Inhibition of Acetylcholinesterase by Derivatives of 1,3,2-Dioxaphosphorinane 2-Oxide

By D. B. COULT

Chemical Defence Establishment, Porton Down, Salisbury, Wilts. SP4 0JQ, U.K.

(Received 1 March 1976)

Cholinesterases are inhibited by 2-fluoro-1,3,2-dioxaphosphorinane 2-oxide by a mechanism that involves a slow association step followed by a very slow phosphorylation step. No phosphorylation step was observed for the interaction between acetylcholinesterase and 2-S-[2'-(*NN*-diethylamino)ethyl]thio-1,3,2-dioxaphosphorinane 2-oxide.

Some 1,3,2-dioxaphosphorinane 2-oxide derivatives with the structural formulae I(a), I(b), I(c) and I(d) are poor anti-cholinesterases compared with their acyclic analogues (Fukuto & Metcalf, 1965; Ashani *et al.*, 1972; Amitai *et al.*, 1975). The suggestion (Ashani *et al.*, 1972) that the low anti-cholinesterase activity of compounds I(a), I(b) and I(c) was a consequence of rapid dephosphorylation ($t_{\frac{1}{2}} = 12$ min) of phosphoryl-acetylcholinesterase prompted other studies with compound I(d) and similar compounds (Amitai *et al.*, 1975), where it was assumed that the phosphorylated enzyme would rapidly dephosphorylate. Compound I(d) and similar compounds were shown to have mammalian toxicities 10^{-3} – 10^{-4} times that of their acyclic analogues (Amitai *et al.*, 1975).

Complete data for the compound I(d)-acetylcholinesterase interaction were not reported, however, and it was therefore considered to be of some interest to examine this interaction in detail and also at the same time to re-investigate the enzyme interactions of compounds I(a)–I(c), since the quoted half-lives for spontaneous dephosphorylation are the lowest yet reported (cf. values quoted by Aldridge & Reiner, 1972).

Experimental

Materials

Bovine erythrocyte acetylcholinesterase (EC 3.1.1.7; 2.6 units/mg), electric-eel acetylcholinesterase (EC 3.1.1.7; 200 units/mg) and horse serum cholinesterase (EC 3.1.1.8; 15.7 units/mg) from Sigma (London) Chemical Co., London S.W.6, U.K., were used. The 1,3,2-dioxaphosphorinane 2-oxides were synthesized as outlined below; all other chemicals (A.R. grade) were from BDH, Poole, Dorset, U.K.

Methods

(a) *Inhibition of enzymes in the presence of substrate.* The enzyme activity (cholinesterase or acetylcholinesterase) was determined titrimetrically by using an automatic titrimer (Radiometer, Copenhagen,

Denmark) fitted with a twin syringe attachment, with one syringe containing 10 mM-NaOH and the other 10 mM-acetylcholine iodide. Enzyme (0.5 unit) and substrate (0.1–1 mM) were incubated under N_2 at either 25° or 37°C in a total volume of 10 ml of NaCl solution (I 0.15, pH 7.4). The rate of acid production was monitored for 2 min before the addition of a known volume of an aqueous solution of the inhibitor. The ensuing rate of acid production, which was proportional to the concentration of free enzyme, was monitored either until a steady-state of acid production was reached, or until the acid production ceased.

(b) *Inhibition of acetylcholinesterase in the absence of substrate.* Acetylcholinesterase (5 units) was incubated with a range of concentrations of inhibitor in 1 ml of 50 mM-Tris/HCl, pH 7.4 (made up to I 0.15 with NaCl), at either 25° or 37°C. Samples were removed after various time-intervals and were used for the assay of free enzyme, either titrimetrically, as above with a substrate concentration of 1 mM, or colorimetrically with acetylthiocholine (1 mM) as the substrate (Ellman *et al.*, 1961). In the latter procedure the increase in E_{410} was recorded continuously with a Perkin-Elmer 402 spectrophotometer in conjunction with an external recorder, and compared with that obtained with samples containing either no enzyme or no inhibitor.

(c) *Preparation of 1,3,2-dioxaphosphorinane 2-oxides.* The 2-fluoro compound was prepared from *NN*-dimethylphosphoramidic difluoride and propane-1,3-diol (Amos *et al.*, 1975), and the 2-S-[2-(*NN*-diethylamino)ethyl]thiolate was prepared from 2-(*NN*-diethylamino)ethanethiol and 2-chloro-1,3,2-dioxaphosphorinane 2-oxide (Fukuto & Metcalf, 1965). Stock solutions of the inhibitors in ethanol were diluted with water before use.

Results

The reaction shown in Scheme 1 and the equations (1)–(4), based on those derived by Aldridge & Reiner (1972), were used for the analysis of the results.

$$k_{\text{obs.}} = k_x = \frac{1}{t} \ln \left(\frac{[E_0]}{[E_0] - [E_t]} \right) \quad (1)$$

$$k_{\text{obs.}} = k_{+1} + k_{-1} = \frac{1}{t} \ln \left(\frac{[E_0] - [E_e]}{[E_0] - [E_t]} \right) \quad (2a)$$

$$\frac{k_{-1}}{k_{+1}} = \frac{[E_0]}{([E_0] - [E_e])} \quad (2b)$$

$$K_1 = \frac{[E_e][I]}{[EI']} = \frac{[I]k_{-1}}{k_{+1}} \quad (3)$$

$$\frac{k_x}{[I]} = \frac{k_2}{K_1} \quad (4)$$

Where $[E_0]$, $[E_t]$ and $[E_e]$ are the enzyme concentrations at time 0, at time t and at equilibrium (steady state).

At 37°C, in the presence of 1 mM substrate, the rate of loss of enzyme activity due to inhibition by the 2-fluoro derivative was exponential, so the first-order rate equation (1) was applied. However, at lower and zero substrate concentrations the rate became biphasic. The rates of the two phases were sufficiently different (approximately by an order of magnitude) to allow the use of eqns. (2a) and (2b), for two opposing first-order rates reaching equilibrium, to the first phase, and to apply eqn. (1) to the second phase. A biphasic rate of loss of enzyme activity was also observed when acetylcholinesterase was inhibited at 25°C, in either the presence or the absence of the substrate. The eqns. (1), (2a) and (2b) were used as before. The calculated rate coefficients, indicating a slowly attained equilibrium followed by a slow phosphorylation step, are shown in Table 1.

Reversible inhibition, with an overall K_1 of 0.37 μM , was observed when acetylcholinesterase was inhibited by the thiolate (Id). No measurable irreversible inhibition was obtained after 6 h incubation of acetylcholinesterase with 0.93 mM-thiolate in the absence of substrate. A plot of v against $v/[S]$ indicates an effect on

K_m and V_{max} , that is a mixture of competitive and non-competitive inhibition.

Discussion

The low anti-cholinesterase activities of the 1,3,2-dioxaphosphorinane 2-oxides (Ia, Ib, Ic) (Fukuto & Metcalf, 1965; Ashani *et al.*, 1972) are consistent with a mechanism of cholinesterase inhibition that involves a slowly reached equilibrium step followed by a slow phosphorylation step. The explanation that the low anti-cholinesterase activity is a consequence of rapid enzyme dephosphorylation is probably erroneous, and was put forward because Ashani *et al.* (1972) measured the rate of dissociation of the

(Ia) X = F

(Ib) X = Cl

(Id) X = $\text{SCH}_2\text{CH}_2\text{N}(\text{C}_2\text{H}_5)_2$

Scheme 1.

Table 1. Inhibition of cholinesterases at pH 7.4 by 2-fluoro-1,3,2-dioxaphosphorinane 2-oxide (Ia)

The experimental conditions for the inhibition of the enzyme and the assay of free enzyme concentration were as described in the text. The kinetic parameters were determined by using the reaction shown in Scheme 1 and eqns. (1)–(4).

Temp. (°C)	$10^4 \times [S]$ (M)	$10^{-3} \times k_{+1}/[I]$ ($\text{M}^{-1} \cdot \text{min}^{-1}$)	$10^2 \times k_{-1}$ (min^{-1})	$10^2 \times k_2$ (min^{-1})	$10^{-3} \times k_x/[I]$ ($\text{M}^{-1} \cdot \text{min}^{-1}$)	$10^6 \times K_1$ (M)	Enzyme source
37	10	—	—	—	0.57	—	Eel
37	10	—	—	—	0.87	—	Eel
37	10	—	—	—	1.46	—	Bovine erythrocyte
37	10	—	—	—	0.85	—	Eel
37	8	—	—	—	0.87	—	Eel
37	6	—	—	—	0.80	—	Eel
37	4	—	—	—	—	—	Eel
37	2	3.3	2.5	—	—	7.5	Eel
37	0	3.7	4.4	2.3	1.9*	12	Eel
25	10	2.5	3.5	3.7	2.6*	14	Eel
25	0	2.8	4.0	—	—	14	Eel
25	10	7.1	1.0	—	—	1.4	Bovine erythrocyte
25	10	8.1	0.65	—	—	0.8	Horse serum

* Values calculated from k_{+2}/K_1 (see eqn. 4).

enzyme-inhibitor complex and not the rate of spontaneous hydrolysis [cf. 0.05 min^{-1} at pH 7.0 and 25°C for spontaneous hydrolysis (Ashani *et al.*, 1972) with 0.04 min^{-1} at pH 7.4 and 25°C for the dissociation of the enzyme-inhibitor complex (Table 1)]. This error results from the assumption that free enzyme plus enzyme-inhibitor complex would be measured by the assay of a diluted enzyme, but this assumption is only valid when the enzyme-inhibitor complex dissociates rapidly. The dissociation has been shown to be slow for 2-fluoro-1,3,2-dioxaphosphorinane 2-oxide and acetylcholinesterase (Table 1). Also, when the spontaneous reactivation of inhibited enzyme was determined, only 90–94% of the enzyme activity was recovered, and this recovery decreased when the enzyme was treated with high concentrations of inhibitor for relatively long periods (Ashani *et al.*, 1972). The slow phosphorylation (k_2 , Table 1) to a stable phosphorylated enzyme could account for this low recovery of enzyme activity.

The observation that the thiolate (Id) is a reversible inhibitor of acetylcholinesterase is consistent with the presently proposed mechanism of inhibition. The thiolate leaving group more closely resembles the substrate for acetylcholinesterase and this allows the equilibrium K_i to be rapidly attained; the thiolate would be expected to be chemically less labile than the fluoridate, by analogy with acyclic analogues (Larsson, 1958), and if a phosphorylation step occurs it is too slow to measure; thus reversible inhibition is observed. The reversible inhibition of acetylcholinesterase by the thiolate would explain the low mammalian toxicity reported for this thiolate and related compounds by Amitai *et al.* (1975). Amitai *et al.* (1975) also reported bimolecular rate constants between 10^3 and $10^4 \text{ M}^{-1} \cdot \text{min}^{-1}$ for the inhibition of electric-eel acetylcholinesterase by this thiolate and related compounds, but presented insufficient details of the treatment of their results for any explanation to be offered for the discrepancy between their results and the findings presented in the present paper.

It is important to emphasize that, if the present studies (i) had only involved experiments carried out

at 37°C in the presence of substrate, an apparent first-order rate of inhibition would have been observed, or (ii) had only involved investigations of inhibition at 25°C in the absence of substrate, an equilibrium would have been measured and possibly interpreted as indicating that phosphorylation of the enzyme succeeded by a dephosphorylation had occurred. However, since the inhibitions were carried out at 37° and 25°C , in both the presence and the absence of substrate, the results not only offered a novel explanation of the low anti-cholinesterase activity and toxicities of the 1,3,2-dioxaphosphorinane 2-oxides, but also point out possible pitfalls in the study of the inhibition of cholinesterases by organophosphorus compounds. For example, it is reasonable to question the mechanism of interaction between acetylcholinesterase and other organophosphorus compounds, in particular the *OO'*-bis-(2-chloroethyl)-, the *OO'*-bis-(2-chloropropyl)- and the *OO'*-bis-(3-chloropropyl)-phosphoryl compounds reported to result in inhibited enzymes with half-lives of dephosphorylation varying from 19 to 36 min (Lee, 1964; Pickering & Malone, 1967).

The 1,3,2-dioxaphosphorinane 2-oxides were prepared by Mr. R. L. Rickard.

References

- Aldridge, W. N. & Reiner, E. (1972) *Enzyme Inhibitors as Substrates*, pp. 55–61, North-Holland, Amsterdam and London
- Amitai, G., Ashani, Y., Grunfeld, Y., Kalir, A. & Cohen, S. (1975) *Abstr. IUPAC Conference 25th, Jerusalem*, p. 240
- Amos, J., Chittenden, R. A., Cooper, G. H. & Rickard, R. L. (1975) *Phosphorus* 6, 35–42
- Ashani, Y., Snyder, S. L. & Wilson, I. B. (1972) *Biochemistry* 11, 3518–3523
- Ellman, G. L., Courtney, K. D., Andres, V. & Featherstone, R. M. (1961) *Biochem. Pharmacol.* 7, 88–95
- Fukuto, R. & Metcalf, R. L. (1965) *J. Med. Chem.* 8, 759–762
- Larsson, L. (1958) *Sven. Kem. Tidskr.* 70, 405–427
- Lee, R. M. (1964) *Biochem. Pharmacol.* 13, 1551–1568
- Pickering, W. R. & Malone, J. C. (1967) *Biochem. Pharmacol.* 16, 1183–1194