Biochemical Pharmacology, Vol. 22, pp. 1875–1881. Pergamon Press, 1973. Printed in Great Britain.

RELATIONSHIPS BETWEEN *IN VIVO* AND *IN VITRO* INHIBITION OF ACETYLCHOLINESTERASE (AChE) AND IMPAIRMENT OF NEUROMUSCULAR TRANSMISSION IN THE RAT PHRENIC-NERVE DIAPHRAGM BY A TERTIARY ANTICHOLINESTERASE AND ITS QUATERNARY ANALOGUE

RICHARD LANCASTER

Department of Pharmacology, The Middlesex Hospital Medical School, London, WIP 7PN, England

(*Received* 6 *November* 1972; *accepted* 12 *February* 1973)

Abstract—The tertiary anticholinesterase agent pinacolyl *S*-(2-dimethylaminoethyl) methylphosphonothioate (compound I) and its quaternary analogue (compound II), were administered subcutaneously to atropinized rats. The animals were killed 30 min later and the tension of the isolated phrenic nerve-diaphragm preparation, stimulated indirectly at 100 Hz for 10 sec, was compared with that of preparations from control animals. A dose of 1·5 μmole/kg of the quaternary compound II, which has a bimolecular rate constant of inhibition twice that of the tertiary compound I, reduced the tetanic response of the diaphragm by 50 per cent; the equivalent dose of the tertiary compound I was 5·2 μmole/kg. At these dose levels, compound II inhibited 65 per cent of the diaphragm acetylcholinesterase (AChE) activity, determined on homogenates, and compound I 92 per cent. When the phrenic nerve-diaphragm preparation from untreated animals was incubated for 20 min with various concentrations of compound I or II, the rate of enzyme inhibition conformed approximately to first order kinetics and equimolar concentrations of the inhibitors reduced tetanic tension to 50 per cent in the same time. There was no discontinuity in the plot of per cent AChE inhibition vs logarithm of the concentration of compound I or II but the slope was much less with the quaternary compound II. The results provide additional evidence that quaternary compounds can reach all sites with AChE activity only *in vitro* and that their distribution when applied *in vitro* is not the same as that *in vivo*.

IN STUDIES on rat brain homogenates[1] it was found that the lipid insoluble quaternary compound pinacolyl *S*-(2-trimethylaminoethyl) methylphosphonothioate, (compound II) was a potent irreversible anticholinesterase and had a bimolecular rate constant for inhibition of acetylcholinesterase (AChE) which was twice that of its lipid soluble tertiary analogue, compound I. When administered *in vivo* to atropinized rats, the quaternary compound II, inhibited only 60 per cent of diaphragm AChE, as determined on homogenates, in doses that produced signs of muscular weakness, whereas the tertiary compound I at doses that produced a similar degree of muscular weakness inhibited over 90 per cent of diaphragm AChE.

To obtain more information on the relationship between the effects on neuromuscular transmission and AChE activity, compounds I and II have been administered to atropinized rats *in vivo* and the response of the phrenic nerve-diaphragm preparation to indirect tetanic stimulation investigated, since anticholinesterases cause a reduction in the height of the tetanic response due to AChE accumulation at the end-plate region.[2–5] The AChE activity was determined on diaphragm muscle homogenates.

In addition, to see if the effects on neuromuscular transmission and on AChE activity are the same when anticholinesterases are administered *in vitro* as when they are administered *in vivo*, compounds I and II have been applied to the phrenic nerve–diaphragm preparation *in vitro* and their effect on AChE activity and the response to indirect tetanic stimulation determined.

METHODS

Male Wistar rats (150–300 g) were used. Animals were killed by a blow on the head. Tissues were perfused *in situ* with 0·15 M-NaCl until blood-free and the left hemidiaphragm with its phrenic nerve and rib cage insertion was removed and suspended in an organ bath containing 40 ml Tyrode's solution of the following composition in mmole/l.: NaCl, 138; KCl, 21·7; $CaCl_2$, 3·6; $MgCl_2$, 2·1; $NaHCO_3$, 11·9; glucose, 11. The organ bath fluid which was maintained at 37°, was gassed with 95% O_2 and 5% CO_2, pH 7·15.

Muscle tension recordings. Muscle tension was recorded on a smoked drum by a spring-loaded lever giving an 18-fold magnification. The phrenic nerve was stimulated with an open Palmer phrenic electrode connected to an electronic square wave generator (Palmer) with a constant voltage output. All stimuli were supramaximal (0·1 msec duration, 5V). The nerve was stimulated every 2 min at 100 Hz for 10 sec and the height of the contraction at the end of the 10 sec period of stimulation was used for comparison throughout.

Compounds I and II administered in vivo. Animals received atropine, 15 μmole/kg i.p., and 30 min later compound I, compound II or 0·15 M-NaCl s.c. in the flank and were killed 30 min later. The left phrenic nerve-diaphragm was set up as described. A tension of 32 g was applied by adjusting the spring-loaded lever, and the preparation was stimulated every 2 min at 100 Hz for 10 sec. The height of the response to tetanic stimulation declined initially but settled down to a constant value in 45 min, and for comparisons, the tension recorded at this time was used. The percentage reduction in height of the response was obtained by comparing the recorded height of the response of the nerve–diaphragm preparation from atropinized rats treated with compound I or II with that from atropinized rats treated with 0·15 M-NaCl. The latter was 122 ± 8 (S.E.M.) mm ($n = 5$).

The right hemidiaphragm was also removed, with the phrenic nerve being cut at the point of contact with the muscle, blotted and homogenized in 10 mM-MeCh (DL-acetyl-β-methylcholine chloride) and its AChE activity determined. In control experiments, no significant difference was found between the AChE activities of the left and right hemidiaphragms.

Effect on the phrenic nerve-diaphragm preparation of compound I *and* II in vitro. The left phrenic nerve-diaphragm from an untreated animal was set up as described. The phrenic nerve was stimulated at 100 Hz for 10 sec every 2 min until the height of contraction became constant. Tyrode's solution containing compound I or II was then added to the organ bath for 20 min and the stimulation continued at 2 min intervals. The percentage reduction in the height of the response was obtained by comparing the height of the contraction at various times after addition of the inhibitor with that of the mean value of the four contractions immediately preceding addition of inhibitor.

After assessment of tension, the diaphragm was removed from the organ bath, the phrenic nerve was cut away from the diaphragm at its point of contact and the muscle

blotted, weighed and homogenized with 10 mM-MeCh. The AChE activity of this homogenate was compared with that of a homogenate from the corresponding right diaphragm, prepared in the same way, which had not been exposed to compound I or II. Control experiments showed that incubation of the diaphragm in Tyrode's solution at 37° for 30 min had no effect on AChE activity.

Preparation of homogenates and determination of their AChE activity. Diaphragm muscle, suspended in 0·15 M-NaCl containing 10 mM-MeCh, using 1 ml of fluid per 60 mg of tissue, was homogenized by grinding in acid-washed sand with a pestle and mortar. The 10 mM-MeCh was used to prevent inhibition of AChE during homogenization by free inhibitor present in the tissue, as it was found that the addition of 10 mM-MeCh to the diaphragm homogenate 5 min before 0·4 μM compound II, which inhibited 50 per cent AChE in 20 min at pH 8·0 in the absence of MeCh, prevented any detectable degree of enzyme inhibition occurring over a period of 20 min.

The automatic titration method of Jensen-Holm *et al.*[6] was used for determination of AChE activity. Assays were carried out at 37°, pH 8·0, in a reaction volume of 5 ml obtained by diluting the enzyme sample 10-fold with 0·15 M-NaCl[7] giving a final concentration of 6 mg tissue/ml. Acetylthiocholine (ATCh), 1 mM, was used as substrate, as previous experiments had shown that under the experimental conditions it was a reasonably selective substrate for AChE in the rat diaphragm.[1] The AChE activity was calculated from initial rates of hydrolysis and all values were corrected for nonenzymic substrate hydrolysis and homogenate blanks. Homogenate blanks were partly due to non specific acid production and partly to hydrolysis of 1 mM-MeCh present in the reaction vessel. At pH 8·0, MeCh was hydrolysed by AChE at 11 per cent of the rate of ATCh[7] and in the presence of 1 mM-MeCh, 1 mM ATCh was hydrolysed by diaphragm homogenate at 88 $\pm$ 8 (S.E.M.) percentage of the rate recorded in the absence of MeCh.

Injections and chemicals used. All injections were given in a volume of 0·1 ml/100 g. The statistical significance of results was assessed by Student's *t*-test. The sources of chemicals were: pinacoly *S*-(2-dimethylaminoethyl) methylphosphonothioate HCl (compound I) and pinacolyl *S*-(2-trimethylaminoethyl) methylphosphonothioate methylsulphate (compound II), Dr. Boskovic, Institute of Toxicology, Belgrade Atropine sulphate, British Drug Houses; ATCh iodide and MeCh chloride, Sigma.

RESULTS

Inhibition of AChE in vivo. Compounds I and II were administered in graded doses to rats pretreated with 15 μmole atropine/kg and the animals killed 30 min later. Figure 1 shows the heights of contraction of the diaphragm in response to indirect tetanic stimulation at 100 Hz for 10 sec and the AChE activities of the diaphragm homogenates of animals treated with compound I or compound II, expressed as a percentage of controls (0·15 M-NaCl injected rats). For both compounds the dose–effect plots were steep but parallel. The dose of the quaternary compound II that produced a 50 per cent reduction in the tetanic response was 1·5 μmole/kg and the equivalent dose of the tertiary compound I, 5·2 μmole/kg. At these doses, compound II produced 65 per cent inhibition of diaphragm AChE, and compound I 92 per cent inhibition.

Inhibition of AChE in vitro. The percentage inhibition of diaphragm AChE, determined on homogenates, produced by compounds I and II *in vitro* was studied by

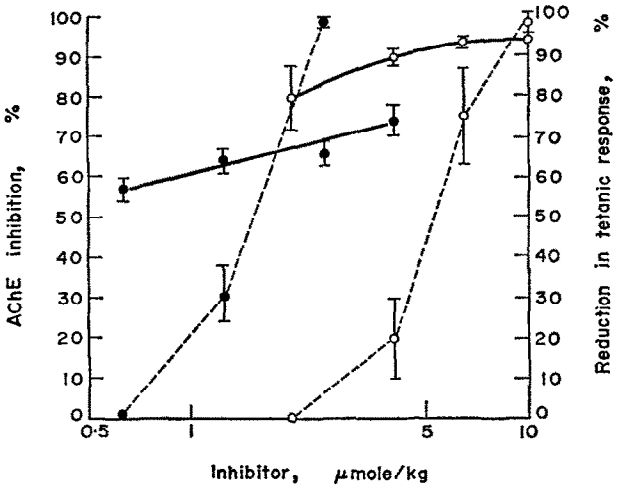

FIG. 1. Relationship between inhibition of AChE and impairment of neuromuscular transmission. The solid lines show the inhibition of AChE, determined on diaphragm homogenates, and the broken lines the percentage reduction in the tetanic response of the phrenic nerve–diaphragm stimulated indirectly at 100 Hz for 10 sec every 2 min, for the tertiary compound I, (○) and for the quaternary compound II (●) treated animals. All values are percentages of values obtained with 0·15 M-NaCl treated animals and reduction was calculated from the tension present at the end of the 10 sec period of stimulation. Mean ± S.E.M. ($n = 4$).

incubating the phrenic nerve–diaphragm preparation from untreated rats with various concentrations of each inhibitor for 20 min at 37°. The results, illustrated in Fig. 2, show that there was no discontinuity in the slope of AChE inhibition plotted against the logarithm of the inhibitor concentration for either compound I or for compound II but that the slope for the tertiary compound I was steeper than that for the quaternary

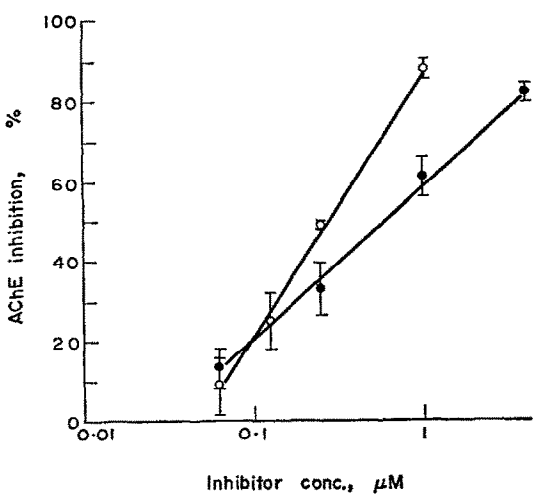

FIG. 2. Inhibition of AChE, determined on diaphragm homogenates, after incubation of the phrenic nerve–diaphragm preparation in Tyrode's solution containing different concentrations of the tertiary compound I (○) and the quaternary compound II (●) for 20 min at 37°. Mean ± S.E.M. ($n = 4$).

nary compound II. At low concentrations, the inhibitors produced similar degrees of inhibition but at a higher concentration, the tertiary compound inhibited more enzyme than did equimolar concentrations of the quaternary compound e.g. 1 μM of compound I inhibited 88 per cent AChE whereas 1 μM of compound II inhibited only 65 per cent AChE ($P < 0{\cdot}001$).

In a control experiment, the influence of tetanic stimulation on AChE inhibition by the quaternary compound II, was investigated. One μM of compound II was applied

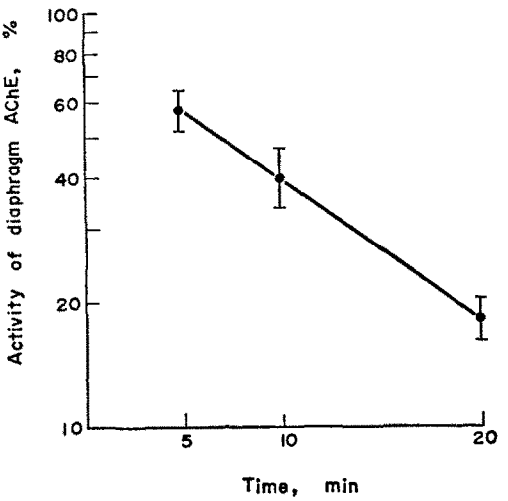

FIG. 3. Time course of inhibition of AChE, determined on diaphragm homogenates, when the phrenic nerve–diaphragm preparation was incubated with 4 μM-compound II in Tyrode's solution at 37°. Mean ± S.E.M. ($n = 4$).

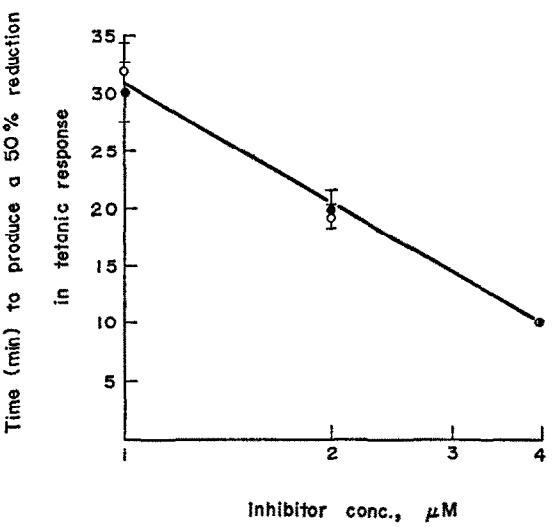

FIG. 4. Time taken for different concentrations of the tertiary compound I (○) and the quaternary compound II (●), incubated with the phrenic nerve–diaphragm preparation in Tyrode's solution at 37°, to produce a 50 per cent reduction in the height of the tetanic response to 100 Hz stimulation. Mean ± S.E.M. ($n = 4$).

to the phrenic nerve–diaphragm preparation which was stimulated at 100 Hz for 10 sec every 2 min. After 20 min, the percentage inhibition of diaphragm AChE was 61 ± 5 (S.E.M.) per cent ($n = 4$). This value was not significantly different from that produced when the inhibitor was applied to the unstimulated preparation, 63 ± 10 (S.E.M.) per cent ($n = 4$).

When the phrenic nerve–diaphragm preparation was incubated with the quaternary compound II, 4 μM, for varying periods of time at 37°, the rate of inhibition of diaphragm AChE conformed approximately to the first order kinetics (Fig. 3). Similar results were obtained with the tertiary compound I.

In a series of experiments, different concentrations of compound I and II were incubated with phrenic nerve–diaphragm preparations from untreated rats and the response to indirect stimulation for 10 sec at 100 Hz was recorded every 2 min until the height of the response had fallen to less than 20 per cent of that recorded before addition of inhibitor. As Fig. 4 shows, 1, 2 and 4 μM-concn of the tertiary compound I produced a 50 per cent reduction in the response to tetanic stimulation in the same time as equimolar concentrations of the quaternary compound II.

DISCUSSION

The results provide further evidence for the view[1] that *in vivo* all AChE sites in the diaphragm are accessible to the highly lipid soluble tertiary compound I, whereas only a fraction of them are accessible to the lipid insoluble quaternary compound II. The dose of compound I (5·2 μmole/kg) that produced a 50 per cent reduction in the tetanic response inhibited 92 per cent of diaphragm AChE whereas the equivalent dose of compound II (1·5 μmole/kg) inhibited only 65 per cent. For both inhibitors the slope of percentage reductions in the height of the tetanic response against logarithm of the dose was steep. Moreover, for compound II a detectable reduction in the tetanic response occurred at doses which were two to three times higher than those which were sufficient to inhibit that fraction of total AChE (50 per cent) that is readily accessible to it *in vivo*,[1] indicating that inhibition of this fraction of enzyme alone does not cause sufficient accumulation of ACh at the neuromuscular junction to affect the tetanic response.

There was no discontinuity in the slope of AChE inhibition plotted against the logarithm of inhibitor concentration for either compounds when incubated with the phrenic nerve–diaphragm preparation *in vitro* but the slope for the tertiary compound I was steeper than that for the quaternary compound II. These results are similar to those obtained on cerebral cortex slices and diaphragm segments[1] and indicate that the distribution of AChE in the diaphragm *in vitro* is a continuum without clearly defined diffusion barriers. A similar conclusion was reached by Goodford,[8] who from studies of the distribution of radioactive cations in smooth muscle preparations *in vitro* concluded that the two compartment model was unduly restrictive for smooth muscle which conformed better to a multicompartment model. *In vitro*, compounds I and II were equipotent in their ability to impair the response of the phrenic nerve–diaphragm preparation to tetanic stimulation. Since the bimolecular rate constant for inhibition by the quaternary compound II, is double that by the tertiary compound I, this finding indicates that *in vitro* the diffusion of compound II to AChE sites on which normal function of the phrenic nerve–diaphragm depends is more restricted than for compound I. Holmes *et al.*[9] reached a similar conclusion when they found that the

rate-limiting step in the response of the phrenic nerve–diaphragm to tubocurarine *in vitro* was diffusion of the drug between the organ bath and its sites of action. These results contrast with the *in vivo* results and are further evidence that the distribution of quaternary compounds in tissues *in vitro* does not correspond to that *in vivo*.

The percentage inhibition of diaphragm AChE produced by compounds I and II (1, 2 and 4 μM) *in vitro*, at the times taken by these concentrations to produce a 50 per cent reduction in tetanic response, can be made by extrapolation from Fig. 2, assuming first order kinetics of AChE inhibition. Such calculations show that all concentrations of the tertiary compound I, a 50 per cent reduction in response only occurred when over 90 per cent of AChE was inhibited. This is in agreement with other workers who have used lipid soluble anticholinesterases.[10–12] With the quaternary compound II however, 1 μM will produce 76 per cent, 2 μM 70 per cent and 4 μM 61 per cent inhibition of diaphragm AChE in the times taken by these concentrations to produce a 50 per cent reduction in the tetanic response. Thus there is no fixed fraction of total AChE that must be inhibited for a given reduction of tetanic response; the higher the concentration of quaternary inhibitor applied, the less AChE inhibition there is at the time the tetanic response is reduced by 50 per cent.

The conclusion to be drawn from these and previous experiments[1] is that the *in vivo* and *in vitro* accessibility of AChE is not identical and that the use of phrenic nerve–diaphragm preparation in studies on the site and mode of action of drugs[13] may not give evidence directly applicable to the *in vivo* situation.

Acknowledgement—I wish to thank Professor F. Hobbiger and Dr. P. F. Heffron for their interest in this work and advice.

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