine of 5 μM that was obtained by Lockridge and La Du (1978), and less than the 24 μM that was reported by Froment *et al.* (1998). The K_m with the non-specific substrate AcTch was about twice that of the specific substrate at 9.1 μM .

The dibucaine-inhibited SChE enzyme in the presence of either BuTch or AcTch substrates and its inhibitory effects reached a maximum within 2 min. It then remained constant (Fig. 1). This is an indication of its reversible nature (Dawson, 1990). Dibucaine was a less potent inhibitor with the specific

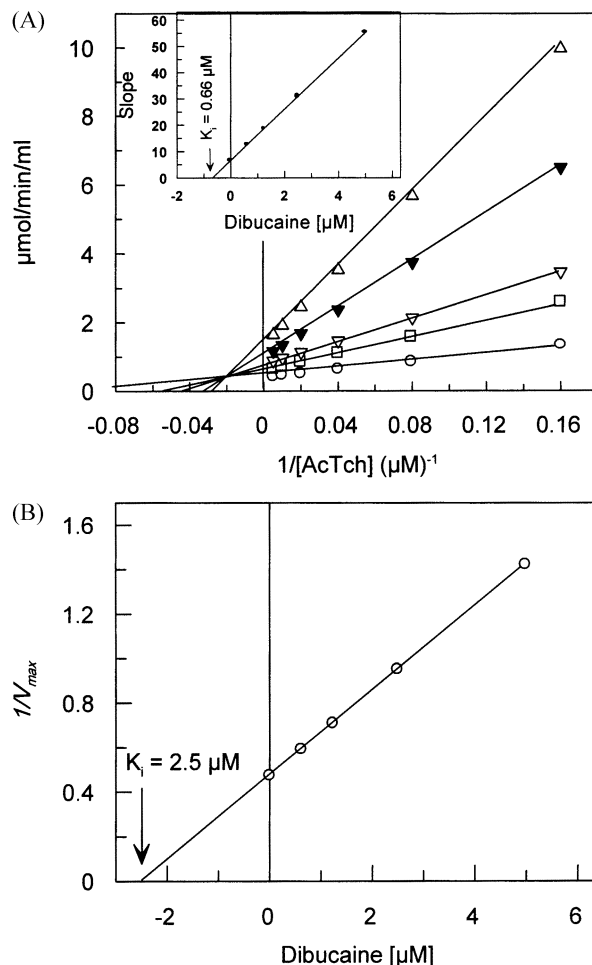


Fig. 4. Kinetic analysis of dibucaine inhibition of the human SChE-catalyzed hydrolysis of AcTch (6.25-200 μM). (A) All of the conditions are essentially as described for Figure 3. (B) Secondary replot of $1/V_{max}$ values derived from the primary double-reciprocal plot vs. dibucaine concentrations for the determination of K_i .

substrate BuTch than with the nonspecific substrate ATch with IC_{50} values of 5.3 μM and 3.8 μM , respectively. The inhibitory effect with BuTch had a competitive nature. This is shown by the double-reciprocal plot (Fig. 3). There was no change in V_{max} and an increase in K_m , reaching about 10 times with 10 μM dibucaine. The replots of the line slopes of the same figure versus the dibucaine concentration gave a straight line that does not pass through the origin. Therefore, the inhibition is partially competitive rather than pure competitive where it should pass through the origin (Segel, 1975). The inhibitory effects of dibucaine in the presence of AcTch were of the linear-mixed type, partially competitive and pure noncompetitive inhibitions with inhibitory constants of 0.66 μM and 2.5 μM , respectively. The replots of the intercepts of the lines in Figure 4A against the dibucaine concentration gave a straight line (Fig. 4B). This indicates a pure noncompetitive inhibition rather than a partial

noncompetitive inhibition that is indicated by a hyperbolic line (Segel, 1975). Dibucaine may bind at a peripheral-binding site that would prohibit the proper positioning of AcTch at the anionic site and lead to a conformational change that may perturb the esteratic site as well, which is similar to the effect of a related compound, tetracaine, on acetylcholinesterase activity (Sussman *et al.*, 1991).

Dibucaine, BuTch, and AcTch all have the quaternary ammonium nature; therefore, they would bind to the anionic-binding site of the enzyme (Kalow and Davies, 1958; Rippol *et al.*, 1993). However, dibucaine possesses a butoxy group that is similar to the acyl butyl group of BuTch. It may then compete at another, nearby hydrophobic site. Vellom *et al.*, (1993), using site-specific mutagenesis, was able to substitute Phe₂₉₅ and Phe₂₉₇ in the acyl pocket of acetylcholinesterase (it shares a 51-54% sequence homology with SChE) to the corresponding amino acids in SChE, Leu₂₈₆, and Ile₂₈₈, respectively. This mutation enhanced the acetylcholinesterase activity to BuTch and diminished its activity to AcTch. Therefore, dibucaine may not only compete at the anionic-binding site, but also at this acyl pocket. This may explain the difference in the mode of inhibition with AcTch as the substrate, which is only short by an ethyl group from BuTch. It appears that dibucaine has twice the chance of competing with AcTch than with BuTch, as inferred by the present data of the competitive-inhibition values of 0.66 μM and 1.33 μM , respectively. The presence of an acyl-binding pocket may also explain the enzyme-substrate specificity, where two binding sites are possible for the specific substrate, BuTch ($K_m = 4.55 \mu\text{M}$), and only one for the nonspecific substrate, AcTch ($K_m = 9.1 \mu\text{M}$). The present results agree with the findings of Vellom *et al.* (1993) on the existence of an additional hydrophobic acyl-binding site for SChE.

The original use of dibucaine at the specified concentration of 10^{-5} M (Kalow and Genest, 1957; then also many others) for the determination of DN was apparently a successful choice for routine surveys of enzyme phenotypes. In the present study, 10 μM dibucaine was found to be the minimum concentration that gave a maximum inhibition of 80% with the "usual" SChE isozyme in the presence of either the specific substrate BuTch or the nonspecific substrate AcTch, the same as benzoylcholine. This inhibition remained constant above the specified dibucaine concentration. It was, therefore, unaffected by the small variations in application by the different laboratories and methodologies.

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