6. M. C. Antunes-Madeira and V. M. C. Madeira, *Pesticide Biochem. Physiol.* **17**, 185 (1982).
7. J. G. Clement, *Fundamental and Appl. Toxicol.* **4**, 96 (1984).
8. M. C. Antunes-Madeira and V. M. C. Madeira, *Biochim. biophys. Acta* **778**, 49 (1984).
9. Z. Kiss, *Eur. J. Biochem.* **95**, 607 (1979).
10. D. M. Neville, *Biochim. biophys. Acta* **154**, 540 (1968).
11. O. H. Lowry, N. J. Rosenbrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
12. J. Codina, J. D. Hildebrandt, R. B. Iyengar, L. Birnbaumer, R. D. Sekura and C. R. Manclark, *Proc. natn. Acad. Sci. U.S.A.* **80**, 4276 (1983).
13. R. A. Cerione, J. Codina, J. L. Benovic, R. J. Lefkowitz, L. Birnbaumer and M. G. Caron, *Biochemistry* **23**, 4519 (1984).
14. E. M. Ross and A. G. Gilman, *Annu. Rev. Biochem.* **49**, 533 (1980).
15. M. Rodbell, *Nature, Lond.* **284**, 17 (1980).
16. R. J. Lefkowitz, R. A. Cerione, J. Codina, L. Birnbaumer and M. G. Caron, *J. Membr. Biol.* **87**, 1 (1985).
17. R. A. Cerione, C Staniszewski, J. L. Benovic, R. J. Lefkowitz, M. G. Caron, P. Gieschik, R. Somers, A. M. Spiegel, J. Codina and L. Birnbaumer, *J. biol. Chem.* **260**, 1483 (1985).
18. G. M. Bokoch, T. Katada, J. K. Northup, E. L. Hewlett and A. G. Gilman, *J. biol. Chem.* **258**, 2072 (1983).
19. D. M. F. Cooper, W. Schlegel, M. C. Lin and M. Rodbell, *J. biol. Chem.* **254**, 8927 (1979).
20. A. G. Gilman, *Cell* **36**, 577 (1984).
21. H. Itoh, F. Okajima and M. Ui, *J. biol. Chem.* **259**, 15464 (1984).
22. T. Katada, A. G. Gilman, Y. Watanabe, S. Bauer and K. H. Jakobs, *Eur. J. Biochem.* **151**, 431 (1985).
23. A. L. Boyton and J. W. Whitfield, *Adv. Cyclic Nucleotide Res.* **15**, 193 (1983).

Biochemical Pharmacology, Vol. 35, No. 9, pp. 1605–1608, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00

Butyrylcholinesterase inhibition by miracil D and other compounds

(*Received* 10 *June* 1985; *accepted* 2 *October* 1985)

Butyrylcholinesterase (BuChE*) is a serine hydrolase of unknown function. It generally occurs in the blood, tissues, and cytosol of organisms and cells, but it also appears to occur specifically in the superior cervical ganglion [1], the central visual pathway, and other areas of the mammalian brain and nervous system [2]. Its structure, ligand and inhibitor patterns resemble those of acetylcholinesterase (AChE), and considerable sequence homology has been detected between human serum BuChE and *Torpedo marmorata* AChE [3]. Both ChEs hydrolyze choline esters, although AChE preferentially hydrolyzes C2 substrates, whereas BuChE preferentially hydrolyzes C4 substrates. Both enzymes possess many inhibitors in common, but preferential inhibition of one enzyme or the other frequently occurs. Among the compounds which preferentially suppress BuChE are many heterocycles and esters which exert significant neuropharmacological effects, most probably through interaction with specific receptors. Included in this group are phenothiazines [4, 5], benzilates [6, 7], butyrophenones [8], imipramines [9], and phencyclidines [10]. We now wish to enlarge this group of preferential BuChE inhibitors through presentation of our findings on the thiaxanthone, miracil D (MD, [lucanthone], Fig. 1). This compound was found to be the most potent reversible BuChE inhibitor tested and was considerably more inhibitory than chlorpromazine. Both competitive and mixed inhibition were detected.

Materials and methods

Human serum BuChE was purified by procainamide affinity chromatography described by Ralston *et al.* [11]. The specific activity of the purified BuChE was approximately 570 units/mg protein. Horse serum BuChE was a product of the Worthington Chemical Co., Freefold, NJ. Catalytic activity was assayed by the method of Ellman *et al.* [12], using butyrylthiocholine (BTC) as substrate. An enzyme activity unit (U) is defined as the hydrolysis of 1 μmole of BTC per min in 0.1 M sodium phosphate buffer, pH 8.0, at 25°. Both BTC and 5,5′-dithiobis-(2-nitrobenzoic acid) were products of the Sigma Chemical Co., St. Louis, MO. Additional drugs and chemicals were obtained from component branches and members of the Walter Reed Army Institute of Research. Enzyme assays were conducted at 25° on a Gilford model 2600 spectrophotometer equipped with a Hewlett–Packard model 7225A plotter and a Gilford Thermal II printer. Enzyme (0.05 units/reaction system) was added last to start the reaction. Absorbance changes at 412 nm during the first minute were linear and were used to calculate reaction rates. Calculated average apparent inhibition constants are presented with their standard deviations, and linear regression analyses were conducted for all data in Figs. 2 and 3.

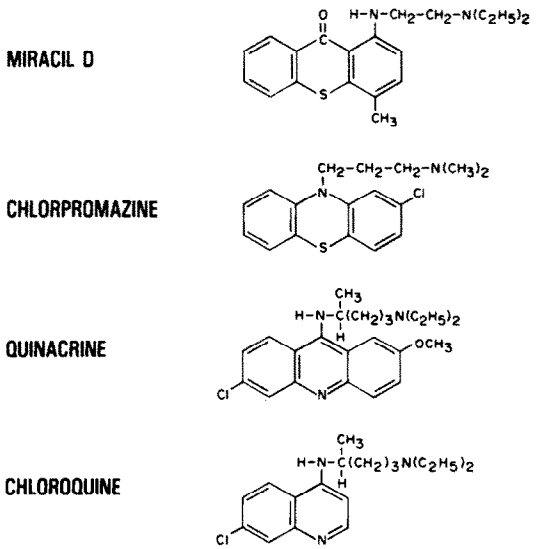

Fig. 1. Structures of miracil D (lucanthone) and additional compounds used in these studies.

* Abbreviations: BuChE, butyrylcholinesterase; AChE, acetylcholinesterase; MD, miracil D; BTC, butyrylthiocholine; and K_m, Michaelis-Menten constant.

Results and discussion

The fifty percent (I_{50}) inhibitory concentrations of selected preferential BuChE inhibitors were determined through analysis of dose–response curves and are presented in Table 1. Miracil D was found to be the most inhibitory compound tested, and its I_{50} was 10-fold lower than that of chlorpromazine, a noted BuChE inhibitor. In addition, proflavine was highly inhibitory. The I_{50} for MD varied with the substrate concentration (not shown), indicating that inhibition was reversible. Preliminary experiments with another thiaxanthone, hycanthone, suggested that these compounds may generally be strong BuChE inhibitors. A concentration of 10^{-5} M MD exerted only 25–40% inhibition of torpedo AChE, thus demonstrating the preferential nature of MD ChE inhibition.

Table 1. Drug concentrations required for 50% inhibition of butyrylcholinesterase

Structural class	Compound	I_{50} (M)
Tropate	Atropine	8.6×10^{-5}
Benzilate	Aprophen	1.5×10^{-6}
Quinoline	Chloroquine	9.0×10^{-6}
Acridine	Quinacrine	1.2×10^{-6}
Acridine	Proflavine	3.7×10^{-7}
Phenothiazine	Chlorpromazine	1.7×10^{-6}
Thiaxanthone	Miracil D	1.5×10^{-7}

I_{50} = 50% inhibitor concentration. Substrate concentration = 1×10^{-4} M BTC. Reaction mixtures contained approximately 0.1 units commercial horse serum BuChE. The structures of the compounds used are: atropine sulfate: endo-(±)-α-(hydroxymethyl)benzeneacetic acid 8-methyl-8-azabicyclo[3.2.1.]oct-3-yl ester sulfate; aprophen: α-methyl-α-phenylbenzeneacetic acid 2-(diethylamino)ethyl ester hydrochloride; chloroquine: N^4-(7-chloro-4-quinolinyl)-N',N'-diethyl-1,4-pentanediamine diphosphate; quinacrine: N^4-(6-chloro-2-methoxy-9-acridinyl)-N^1,N^1-diethyl-1,4-pentanediamine dihydrochloride; proflavine: 3,6-diaminoacridinium sulfate; chlorpromazine: 2-chloro-*N*,*N*-dimethyl-10*H*-phenothiazine-10-propanamine hydrochloride; and miracil D: 1-[(2-diethylaminoethyl)amino] - 4 - methylthioxanthen - 9 - one hydrochloride.

MD inhibition of BuChE was analyzed in detail by plotting V vs $[V/S]$ [13], and $[S/V]$ vs I [14]. These plots, in contrast to reciprocal V vs S plots (Lineweaver–Burk) have been regarded as sensitive indicators of deviations from linearity, and the Hofstee plot has proven highly useful in analysis of substrate and inhibitor modulation of enzyme activity [14, 15]. Figure 2, therefore, presents data in terms of V vs (V/S) in the absence of MD and at selected MD concentrations. The uppermost curve in Fig. 2 depicts the velocity of BTC hydrolysis in the absence of inhibitor. The curve is biphasic, an increase in slope, indicative of substrate activation [15], occurring at higher BTC concentrations. Such substrate modulation of BuChE activity indicates that Michaelian kinetics is not fully applicable throughout the substrate concentration range tested. In the high substrate region, the principal influence of MD was to reduce the K_m (slope) rather than the V_{max} (Y intercept), indicative of competitive inhibition. Use of lower concentrations of BTC, and extrapolation of curves to the ordinate, reveal that both the K_m and V_{max} change. Curves with these characteristics suggest the occurrence of mixed inhibition. Lineweaver–Burk plots (not shown) for both low and high substrate regions yielded similar results. An average competitive K_{iapp} of 1.3×10^{-8} M ± 3.5×10^{-9} M was calculated from slopes of the low substrate region of the Hofstee plot using the formula

$$K_p = K_m(1 + i/K_i)$$

where K_p is the inhibitor-modified K_m. Replots of the slopes vs MD concentration yielded a competitive $K_{i\,app}$ of 1.4×10^{-8} M in the low substrate region and 1.5×10^{-8} M in the high substrate region. A separate series of experiments produced the data shown in Fig. 3, a plot of S/V vs I. One specific advantage of this plot is the graphic determination of the mixed $K_{i\,app}$ [13], which is inferred to be 5.5×10^{-7} M from the intersection of the two curves in

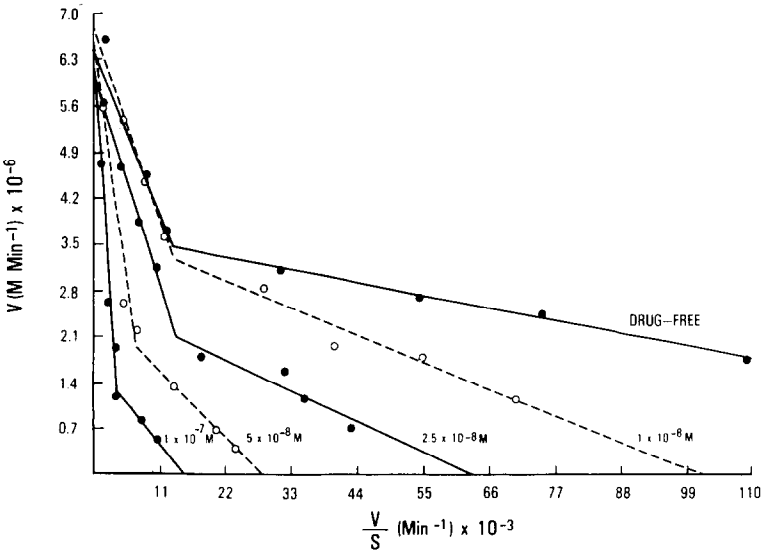

Fig. 2. Hofstee plot showing miracil D inhibition of human serum BuChE. The reaction velocity (V), is plotted against the velocity/substrate concentration, (V/S). Miracil D concentrations appear on the diagram; BTC concentrations, (mM), in the absence of MD were as follows (from left to right): 3.0, 1.0, 0.5, 0.3, 0.1, 0.05, 0.033, and 0.0165. In the presence of 1×10^{-8} M MD and 2.5×10^{-8} M MD the systems containing 0.0165 mM BTC were omitted, and in the presence of 5×10^{-8} M and 1×10^{-7} M MD both 0.0165 and 0.033 mM BTC were omitted. Points are the averages of duplicate determinations.

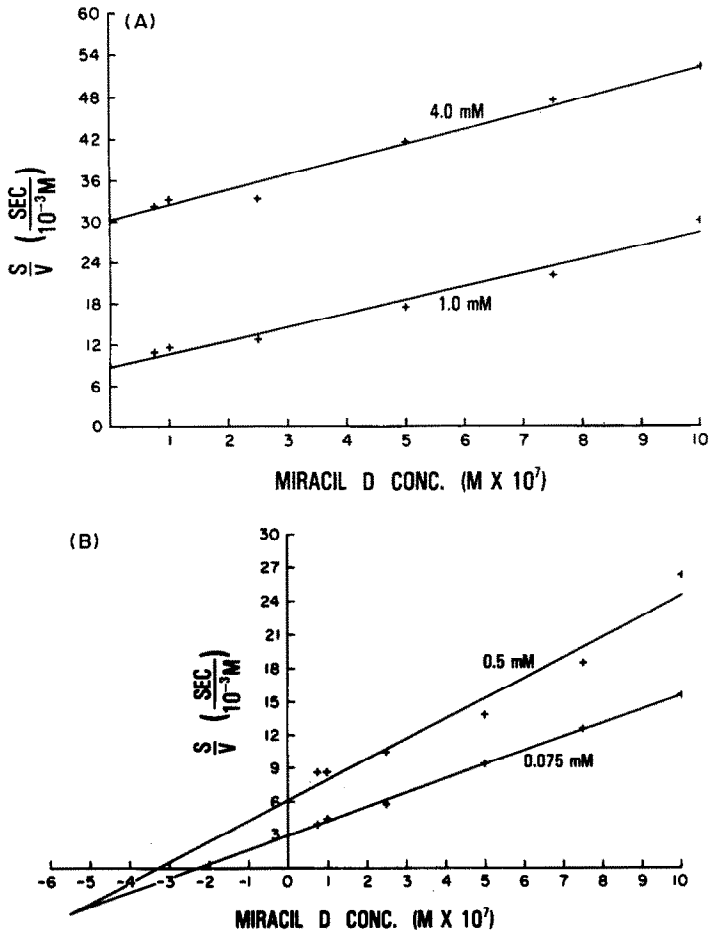

Fig. 3. Plot of S/V vs I for the higher substrate, activated region (Fig. 3A) and for lower substrate concentrations (Fig. 3B). Extrapolation to the abscissa at the intersection of the two curves yields a mixed $K_{i\,app}$ of 5.5×10^{-7} M. The reaction systems contained 0.05 U human serum BuChE.

the lower substrate region. Parallel curves in this analytical form indicate competitive inhibition but do not yield inhibition constants. Such parallel curves were obtained in the higher substrate region.

This communication presents results from continuing investigations on the structure, function, and inhibition of cholinesterases. MD was revealed to be a strong, reversible, preferential BuChE inhibitor which blocked catalysis by a predominantly competitive mechanism at high substrate concentration and by a mixed competitive, non-competitive mechanism at low substrate concentration. Both the I_{50} values listed in Table 1 and comparison with published [14] competitive inhibition constants suggest MD to be the strongest heterocyclic BuChE inhibitor yet discovered. Thiaxanthones, more generally, appeared to be strong BuChE inhibitors. Comparison of results depicted in the Hofstee and Cornish–Bowden plots suggests the validity of the kinetic conclusions, but the complexity of enzymes which undergo substrate modulation as well as mixed inhibition renders mechanistic interpretation tenuous.

The structural properties which make thiaxanthones such strong inhibitors of BuChE are not readily apparent, although the ketonic ring oxygen is an unusual substituent of ChE inhibitors and perhaps analogous to the substrate carbonyl group. However, the structural requirements for aromatic or heterocyclic binding to specific macromolecules have been elucidated by Albert *et al.* [16], and by Lerman [17], for nucleic acids, and by Wallace *et al.* [18] for α-chymotrypsin. In each instance, increasing planar area and positive charge were important binding parameters. It is noteworthy that the present results and those of Wright and Sabine [19] show quinacrine, an acridine, to inhibit BuChE more effectively than chloroquine, a quinoline. Both compounds possess the same side chain, but differ in planar area. In rabbit skeletal troponin C, a calcium-binding protein, biophysical and biochemical studies upon natural and synthetic peptides [20] have indicated the phenothiazine, trifluoperazine, to intercalate, or to stack, to a helical amino acid sequence in which phenylalanine, alanine, leucine, and isoleucine residues provided the opportunity for hydrophobic interaction. Concurrently, a proximal, negatively charged glutamic acid residue attracted the positively charged nitrogen of the piperazine ring. Thus, a potential model for heterocycle binding to proteins has been described, and homology studies comparing heterocycle binding regions will be of interest. It is inviting to speculate that BuChE, α-chymotrypsin, and troponin C possess a similar hydrophobic ligand binding site, in contrast to AChE. More generally, the studies of Albert *et al.* [16] and those of Wallace *et al.* [18] suggest that helices containing charged groups and hydrophobic regions in nucleic acids and proteins may serve to bind similar compounds by potentially analogous modes.

Acknowledgements—The authors thank Lisa Baker for her contributions to this research.

Department of Applied Biochemistry
Division of Biochemistry
Walter Reed Army Institute of Research
Washington, DC 20307-5100, U.S.A.

JEFF S. VERDIER
ALAN DAVID WOLFE*

REFERENCES

1. R. Davis and G. Koelle, *J. Cell Biol.* **88**, 581 (1981).
2. A. M. Graybiel and C. W. Ragsdale, Jr., *Science* **299**, 439 (1982).
3. K. MacPhee-Quigley, P. Taylor and S. S. Taylor, *Fedn Proc.* **44**, 1068 (1985).
4. K. B. Augustinsson, *Acta physiol. scand.* **15** (Suppl. 52), 1 (1948).
5. J. J. Gordon, *Nature, Lond.* **162**, 146 (1948).
6. L. Baker and A. D. Wolfe, *Fedn Proc.* **42**, 2112 (1983).
7. R. S. Rush, J. S. Ralston and A. D. Wolfe, *Biochem. Pharmac.* **34**, 2063 (1985).
8. H. Michalek, *Biochem. Pharmac.* **22**, 1067 (1973).
9. E. Perkinson, R. Ruckert and J. P. Da Vanzo, *Proc. Soc. exp. Biol. Med.* **131**, 685 (1969).
10. S. Maayani, H. Weinstein, N. Ben-Zvi, S. Cohen and M. Sokolovsky, *Biochem. Pharmac.* **23**, 1263 (1974).
11. J. S. Ralston, A. R. Main, B. F. Kilpatrick and A. L. Chassen, *Biochem. J.* **211**, 243 (1983).
12. G. L. Ellman, K. D. Courtney, V. Andres, Jr., and R. M. Featherstone, *Biochem. Pharmac.* **7**, 88 (1961).
13. B. H. J. Hofstee, *Science* **116**, 329 (1952).
14. A. Cornish-Bowden, *Biochem. J.* **137**, 143 (1974).
15. R. S. Rush, A. R. Main, B. F. Kilpatrick and G. D. Faulkner, *J. Pharmac. exp. Ther.* **216**, 586 (1981).
16. A. Albert, S. D. Rubbo and M. I. Burville, *Br. J. exp. Path.* **30**, 159 (1949).
17. L. S. Lerman, *Proc. natn. Acad. Sci. U.S.A.* **49**, 94 (1963).
18. R. A. Wallace, A. N. Kurtz and C. Niemann, *Biochemistry* **2**, 824 (1963).
19. C. I. Wright and J. C. Sabine, *J. Pharmac. exp. Ther.* **93**, 230 (1948).
20. J. Gariepy and R. S. Hodges, *Biochemistry* **22**, 1586 (1983).

* Author to whom correspondence should be addressed.

Biochemical Pharmacology, Vol. 35, No. 9, pp. 1608–1611, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00

Effects of ethanol and secretagogues on the composition of phosphatidylinositol in pancreas and submaxillary gland

(*Received* 13 *June* 1985; *accepted* 4 *November* 1985)

Acute and chronic pancreatitis are severe complications of alcoholism, but the pathogenesis is incompletely understood [1]. Rats fed an ethanol-containing diet for 24 days had a lower content of arachidonoyl-containing phosphatidylinositols in pancreas and submaxillary gland than the pair-fed controls [2, 3].This might reflect changes in the stimulus-secretion coupling, since phosphatidylinositols or polyphosphoinositides are involved in this process [4–8]. No effect of acute ethanol administration was found in the previous study [2], and the time-course of the appearance and disappearance of the change was therefore investigated both in pancreas and submaxillary gland. Since the changes observed in both organs might be due to chronic hyperstimulation, this was simulated by administration of secretagogues.

Materials and methods

Male Sprague–Dawley rats were used. Rats weighing about 100 g were fed a liquid diet for 24 days [9]. The alcohol diet, which provides 36% of the energy and the control diet with carbohydrate replacing ethanol, were obtained from Bioserv, Inc. (Frenchtown, NJ). Alcohol was introduced stepwise, and the controls were pair-fed [9] also when control diet was substituted for ethanol diet for 3 days after the 24 days of ethanol administration. In another experiment the liquid diet was given to 170 g rats for 6 days followed by one day of starvation. Rats weighing about 200 g were used in the other experiments.

Intravenous infusion (1 ml/hr) of solutions in saline (9 g/l) were performed in the femoral vein with a thin polyethylene catheter. Caerulein (Sigma Chemical Co.) was infused for 18 hr at a rate of 50 ng/hr, known to cause maximal stimulation of the pancreas [10]. Carbamyl-β-methylcholine (Sigma Chemical Co.) was infused for 18 hr or 4 days at a rate of 150 μg/hr that causes significant stimulation of pancreatic protein secretion [11]. The rats were kept in restraining cages with free access to water, and, in the 4-day experiments, free access to food pellets.

The rats were killed by cervical dislocation, and the pancreas and submaxillary gland were taken out, avoiding visible fat. The phosphatidylinositols were isolated and hydrolysed, and the diacylglycerols were analyzed as trimethylsilyl ethers as previously described [2, 3].

Student's *t*-test was used for statistical analysis.

Results

Identification of phosphatidylinositol species. The trimethylsilyl ethers of the diacylglycerols obtained from the phosphatidylinositols of pancreas and submaxillary gland were not completely separated by the gas chromatographic method. The major components in the gas chromatographic peaks were identified by gas chromatography/mass spectrometry (Table 1). It is assumed that the more unsaturated acyl group was in the 2-position.

Effects of ethanol. Rats fed an ethanol-containing liquid diet for 24 days had not the same phosphatidylinositol composition in the submaxillary gland as the corresponding pair-fed controls (Table 1). Thus, the arachidonoyl-containing species (36:4 and 38:4) were significantly less abundant whereas more saturated di-C_{18}-acyl-containing species contributed to a larger extent in the ethanol-fed animals. The fractions of both di-C_{16}-containing species were lower in the ethanol-fed rats. Differences in the same direction but smaller and less significant were seen when the diets were given for only 6 days followed by a day of starvation (Table 2). The difference between ethanol-fed and control groups was only slightly decreased by feeding control diets to both groups for three days after 24 days of pair-feeding the test diets.

Differences were also seen in the phosphatidylinositol composition in pancreas of rats pair-fed the ethanol-containing and control diets for 6 days followed by a day of