NMR STUDIES OF THE INTERACTION OF ESERINE AND ATROPINE WITH ACETYLCHOLINESTERASE

Gabor Kato, James Yung and Milan Ihnat
Department of Research in Anaesthesia, McGill University, Montreal, Canada.

Received May 11, 1970

Summary - In vitro binding of atropine or eserine to acetylcholinesterase derived from squid head ganglia was studied by high resolution ^1H nuclear magnetic resonance spectroscopy. Increased immobilization of the inhibitors due to binding by the enzyme is manifested in the altered proton transverse relaxation times of the inhibitor protons. Tetraethylpyrophosphate diminished the binding of eserine but had little effect on the binding of atropine Kinetic studies showed that eserine is a potent anticholinesterase ($I_{50}=10^{-7}M$) while atropine is a poor anticholinesterase ($I_{50}=10^{-1}M$). These results indicate that whereas eserine binds to the catalytic site of acetylcholinesterase, atropine binds to a second site distinct from the active centre.

INTRODUCTION

Recently, Changeux, Leuzinger and Huchet have shown the reversible binding of acetylcholine to acetylcholinesterase which was inhibited by eserine (1), and also the binding of flaxedil and d-tubocurarine, two receptor inhibitors which only partially inhibited the enzyme (2). They suggested that acetylcholinesterase contains regulatory sites which are distinct from the active site.

In this communication we report the results of studies, by ^1H nuclear magnetic resonance (^1H nmr) methods, of the association of atropine and eserine with acetylcholinesterase extracted from head ganglia of squid (loligo apalescens). These two compounds were chosen since it is known that eserine has a high affinity for the catalytic site and may be considered as an ideal compound to assay hydrolytic sites whereas atropine may be considered as a suitable compound to assay receptor sites since it binds weakly to known macromolecules (3) and has a high affinity for muscarinic receptors(4).

METHODS AND MATERIAL

The application of nmr spectroscopy to the study of interactions between small and large molecules by observing the spectrum of the small molecule, has been described (5,6,7). All nmr measurements were made on a Varian A-60D high resolution spectrometer. Measured line widths were corrected for instrumental broadening (0.4Hz) and transverse relaxation rates $1/T_2$ were calculated from $1/T_2=\pi\Delta\nu_{\frac{1}{2}}$ where $\Delta\nu_{\frac{1}{2}}$ is the line width at one half maximum peak height.

Acetylcholinesterase was extracted from squid head ganglia (loligo apalescens) with phosphate buffered saline (0.1M sodium phosphate buffer, pH7.3, 0.1M NaCl) and was purified by ammonium sulphate fractionation. The 20-40% ammonium sulphate fraction was dissolved in the phosphate buffered saline, dialyzed against distilled water for 24h, centrifuged and the supernatant lyophilized to dryness. The specific activity of the acetylcholinesterase was 20 mmoles ACh hydrolyzed/mg/hr.

RESULTS AND DISCUSSION

Direct coordination of atropine and eserine by the enzyme is demonstrated by the examination of the nmr spectra (at 60MHz) of the N-methyl and phenyl protons of atropine, and the N-methyl and C-methyl protons of eserine in the presence and absence of acetylcholinesterase. Typical results are shown in Figure 1A and B. At 39°C and pD7.8 in the presence of enzyme (4 mg/ml), the resonance lines of atropine and eserine undergo broadening owing to association with the enzyme. However, within the experimental error, no significant changes in the positions of the resonance lines could be detected in the presence of acetylcholinesterase.

Under these conditions, the enzyme-inhibitor complex

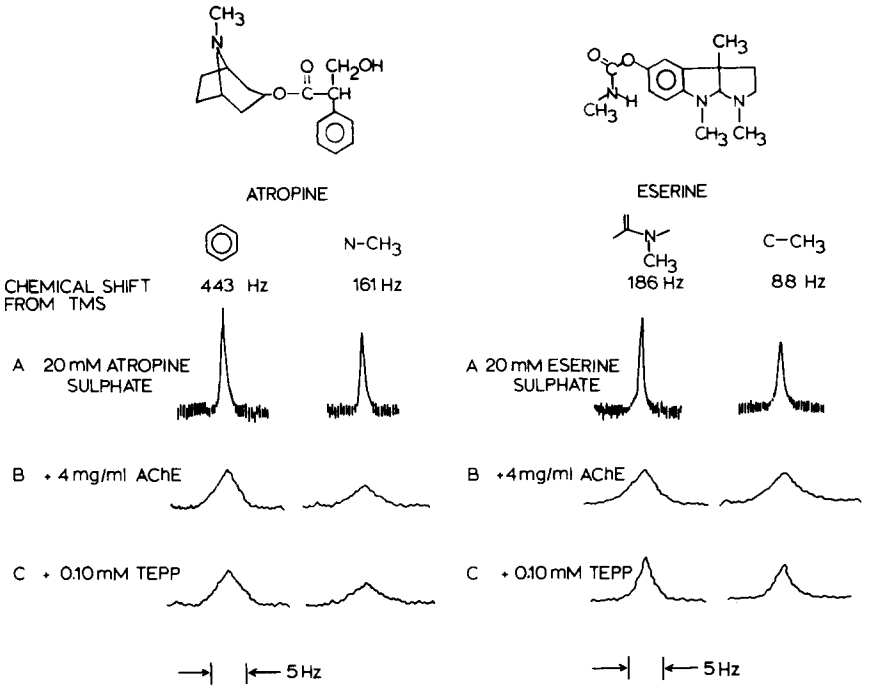

Fig. 1. The effect of Acetylcholinesterase (AChE) on the nmr spectra of atropine and eserine and of TEPP on the nmr spectrum of atropine + AChE and eserine + AChE. The solvent was 99.7% D_2O containing 0.01 phosphate buffer, pD7.8. T = 39°C.

was short-lived and yielded a spectrum representative of a weighted average of the enzyme-bound and free forms of inhibitor. This resulted in sharper resonances at higher inhibitor concentrations. The line widths of the N-methyl and phenyl groups of atropine and of the N-methyl and C-methyl groups of eserine increased in a continuous fashion as the inhibitor concentration was decreased at constant enzyme concentration (Table 1). As pointed out earlier (8) this is the opposite to what one would expect if the broadening were due to nonspecific mechanisms. Values of the relaxation rates are given in Table II. This shows that on binding to acetylcholinesterase the relaxation rate of the N-methyl group of atropine increased from 3.2 to $21.2 \times 10^3 sec^{-1}$, by a factor of

Table 1

Comparison of the Effects of DFP and TEPP on the Line Widths of Atropine and Eserine in the presence of Acetylcholinesterase

	Atropine Sulphate mM	DFP mM	TEPP mM	$\Delta\nu_{\frac{1}{2}obs}$ (Hz) PHENYL	NCH_3
EXPT. A	5	-	-	4.71	6.55
	10	-	-	3.83	4.56
	20	-	-	2.86	3.97
	30	-	-	2.37	3.15
EXPT. B	20	0	-	3.10	3.50
		0.005	-	3.00	3.50
		0.10	-	3.00	3.60
		-	0	3.15	3.61
		-	0.005	3.10	3.70
		-	0.10	3.15	3.54
	Eserine Sulphate mM			NCH_3	CCH_3
EXPT. A	10	-	-	6.92	9.40
	20	-	-	4.25	5.43
	40	-	-	3.17	4.23
	60	-	-	2.93	3.21
EXPT. B	40	0	-	3.35	4.20
		0.005	-	2.90	3.60
		0.10	-	2.50	3.20
		-	0	3.15	4.00
		-	0.005	2.70	3.20
		-	0.10	2.40	3.00

Condition: 0.01 M phosphate buffer in 99.7% D_2O; pD = 7.9, T=39°C; AChE concentration 4 mg/ml; $\Delta\nu_{\frac{1}{2}obs}$ is the line width at half height and is the average of ten measurements; sweep width 250 Hz, sweep time 500 sec.

6,600, and that of the phenyl group by 10,900; the relaxation rate of the N-methyl group of eserine increased from 0.9 to $12.2 \times 10^3 sec^{-1}$, by a factor of 13,600 and that of the C-methyl group by 10,100. The large and similar changes in$(1/T_2)$ indicate that both inhibitors are

Table II

Relaxation Rates and I_{50} for Atropine and Eserine

Inhibitor	Resonance peak	$\left(\frac{1}{T_2}\right)_{free}$ sec^{-1}	$\left(\frac{1}{T_2}\right)_{bound}$ sec^{-1}	$\frac{T_2 \text{ free}}{T_2 \text{ bound}}$	I_{50} (M)
Atropine	N-CH_3	3.2	21.2×10^3	6.6×10^3	10^{-1}
Sulphate	phenyl	1.5	16.4×10^3	10.9×10^3	
Eserine	N-CH_3	0.9	12.2×10^3	13.6×10^3	10^{-7}
Sulphate	C-CH_3	1.6	16.2×10^3	10.1×10^3	

$(1/T_2)_{free}$ and $(1/T_2)_{bound}$ are the relaxation rates of the free and bound nuclei respectively. $(1/T_2)_{bound}$ was obtained from a plot of $(1/T_2)_{obs}$ versus $[E]_T$(6). $[E]_T$ is the total concentration of enzyme and $(1/T_2)_{obs}$ is the observed transverse relaxation rate. I_{50} is the concentration of inhibitor required to produce 50% inhibition of the enzyme activity.

strongly bound to the enzyme.

In the second series of experiments the effects of two irreversible anticholinesterases, tetraethyl pyrophosphate (TEPP) and diisopropyl fluorophosphate (DFP) on the binding of atropine and eserine to acetylcholinesterase were studied. The enzyme was preincubated with varying concentrations of TEPP or DFP for sufficient duration to permit completion of the reaction between the phosphate inhibitor and the enzyme. The effects of TEPP and DFP on line widths of both atropine and eserine are shown in Table 1 (Expts B) and Figure 1C. The addition of TEPP or DFP to acetylcholinesterase partially abolished the increases in line widths of the N-methyl and C-methyl groups of eserine in the presence of acetylcholinesterase, but the line widths of the atropine peaks remained unchanged. The selectivity in inhibiting the binding

of eserine without preventing the binding of atropine suggests the existence of two distinguishable binding sites on acetylcholinesterase.

A direct test of this postulate was made by studying the effect of atropine and eserine on the catalytic activity of the enzyme. Eserine sulphate and atropine sulphate were individually incubated in varying concentrations (10^{-8}M-10^{-1}M) with acetylcholinesterase (2.0mg/ml) for 20 min. at pD7.9 and 39°C. Acetylcholine chloride (10mM) was then added and the rate of hydrolysis was followed by nmr methods as described earlier for serum cholinesterase (9). The activity without inhibitors was also determined. The results are summarized in Table II. While eserine is a potent anticholinesterase (I_{50}=10^{-7}M) atropine is a poor anticholinesterase (I_{50}=10^{-1}M). If both compounds bind at the catalytic site, both should inhibit acetylcholine hydrolysis. Yet atropine which strongly binds to the enzyme is an ineffective anticholinesterase. Consequently, it must bind to a site other than the catalytic site.

Our results indicate that there are at least two distinguishable binding sites on the enzyme. One is the catalytic site (where eserine binds) which consists of an anionic and an esteratic site; the other is an anionic site (where atropine binds) which is distinct from the catalytic site. These results are in good agreement with those for the binding of several other inhibitors to this enzyme (2) and the binding of acetylcholine in the presence of eserine (1). The physiological significance of this property of acetylcholinesterase, however, is not yet clear. Whether or not acetylcholinesterase functions as the post-synaptic receptor for acetylcholine remains to be shown.

With support from the Canadian Medical Research Council and the Muscular Distrophy Association.

REFERENCES

1. Changeux, J.P., Leuzinger, W., and Huchet, M., F.E.B.S. Letters 2, 77 (1968).
2. Changeux, J.P., Mol. Pharmacol. 2, 369 (1966).
3. Oroszlan, S.I., and Maengwyn-Davies, G.D., Biochem. Pharmacol. 11, 1203 (1962).
4. Paton, W.D.M. and Rang, H.P., Proc. Roy. Soc. B. 163, 1 (1965).
5. Jardetzky, O., Adv. Chem. Phys. 7, 499 (1964).
6. Jardetzky, O., and Wade-Jardetzky, N.G., Mol. Pharmacol. 1, 214 (1965).
7. Gerig, J.T., J. Am. Chem. Soc. 90, 2681 (1968).
8. Fischer, J.J. and Jardetzky, O., J. Am. Chem. Soc. 87, 3237 (1965).
9. Kato, G., Mol. Pharmacol. 4, 640 (1968).