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Inhibition of Cholinesterases by the Zwitterionic Detergent 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS)

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3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), a zwitterionic detergent, behaves as a reversible inhibitor of several cholinesterases. Human and horse serum cholinesterases were more sensitive to inhibition by the detergent than the enzyme from *Electrophorus electricus* or human erythrocyte. Thus, CHAPS binds with butyrylcholinesterase in preference to acetylcholinesterase. Noncompetitive inhibition kinetics were observed with all enzymes tested; the apparent inhibition constants were 0.4, 2.5, 5.8, and 7.5 mM for the cholinesterases from human serum, horse serum, *Electrophorus electricus*, and human erythrocyte, respectively. The rate of phosphorylation of the cholinesterases by diisopropyl phosphorofluoridate was significantly reduced in assays performed in the presence of CHAPS; however, the detergent was unable to fully protect the cholinesterases from the inactivation by the organophosphate. These results indicate that the detergent binds in a noncompetitive manner to cholinesterases and this reduces the capacity of the enzymes to react with both the substrate and the organophosphate.

INTRODUCTION

Cholinesterases (acetylcholinesterase, EC 3.1.1.7; butyrylcholinesterase, EC 3.1.1.8) are ubiquitously serinė hydrolases which specifically hydrolyze esters of choline. Acetylcholinesterase (AChE)[2] acts in cholinergic transmission by rapidly splitting the neurotransmitter acetylcholine (*1–3*). Unlike AChE, no clear physiological function has been assigned to butyrylcholinesterase (BuChE).

Although clearly optimized with esters of choline as substrate molecules, these enzymes also react with other esters of carboxylic acids and acyl halides of substituted carbamic, phosphoric, and sulfonic acids (*4, 5*). In addition to the "estearic site," which contains the reactive serine, the occurrence of an "anionic site" and a "hydrophobic site" has also been established. The anionic site binds ammonium derivatives, which act as substrates or inhibitors (*6*), and metal cations

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[2] Abbreviations used: AChE, acetylcholinesterase; BuChE, butyrylcholinesterase; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DFP, diisopropyl phosphorofluoridate; EGTA, ethylene glycol bis(β-aminoethyl ether) *N, N, N', N'*-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; DTNB, 5-5'dithiobis-2-nitrobenzoic acid.

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(*7, 8*). Furthermore, several peripheral anionic sites seem to regulate their catalytic mechanism (*9, 10*). The hydrophobic site binds aromatic substrates (e.g., phenyl acetate), branched-chain aliphatic esters (e.g., isoamyl acetate) (*10*), and aromatic hydrocarbon (*11*) to these enzymes.

Cholinesterases are found in tissues in both soluble and membrane-bound forms (*12–14*). To investigate the molecular properties of these enzymes in excitable tissues they must be brought into solution by means of nonionic detergents, biliary salts, proteases, and phospholipases (*15–17*).

Since CHAPS had been proven to be very efficient in extracting proteins from membranes (*18, 19*), attempts were made to solubilize AChE from erythrocyte and muscle microsomes with this zwitterionic detergent.

In this paper we report the inhibitory action of CHAPS on cholinesterases of different sources. The detergent behaves as a noncompetitive inhibitor of these enzymes. Furthermore, the detergent is able to protect these enzymes against the inhibition produced by diisopropyl phosphorofluoridate (DFP). A plausible mechanism for the interaction of the detergent with cholinesterases is also given.

MATERIALS AND METHODS

Materials. All reagents were of analytical grade. Special chemicals were obtained as follows: acetylthiocholine iodide, 5-5′-dithiobis-2-nitrobenzoic acid (DTNB), pepstatin, and EGTA were obtained from Sigma Chemical Company. Triton X-100 and EDTA were purchased from Merck. Diisopropyl phosphorofluoridate (DFP) was from Aldrich Chemical Company, and 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) from Boehringer-Mannheim.

Acetylcholinesterase from the electric organ of *Electrophorus electricus* was obtained from Boehringer-Mannheim. Cholinesterase from horse serum was purchased from Sigma Chemical Company.

Cholinesterase activity was measured in human plasma samples. These were obtained after withdrawal by spinning a heparinized blood sample at 1000*g* for 10 min in a refrigerated centrifuge.

Extraction of AChE from human erythrocyte. Recently prepared human erythrocytes, 7 ml, were washed three times with cold 150 mM NaCl in 5 mM phosphate buffer, pH 7.5. Lysis of the cells was achieved by pouring and stirring into 20 times its volume of cold 5 mM phosphate buffer, pH 7.5. Membranes were separated by centrifuging at 100,000*g* for 30 min, at 4°C, washed, and resuspended in the initial volume with 10 mM Tris buffer, pH 7.0, containing 1 M NaCl, 50 mM $MgCl_2$, 5 mM EGTA, 3 mM EDTA, 5 mg/ml pepstatin, and 1% Triton X-100. The mixture was stirred overnight at 4°C. After centrifugation as before, the soluble fraction was collected and assayed for AChE activity and protein content.

Assay of cholinesterase. The Ellman assay solution contained 0.1 M phosphate buffer, pH 8.0, 1 mM acetylthiocholine, 0.33 mM DTNB, and 50 μl of enzyme solution in a total volume of 3 ml. The initial increase in absorption was recorded at 412 nm, $\varepsilon = 1.36 \times 10^4\ M^{-1}\ cm^{-1}$, against a reference cell containing all components except the enzyme (*20*).

One unit of cholinesterase activity represents the amount of enzyme which hydrolyzes 1 μmol of the substrate per hour at 37°C.

Reversible inhibition by CHAPS. The enzyme activity was followed in samples preincubated for 10 min at 37°C with increasing concentrations of CHAPS, 0.5–20 mM. The reaction medium was prepared both without and with detergent added. I_{50} values and Hill coefficients were calculated from a Hill plot, log $(V/V_0\text{-}V)$ vs log I, where V_0 is the velocity measured in the absence of inhibitor, V is the velocity in assays with the inhibitor added, and I represents a fixed concentration of CHAPS in both preincubation and reaction medium.

Values of K_i were determined from Dixon plots of $1/v$ vs I in assays performed at variable substrate concentrations, 0.1–1 mM for AChE and 0.1–5 mM for BuChE.

Irreversible inhibition. Inactivation of cholinesterases by DFP was studied in 0.1 M phosphate buffer, pH 8.0, by Ellman assay of residual activity after incubation periods of 1–30 min at 37°C. Assays were performed by incubating the enzymes for 5 min with variable amounts of CHAPS in the incubation medium, prior to the addition of a fixed concentration of DFP. Assays were also carried out in samples preincubated with a fixed concentration of CHAPS, 1–5 mM, and a range of DFP concentrations. All inhibition reactions were performed in the absence of substrate.

The pseudo-first-order constants for the reaction of DFP with cholinesterases, k_1, were calculated from the slopes of the plots of $\ln(V/V_0 \times 100)$ versus t, V_0 and V being the velocity of the reaction without and with DFP after incubation for t min. The slopes were determined by a least-squares method, and used for further analysis by applying the double-reciprocal method (*21*). Thus, the bimolecular reaction constants for the phosphorylation reaction, k_i, were calculated both in the absence and in the presence of a fixed amount of CHAPS.

RESULTS

Incubation of cholinesterases of different origin with progressive concentrations of CHAPS produced an inhibition of these enzymes. The inhibitory action was not dependent on the incubation time. The extent of inhibition was higher for human and horse serum BuChE than for the enzyme in human erythrocyte and *Electrophorus* (Fig. 1, Table 1). Hill plots of data on inhibition of cholinesterases by the detergent yielded, in all cases, Hill coefficients close to 1 (Table 1). Since BuChE is predominant in mammalian serum, these results indicate that CHAPS binds with BuChE in preference to AChE. In all cases, the inhibition phenomenon was reversible as the original activity was almost completely restored in assays performed in buffer depleted of the detergent.

The rates of acetylthiocholine hydrolysis at variable substrate concentrations in the absence and in the presence of several concentrations of CHAPS were determined. Under the conditions employed all Dixon plots were linear (Fig. 2), the detergent behaving as a weak noncompetitive inhibitor of all cholinesterases tested. For each enzyme, the inhibition by CHAPS yielded similar I_{50} and K_i values (Table 1), which is as might be expected for a noncompetitive inhibitor for which

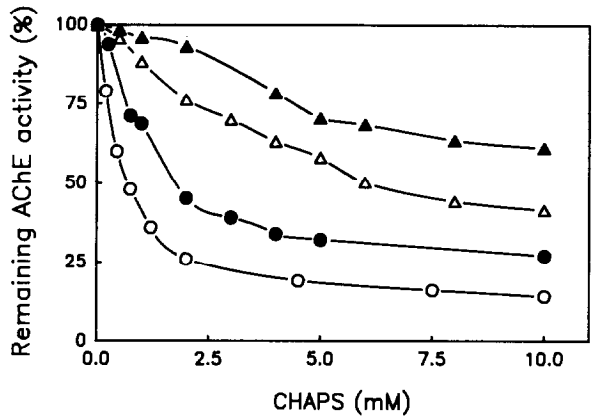

FIG. 1. Inhibition of cholinesterases of different origin by CHAPS: human serum BuChE (○), horse serum BuChE (●), *Electrophorus electricus* AChE (△), and human erythrocyte AChE (▲). When using the enzyme from erythrocyte the concentration of CHAPS was increased to 20 mM. The values are means of three different experiments, the assays being performed in triplicate.

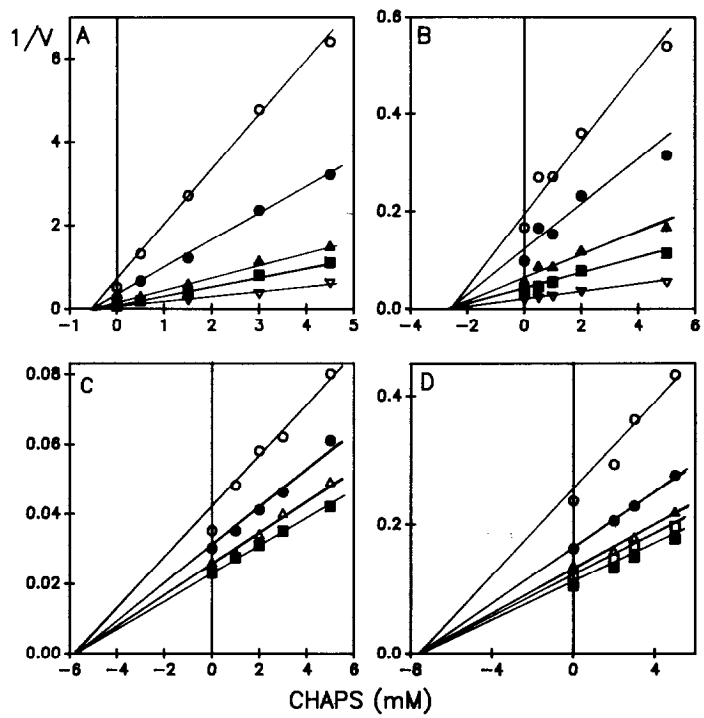

FIG. 2. Dixon plots for the inhibition of cholinesterases by CHAPS: human serum (A), horse serum (B), *Electrophorus electricus* (C), and human erythrocyte (D). The concentrations of acetylthiocholine used were 0.1 (○), 0.2 (●), 0.4 (△), 0.5 (▲), 0.6 (□), 1 (■) and 5 (▽) mM.

TABLE 1

Inhibition of Cholinesterases of Different Origin with CHAPS

	K_m (μM)	K_i (mM)	I_{50} (mM)	n_H
BuChE, human serum	1453	0.4	0.7	0.96
BuChE, horse serum	779	2.5	1.9	1.00
AChE, *Electrophorus*	102	5.8	5.0	1.05
AChE, human erythrocyte	147	7.5	13.0	1.07

Note. Inhibition constants (K_i) were calculated from Dixon plots shown in Fig. 2. K_m values were calculated from Lineweaver–Burk plots (not shown). I_{50} and n_H values were calculated from Hill plots as detailed in the text. Data are average values of three separate experiments.

I_{50} is nondependent on the substrate concentration. In all cases Lineweaver–Burk plots, both without and with added CHAPS, were linear and from them K_m values were calculated (Table 1). Secondary plots of slopes and intercepts versus detergent concentrations also showed a linear relationship (not shown). Therefore, the occurrence of cooperativity for binding the substrate and/or the detergent was ruled out.

To test the accessibility of DFP to the active site of cholinesterases in the presence of CHAPS, the time course of the phosphorylating reaction was followed with progressive concentrations of the organophosphate (Fig. 3). This allowed us to calculate the bimolecular rate constants for the phosphorylation of cholinesterases (k_i), both in the absence and in the presence of the detergent (Fig. 3C, Table 2). BuChE from human and horse serum seems to be more sensitive to inactivation by DFP than AChE, in agreement with previous reports (*22, 23*). Furthermore, it has been reported that BuChE from human plasma is more sensitive to inhibition by DFP than the enzyme from horse plasma (*24*), this being in agreement with the results reported here.

The k_i values were significantly reduced in assays of inactivation performed in

TABLE 2

Bimolecular Reaction Constants for the Phosphorylation Reaction of Several Cholinesterases for DFP in the Absence and in the Presence of CHAPS

	k_i (M^{-1} min^{-1})	
	− CHAPS	+ CHAPS
BuChE, human serum	6.0×10^6	2.06×10^6
BuChE, horse serum	8.96×10^5	2.85×10^5
AChE, human erythrocyte	1.28×10^4	0.58×10^4
AChE, *Electrophorus*	1.95×10^3	1.63×10^3

Note. In all cases the concentration of CHAPS in the assay medium, 5 mM, was maintained constant.

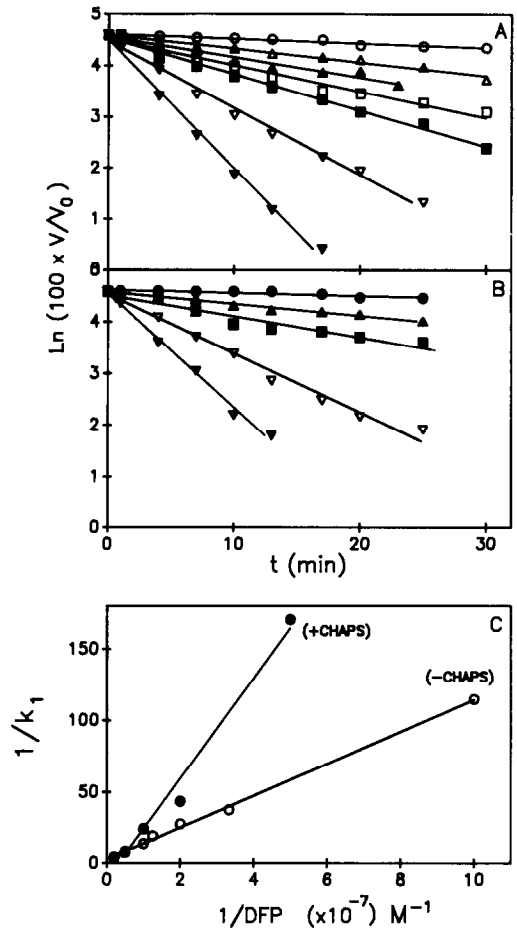

FIG. 3. Time course of inactivation of horse serum cholinesterase by DFP. (A), The incubation medium contained 0.1 M phosphate buffer, pH 8.0, and progressive concentrations of DFP: 10^{-8} (○), 2×10^{-8} (●), 3×10^{-8} (△), 5×10^{-8} (▲), 8×10^{-8} (□), 10^{-7} (■), 2×10^{-7} (▽) and 5×10^{-7} (▼) M. (B) 5 mM CHAPS was added to the reaction mixture. (C) Modification of the bimolecular rate constant for the phosphorylation of the enzyme (k_i) in assays performed without (○) and with 5 mM CHAPS (●).

the presence of a fixed concentration of CHAPS (Fig. 3C, Table 2). Furthermore, k_I values were reduced if inactivation was followed in medium containing progressive concentrations of the detergent (Fig. 4). This indicates that the detergent binds to the enzyme and, in doing so, protects the enzyme against the inhibition by DFP. From Table 2, it appears that protection was more efficiently gained for BuChE than for AChE and this is in agreement with previous data on the interaction of the detergent with the enzymes tested. However, CHAPS is unable to fully protect the enzyme from the inactivating action of DFP (Fig. 4B). This agrees with the expected data from a noncompetitive inhibitor of cholinesterases. Therefore, it

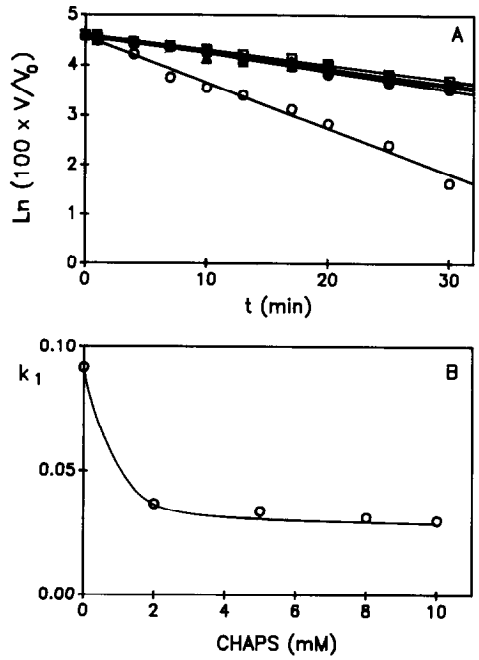

FIG. 4. (A) Time course of inactivation of horse serum cholinesterase by 10^{-7} M DFP in the presence of varying concentrations of CHAPS: 0 (○), 2 (●), 5 (△), 8 (▲), and 10 (□) mM. (B) Calculated K_1 values were plotted versus the concentration of CHAPS.

seems likely that the detergent is associated to some hydrophobic area of these enzymes. This would reduce the capacity of the enzymes to react with both the substrate and the organophosphate.

DISCUSSION

The results presented here unambiguously show that CHAPS has an inhibitory action on cholinesterases. The structural characteristics of this detergent involve a quaternary ammonium head, in common with cationic substrates and reversible inhibitors of cholinesterases, and a hydrophobic area lent by the cholic acid derivative moiety of the molecule. Therefore, the capacity of CHAPS for inhibiting the cholinesterases would depend on the relative importance of ionic or hydrophobic interactions for binding substrates or inhibitors to the active sites of these enzymes. The cholinesterases tested were inhibited by the detergent at concentrations below its critical micellar concentration [CMC = 8 mM (*18*)]. Therefore, it seems likely that the inhibition process is performed before the monomeric tenside molecules begin to build aggregates or micelles and the enzymes are incorporated into detergent micelles. The catalytic mechanism of cholinesterases involves the fixation of cationic substrates on both "anionic" and "esteratic" subsites. Studies

on the reactivity of series of (i) acetic esters with a nonionic leaving group (*25, 26*), (ii) trimethyl-*N*-alkylmethyl ketones and uncharged isosteric inhibitors structurally related to acetylcholine (*27*), (iii) β-substituted ethyl acetates (*28*) and (iv) *N*-alkanesulfonyl chlorides (*29*) have yielded a model in which the catalytic active groups of AChE are located on the bottom of a protein crevice with a limited range of hydrophobic walls. This hydrophobic domain provides the area for the binding of the substrate and leaves groups not longer than those in *n*-butyl acetate. In contrast, the hydrophobic pocket of BuChE should be large enough to accommodate aromatic cholinesters, e.g., benzoylcholine, and aromatic hydrocarbons e.g., anthracene and biphenyl (*11*). According to these premises, CHAPS behaves as a poor inhibitor of AChE with K_i values in the range of those found with tetramethylammonium (*27*). The relative affinity of CHAPS for AChE from different sources may reflect differences in the hydrophobicity of the anionic subsites of these enzymes or in the overall hydrophobic character of the enzyme molecule.

The detergent possesses higher efficiency for binding to plasma cholinesterase than AChE. This situation has also been reported with other positively charged aromatic compounds, e.g., bencyclan (*30*) and aprophen (*31*); and allycyclic compounds, e.g., trimethaphan (*32*). Local anesthetics are also selective in inhibiting plasma cholinesterase (*33, 34*). Plasma BuChE is also more sensitive to inhibition by steroidal glycoalkaloids, e.g., α-solanine, than is erythrocyte AChE (*35, 36*). A characteristic of most of these compounds is that they display a mixed or pure noncompetitive type of inhibition of cholinesterases (*30–34*). It is generally accepted that a noncompetitive cationic inhibitor would interact with the anionic subsite of the acetylated cholinesterase without affecting the hydrolysis of other substrate molecules. The occupation of this subsite by the inhibitor must inhibit the deacetylation step of the enzyme hydrolysis to fully satisfy the theoretical requirement of a noncompetitive mode of inhibition (*37*). Therefore, it can be reasonable assumed that the detergent is associated with the anionic subsite of cholinesterases in such a way as to produce effects in areas remote from their binding sites through induced conformational changes (*38*). This could be the reason for the decreases measured in the values of the bimolecular rate constants for inhibition of cholinesterases by DFP when the enzymes were preincubated with CHAPS. Even compounds which apparently do not show any effect on the cholinesterase activity can be attached to the enzyme, in such a way that they protect the active site from irreversible phosphorylation by organophosphorous compounds (*39, 40*).

To summarize, it has been clearly demonstrated that the zwitterionic detergent CHAPS reversibly inhibits cholinesterases in a noncompetitive manner. The enzyme inhibited by CHAPS is less sensitive to DFP inactivation than the uninhibited enzyme. Other zwitterionic detergents should be tested before being used in tissue extraction of membrane-bound cholinesterases.

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