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INHIBITION OF HUMAN SERUM AND RABBIT MUSCLE CHOLINESTERASE BY LOCAL ANESTHETICS

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Abstract—The effects of tertiary amine local anesthetics (procaine, mepivacaine, lidocaine, tetracaine, dibucaine, and bupivacaine) and chlorpromazine were investigated for rabbit muscle acetylcholinesterase and human serum cholinesterase. The muscle enzyme was poorly inhibited by local anesthetics containing an amide linkage. The serum cholinesterase was inhibited by all those compounds, their relative potencies being proportional to their octanol/water partition coefficients. The dissociation constants of tetracaine and procaine, ester anesthetics, were 1000-fold and 100-fold, respectively, that which would be expected from their partition coefficient basis respective to the other amide anesthetics. Procaine showed competitive inhibition of serum cholinesterase, whereas for most anesthetics a mixed type of inhibition was observed. Procaine probably binds at the main anionic site, while the other positively charged anesthetics bind to either the catalytic centre or to the peripheral or modulator anionic site, modifying the kinetic behaviour of cholinesterase as has been demonstrated by the appearance of negative cooperativity for binding to the substrate.

Local anesthetics constitute a biochemically and medically important class of compounds. Most have an aromatic ring system joined by an ester or amide linkage to a tertiary amine grouping, but the structural details differ considerably. While their primary clinical usage depends upon their ability to block peripheral nerve conduction, studies have shown that they also affect a wide variety of other cellular processes. Many of these effects are on membrane systems, and for most effects the relative potencies of the various compounds can be related to their octanol/water partition coefficients [1, 2]. Some of the systems which have been shown to be inhibited or otherwise affected by local anesthetics are microtubule polymerization [3], lymphocyte capping [4], various Ca^{2+}-regulated systems [5, 6], sarcoplasmic reticulum Ca^{2+}-ATPase [7], mitochondrial electron transport [8], acetylcholine receptor [9, 10]. Mitochondrial F_1-ATPase [11], luciferase [12], and membrane-bound acetylcholinesterase from rat brain synaptosomes [13]. The inhibitory effect of local anesthetics on acetylcholinesterase might be related to the structural similarities between these tertiary amine compounds and the natural substrate acetylcholine.

It has been reported that, except for the acetylcholine receptor where there is a specific binding site [9, 10], for most systems the correlation of potency with the octanol/water partition coefficient points to an interaction of a relative nonspecific, nonstructured, hydrophobic site which may be either lipoidal or proteinaceous.

In the present study, we have analyzed the interaction of local anesthetics with human serum cholinesterase (ChE, EC 3.1.1.8) and Triton-solubilized acetylcholinesterase (AChE, EC 3.1.1.7) from rabbit white muscle in order to compare the sensitivity of these two enzymes to the anesthetics. The relationship between the hydrophobic character of these compounds and their relative potency to inhibit the cholinesterase from these two sources has also been investigated. Finally, we have studied the type of inhibition produced by the different anesthetics on both serum and muscle cholinesterases.

MATERIALS AND METHODS

Chemicals

Acetylcholine iodide, 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB) and all anesthetics were obtained from Sigma Chemical Co. (London). Triton X-100 from Merck (Darmstadt). All solutions were prepared in distilled water, redistilled in an all glass still.

Solubilization of muscle AChE

Solubilization of rabbit muscle AChE was performed by homogenization of the tissue in a mortar, using sand, with 15 mM Hepes buffer, pH 7.5, to obtain a 20% homogenate. After centrifugation at 100,000 *g* for 1 hr at 4° (Kontron, model T2050; TFT65 rotor) the supernatant containing the "naturally soluble" form of the enzyme was separated. The pellet from the first centrifugation was resuspended in the above Hepes buffer, containing 0.5% Triton X-100, to obtain a 40% homogenate. After 10 min the suspension was centrifuged at 100,000 *g* for 1 hr to separate the "Triton-solubilized" fraction of AChE.

Determination of the partition coefficients

Phosphate buffer, 0.1 M, pH 8, was saturated with octanol in a separating funnel (saturated buffer).

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Then, 50 μl of the appropriate inhibitor was added to 20 ml of the saturated buffer and 4 ml of octanol. After shaking for 10 min the mixture was left for another 10 min to separate the inorganic and organic phases and centrifuged at 3000 rpm for 5 min in a bench centrifuge. Afterwards, the organic phase (OP_1) was separated and 4 ml of octanol was added to the inorganic phase, the above protocol being repeated to obtain another organic phase (OP_2). The absorbance value in the maximum of absorption of each anesthetic was measured in both OP_1 and OP_2. The partition coefficients were determined applying

$$P = \frac{OP_1 - OP_2}{OP_2} \frac{V_{AP}}{V_{OP}}$$

where V_{AP} and V_{OP} are volumes of the aqueous (20 ml) and organic phase (4 ml), respectively.

Assays. The spectrophotometric method of Ellman *et al.* [14] was used to assay both muscle AChE and serum ChE. In this method, the substrate analogue acetylthiocholine is hydrolyzed by the enzyme, and the sulphydryl group thus produced reacts with the reagent 5,5'-dithio-bis-(2-nitrobenzoate) (DTNB) to give a yellow colour. The reaction rate was followed as the absorbance increased at 412 nm. The standard reaction mixture was made up with 0.1 M phosphate buffer (pH 8.0), 1 mM acetylthiocholine iodide, 0.33 mM DTNB prepared as described by Ellman *et al* [14], 100 μl of Triton-solubilized AChE (6 mg/ml) or 50 μl of serum (2 mg/ml) and a volume of the selected anesthetic to reach the desired concentration, the total volume being 3.0 ml. After 3 min of equilibration period, in the presence of the chosen anesthetic, the reaction was started by addition of the substrate and the non-enzymatic hydrolysis of the substrate was discounted from that obtained in the presence of the enzyme. All assays were carried out in duplicate at 25°. The set of experiments with each anesthetic was repeated at least three times. One unit of AChE or ChE activity represents the amount of enzyme which hydrolyzes 1 nmol of substrate per hour in our assay conditions.

The protein content of enzyme preparations was measured by a modified biuret method [15].

The enzyme-inhibitor dissociation constant (K_i) for each anesthetic was calculated from a Hunter–Down plot where the ratio $v_i\ I/v_0 - v_i$ was plotted against the substrate concentration. Thus, K_i was obtained by extrapolating the line to zero substrate using those data obtained at substrate concentrations below 0.2 M since above that the plot was non-linear for most of the anesthetics assayed. The lines were fitted by a linear-regression programme, the regression coefficients oscillated between 0.965 for mepivacaine and 0.997 for dibucaine.

RESULTS

Solubilization of AChE from rabbit muscle

Extraction of AChE from rabbit white muscle with dilute buffer solubilized from 50 to 55% of total activity on the basis of the activity found in the starting homogenate. The pellet from the first centrifugation was homogenized in buffer containing Triton X-100 (0.5% w/v) and, after centrifugation, an additional 120% of the activity measured in the initial homogenate was brought into solution, indicating that most of the AChE in the muscle was in an occluded form as has been reported by others [16].

All the experiments with anesthetics were performed on this Triton-solubilized AChE from muscle.

Anesthetic effects on cholinesterases

The effect of local anesthetics on inhibition of rabbit muscle acetylcholinesterase was studied using the Triton-solubilized enzyme extract. Only those agents with the ester function were able to inhibit significantly the muscle enzyme. The calculated K_i values were 252 and 120 μM for procaine and tetracaine respectively (Table 1). Lineweaver–Burk plots were prepared to establish which types of inhibitor these two agents were, when acting on muscle AChE. Both of them showed a mixed type of inhibition (Fig. 1) as would be expected if these compounds were able to occupy both the main anionic site and some of the peripheral anionic sites on the surface of the enzyme molecule.

The serum enzyme was inhibited by all local anesthetics and chlorpromazine, their inhibition constants (K_i) being shown in Table 1, and plotted as function of their octanol/water partition coefficients in Fig. 2. The inhibitory potencies of mepivacaine, lidocaine, chlorpromazine, dibucaine and bupi-

Table 1. Concentration of anesthetics agents required for 50% inhibition and K_i values for rabbit muscle and serum cholinesterases

Substance	Log *P*	Muscle AChE		Serum ChE	
		I_{50} (M)	K_i (10^{-6} M)	I_{50} (M)	K_i (10^{-6} M)
Procaine	0.84	5.0×10^{-4}	252	8.0×10^{-5}	4.86
Mepivacaine	1.64	N.I.	—	6.3×10^{-4}	180.90
Lidocaine	1.86	N.I.	—	2.2×10^{-4}	57.86
Tetracaine	2.42	3.6×10^{-4}	120	1.1×10^{-7}	0.012
Chlorpromazine	3.40	—	—	1.0×10^{-5}	5.54
Dibucaine	3.87	N.I.	—	3.1×10^{-6}	0.84
Bupivacaine	4.05	N.I.	—	1.6×10^{-6}	1.35

P, octanol/water partition coefficient. The I_{50} values were calculated at 1 mM substrate concentration. *P* and K_i values were calculated as indicated in the text. The muscle ChE was not inhibited (N.I.) by local anesthetics (up to 1 mM) except for those with the ester function.

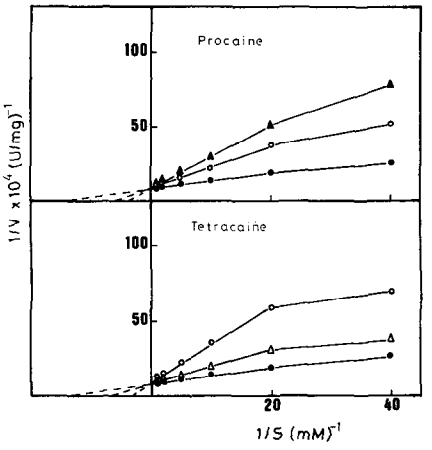

Fig. 1. Lineweaver–Burk plot for rabbit muscle acetylcholinesterase. ●, No inhibitor; △, with 0.05 mM tetracaine; ○, with 0.2 mM tetracaine or procaine; ▲, with 0.3 mM procaine.

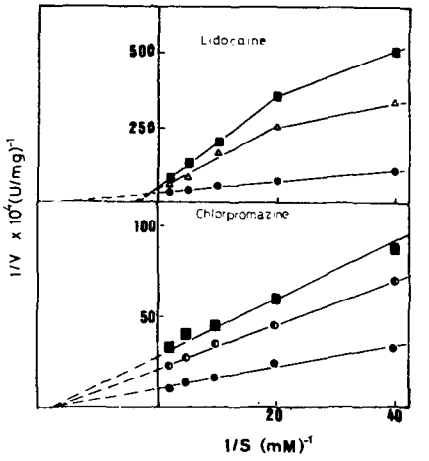

Fig. 3. Lineweaver–Burk plot for human serum cholinesterase in the absence of inhibitor (●) and in the presence of 0.15 mM (△) and 0.25 mM (■) lidocaine and 5 μM (◐) and 8 μM chlorpromazine (■).

vacaine were linearly related to their partition coefficients, but the K_i values obtained with tetracaine and procaine, which are esters, were 1000-fold and 100-fold, respectively, that which would be expected from their partition coefficient basis relative to the other compounds.

Lineweaver–Burk plots were prepared for the inhibition of serum ChE by local anesthetics and chlorpromazine in order to see if the type of inhibition varied between them. The behaviour of procaine and tetracaine, both with ester linkages, was different in that the former displayed a mixed type inhibition whereas the latter drastically modified the kinetic behaviour of serum ChE, non-linear plots being obtained (Fig. 3). From those compounds with an amide linkage, both mepivacaine and lidocaine proved to behave in a similar way yielding non-linear plots (Fig. 3), whereas dibucaine displayed also a mixed type of inhibition as it was seen with procaine (Fig. 4). The enzyme incubated with either bupivacaine or tetracaine yielded non-linear plots but in both cases a negative cooperativity was also observed (Fig. 4). Chlorpromazine tended to behave as a non-competitive inhibitor (Fig. 3).

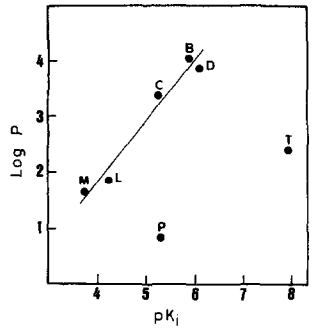

Fig. 2. Logarithmic plot of P vs pK_i ($-\log K_i$) for human serum cholinesterase. The inhibitory agents were the following: P, procaine; M, mepivacaine; L, lidocaine; T, tetracaine; C, chlorpromazine; D, dibucaine; B, bupivacaine.

DISCUSSION

The results presented here show clearly that the tertiary amine anesthetics have a pronounced effect on the activity of human serum ChE. At the pH of our experiments, the tertiary amines used are expected to be in a predominant cationic monomeric state, since the pK_a values range from 7.85 for lidocaine to 8.95 for procaine [17], the critical micellar concentration of tetracaine (for example) being 61 mM at pH 6.5 [18].

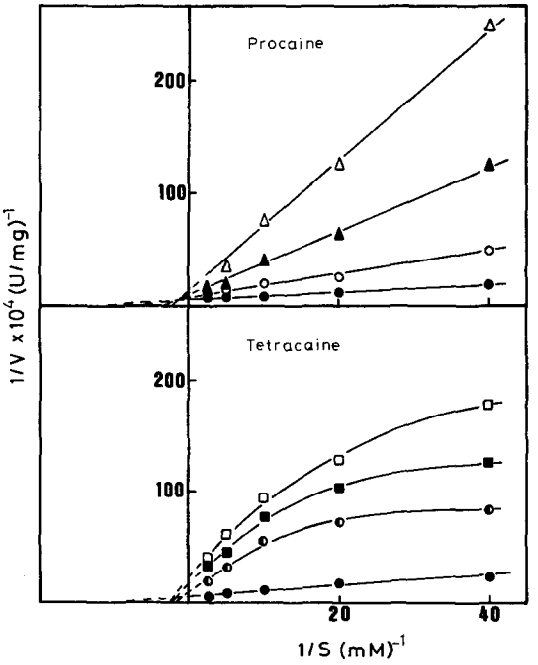

Fig. 4. Lineweaver–Burk plot for human serum cholinesterase in the absence of inhibitor (●) and in the presence of 0.1 mM (○), 0.14 mM (▲), 0.18 mM (△) procaine or 0.1 μM (◐), 0.2 μM (■), 0.3 μM (□) tetracaine.

The data shown in Fig. 2 for human serum ChE illustrate the usefulness of partition coefficient plots as a simple means for distinguishing between different types of inhibitor binding. Since the inhibitory potencies of mepivacaine, lidocaine, chlorpromazine, dibucaine and tetracaine are linearly related to their partition coefficients, it is clear that the hydrophobic site at which these compounds bind into the enzyme is not very specific with regard to ligand, size and shape. This is evident since, among these local anesthetics, there are single-lidocaine, double-dibucaine and triple-tetracaine fused aromatic ring systems, and compounds which are structurally very similar, such as lidocaine, bupivacaine and mepivacaine. However, those compounds with the ester linkage—procaine and tetracaine—were able to inhibit serum ChE at a concentration much lower than would be expected on the basis of their partition coefficients (relative to the other compounds with amide linkage). This indicates that the ester agents bind to the enzyme in a different way to the amide compounds. Moreover, the muscle AChE was not inhibited up to 1 mM by those compounds with an amide linkage, whereas the enzyme seems to be affected by the ester agents, procaine and tetracaine. It has been found [13] that AChE-bound to synaptosomes isolated from rat brain is inhibited by local anesthetics but the concentration needed to inhibit the membrane-bound enzyme is ten times higher than that reported here. This could imply that brain enzyme is less sensitive than the serum ChE to inhibition by local anesthetics and from the results reported here this also appears to apply to muscle AChE. The site of action of anesthetics, in this case, is in the protein, although for membrane systems, this site might be the boundary lipid to which the integral protein is attached.

It has been found that AChE isolated from several sources has both a catalytic anionic site and a peripheral or modulator anionic centre [19, 20]; some modulators bind at the catalytic centre but many others interact with the peripheral site. Those compounds which bind to the peripheral anionic centre produce "mixed" inhibition kinetics [21] with the intersection point of Lineweaver–Burk plots occurring at an off-axis location, as shown in Figs. 3–4.

In a previous report [22] we have shown that at suboptimum substrate concentration the detergent-solubilized AChE rabbit muscle displayed a negative cooperativity which is modified when the assays are carried out in the presence of variable concentrations of NaCl. We have explained this phenomenon assuming that the substrate can bind to both the main and peripheral anionic site, the salt showing the capacity to compete with the substrate for these two binding sites. Similarly, these cationic inhibitors will compete for the substrate both in the anionic and peripheral sites. Thus the negative cooperativity would appear as the result of a shift from a form of the enzyme with high activity, at low substrate concentration, to a less active form, the inhibitor being able to increase the enzyme activity, at low substrate concentration, relative to that obtained at higher substrate. The inhibitor will then be able to occupy either the main anionic, the peripheral or modulator anionic site, or both of them. Therefore, for most of these inhibitors a mixed type of inhibition is observed depending on their relative affinity for these two anionic sites.

It is hard to establish any relationship between the extent of negative cooperativity and the size and shape of the inhibitors since in most of them, the predominant spatial structure is unknown and also because the size of the hydrophobic pocket of the serum ChE has not been fully clarified.

Summarizing, the ability of local anesthetics to inhibit serum ChE is related to their octanol/water partition coefficients, indicating that hydrophobic forces are involved in binding to the enzyme and in doing so they will produce competitive, non-competitive or mixed inhibition of the serum enzyme depending on their relative capacity for binding to the main anionic site or the peripheral or modulator site.

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