INHIBITION OF PLASMA CHOLINESTERASE BY *O*-ALKYLFLUOROPHOSPHONATES

Jiri CABAL, Jiri KASSA and Jiri PATOCKA

Department of Toxicology, Military Medical Academy, 500 01 Hradec Kralove, Czech Republic

Received June 10, 1996
Accepted November 19, 1996

Inhibition of plasma cholinesterase by three methylfluorophosphonates (MFF), sarin, soman and cyclosin, and by the products of their hydrolysis and alcoholysis was examined. Inhibition by phosphonic acids and by methyl esters derived from MFF was purely competitive while that by MFF was irreversible. The rate of phosphorylation of cholinesterase by MFF differs, depending on the structure of the alkoxy group in the MFF and decreases in the sequence soman–sarin–cyclosin. The affinity values of MFF, phosphonic acids and methyl esters of phosphonic acid for cholinesterase are comparable. The *in vitro* kinetic parameters suggest that plasma cholinesterase might act as a natural detoxicating agent in cases of poisoning with the above inhibitors of acetylcholinesterase.
Key words: Sarin; Soman; Acetylcholinesterase; Inhibitors.

Some *O*-alkylmethylfluorophosphonates belong to effective inhibitors of cholinesterases. The inhibition of acetylcholinesterase which takes part in the transmission of nerve impulses in living organisms may very rapidly lead to a cessation of viral functions of the body. However, the amount of organophosphate that brings about this effect is higher by several orders of magnitude than the amount sufficient for completely inactivating all the acetylcholinesterase present in the organism[1]. This difference is mainly due to the reactions of organophosphates with other enzymes, particularly of the hydrolase group. These enzymes are either irreversibly inhibited (*e.g.*, serine hydrolases) or they catalyze detoxication of organophosphates (*e.g.*, diisopropylfluorophosphate hydrolase, sarinase, and other alkylphosphofluoridases, as well as alkaline phosphatases). Plasma cholinesterase (EC 3.1.1.8; butyrylcholinesterase, pseudocholinesterase), the function of which in the organism is not completely understood[2], belongs to the group of serine hydrolases. Its inhibition and even an inborn deficiency are not lethal. In view of the fact that this enzyme is not associated with any particular tissue or organ and that it is known that reactions of organophosphates with serine hydrolases are generally much faster than their enzymatically catalyzed hydrolysis[3], the enzyme might play an important role in the detoxication of the organism after exposure to organophosphates.

The idea was examined by using three highly toxic organophosphates, *viz*. sarin (*O*-isopropylmethylfluorophosphonate), soman (*O*-1,2,2-trimethylpropylmethylfluorophosphonate) and cyclosin (*O*-cyclohexylmethylfluorophosphonate), as well as some products of their degradation.

EXPERIMENTAL

Kinetic Studies

The measurements were done in a Specord M42 (Zeiss, Jena) spectrophotometer, using cuvettes of 1 cm thickness at 412 nm and 25 °C. The reaction of the enzyme with the inhibitor was started by injecting the solutions of plasma cholinesterase, Tris buffer (pH 7.6), butyrylthiocholine iodide and Ellman's reagent, *i.e.* 5,5′-dithiobis(2-nitrobenzoic acid), into the cuvette with inhibitor solution. The increase in absorbance with time was then monitored. Cholinesterase hydrolyzes butyrylthiocholine iodide, giving rise to butyric acid and thiocholine iodide. The released thiocholine iodide splits the S–S bond in Ellman's reagent[4]. The reaction products are yellow and display an absorption maximum at 412 nm. The starting concentrations of the reagents after injection were as follows: plasma cholinesterase 0.021 mg/ml (freeze-dried concentrate of horse plasma cholinesterase, USOL, Prague), Tris buffer 30 mM (Lachema, Brno), butyrylthiocholine iodide 10 mM (Lachema, Brno), Ellman's reagent 50 μM (Merck, Darmstadt). The inhibitor concentrations used were ten times higher than the concentration required to fully block all of the enzyme.

The rates of reaction v_i for inhibitors of the type of *O*-alkylmethylphosphonic acids and *O*-alkyl-*O*-methylmethylphosphonates were calculated from the slopes of time dependence of absorbance. The inhibition rate constants k_i for MFF were calculated from the curves of time dependence of absorbance using a PC iteration program[5] according to:

$$A_t = A_{lim}[1 - \exp(-k_i t)] , \qquad (1)$$

where A_t is the absorbance at time t and A_{lim} is the limiting (maximum) absorbance at infinite time.

The dissociation constants K_d of the enzyme–inhibitor complexes were determined again with the PC iteration program[5] using the following expressions:

$$v_i = Vc_s/[c_s + K_m (1 + c_i/K_d)] \qquad (2)$$

for competitive inhibition and

$$k_i = k_{lim}c_i/[c_i + K_d (1 + c_s/K_m)] \qquad (3)$$

for irreversible inhibition, where v_i is the rate of the inhibited reaction, V the limiting rate of the noninhibited reaction, c_s the molar concentration of substrate, K_m the Michaelis constant, c_i the molar concentration of the inhibitor, K_d the dissociation constant of the enzyme–inhibitor complex, k_i the rate constant of phosphorylation, k_{lim} the limiting (maximum) rate constant of phosphorylation. The K_m value for butyrylthiocholine iodide and plasma cholinesterase was found to be 0.58 mM.

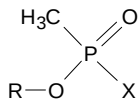
Inhibitors Used

Methylfluorophosphonates, *viz*. sarin (**1a**), soman (**2a**) and cyclosin (**3a**) were prepared at VOZ 072 in Zemianske Kostolany. The inhibitor content was checked in every case – in no case it was lower

than 97%. The remaining 3% consisted of the hydrolysis products, *i.e.* phosphonic and hydrofluoric acids.

Phosphonic acids derived from methylfluorophosphonates (MFF, **1b**, **2b**, **3b**) were prepared *in situ* by hydrolysis of 10 mM solutions of MFF in a pH-stat at pH 10 and at 25 °C. The titrating agent in the pH-stat was 0.5 M NaOH. Hydrolysis was monitored in the pH-stat for the duration of ten half-lives. The consumption of the titrating agent corresponded to about one equivalent of MFF. After the reaction was terminated the pH of the phosphonic acid solution was set to 7.6 by adding 0.1 M HCl.

The methyl esters derived from MFF (**1c**, **2c**, **3c**) were prepared in a reaction of MFF with sodium methanolate in anhydrous methanol at room temperature. Fifty ml of anhydrous methanol was combined with metallic sodium in an amount corresponding to 50 ml 15 mM sodium methanolate. To this solution 1 ml of 0.5 M MFF in methanol was added dropwise. After 3 h the methanol solution was diluted 1 : 1 with water and placed in the pH-stat. The pH was set to 7.6 by adding 0.1 M HCl. The purity of the product after alcohololysis was checked for all MFF by thin-layer chromatography on Silufol using hexane–acetone–ethyl acetate (8 : 3 : 9) and detected with ammonium molybdate after hydrolysis.



	R	X
1a	2-propyl	F
1b	2-propyl	OH
1c	2-propyl	OCH_3
2a	1,2,2-trimethylpropyl	F
2b	1,2,2-trimethylpropyl	OH
2c	1,2,2-trimethylpropyl	OCH_3
3a	cyclohexyl	F
3b	cyclohexyl	OH
3c	cyclohexyl	OCH_3

RESULTS AND DISCUSSION

The basic kinetic parameters of reactions of plasma cholinesterase with organophosphates are shown in Table I. The binding of the inhibitor to the enzyme is characterized by a dissociation constant K_d of the enzyme–inhibitor complex. The calculated values show that plasma cholinesterase binds rather nonselectively all the tested compounds with the exception of alcohols. Insignificant differences in the binding of the organophosphates, of their methyl esters and of phosphonic acids are in agreement with the assumptions of Adams and Whithaker[6] who predicted on the basis of their experiments that, in contrast with acetylcholinesterase, the plasma cholinesterase does not contain any anionic residues in its binding site. Otherwise the binding of phosphonic acids which, at physiological pH values, are anionic would be substantially weaker. Plasma choli-

nesterase, like acetylcholinesterase, can bind lipophilic substances in its hydrophobic regions. If one takes the lipophilicity of a substance to be related to its distribution coefficient between oil and water, changes of this property should be reflected in different affinity of the inhibitor for the enzyme. Rosenthal *et al.*[7] found the following distribution coefficients for a system benzene–water: sarin 2.8, soman 41 and cyclosin 42. However, these differences were not reflected in the values of the dissociation constant. Apparently then in this case the hydrophobic region of these substances does not play a significant role. This is supported by an experiment where it was attempted to inhibit the enzyme by alcohols originating during hydrolysis of organophosphates after their covalent binding to the active site of the enzyme. These alcohols which form in fact the basis of the lipophilicity of the organophosphates studied displayed a negligible inhibitory power. Inhibition could only be observed at concentrations that may have caused a disturbance of the hydrolytic function of the enzyme due to changes in environmental conditions[8] rather than through binding to the enzyme.

It followed from the kinetic behavior of the inhibitors that phosphonic acids and their methyl esters are reversible inhibitors of cholinesterase. In their case the degree of inhibition could be affected by substrate concentration. The character of the reversible inhibition was tested according to Kotyk and Horak[9]. In Dixon's plot all the esters and

TABLE I
Dissociation constants K_d (mol l^{-1}) of enzyme–inhibitor complexes, the limiting rate constants of phosphorylation k_i (s^{-1}) and second-order rate constants of phosphorylation k_2 (mol^{-1} l s^{-1}) of reactions of plasma cholinesterase with *O*-alkylmethylfluorophosphonates and their products of hydrolysis

Inhibitor	$K_d \cdot 10^5$	k_i	$k_2 \cdot 10^7$
1a	10.0	3.60	72.0
1b	10.0		
1c	8.9		
Isopropanol	>1		
2a	8.5	11.80	240.0
2b	8.5		
2c	7.1		
3,3-Dimethyl-2-butanol	>1		
3a	3.1	0.17	3.4
3b	7.6		
3c	6.3		
Cyclohexanol	>1		

phosphonic acids yielded a linear dependence. The binding coefficients α were found to lie between 500 and 20 000, the rate constants β lay between 0.0005 and 0.03. The high values of α and low values of β might underlie the assumed purely competitive inhibition. This was supported in a Lineweaver–Burk plot where the straight lines for different concentrations of a given inhibitor intersected at a point on the *y*-axis (reciprocal of reaction rate).

For the MFF themselves the substrate concentration only affected the rate at which the otherwise constant degree of inhibition was achieved.

In general it may be stated that the binding of organophosphates to plasma cholinesterase is apparently most affected by the overall shape of the inhibitor molecule and much less by its various properties, such as lipophilicity or polarity.

The rates of phosphorylation k_i of the active site of the enzyme when a covalent bond is formed between the enzyme and the inhibitor differ, in contrast with the dissociation constants K_d, in the sequence soman–sarin–cyclosin always by an order of magnitude. These differences in the rate of reaction can be attributed to the shape of the *O*-alkoxy group of the organophosphates since this is the only property in which the three inhibitors differ. The lipophilicity apparently plays no role as indicated by the K_d values. The shape of the alkoxy group apparently determines the orientation of the organophosphate molecule with respect to the active site of plasma cholinesterase and hence also the ease with which the phosphorylation reaction will proceed. The highest rate of phosphorylation of plasma cholinesterase by soman supports this assumption since the alkoxy part of the molecule is structurally isomeric with the choline moiety of the native substrate of the enzyme. The effect of the alkoxy group on the electron density distribution in the organophosphate molecule and the consequent readiness to undergo nucleophilic substitutions is probably insignificant. If these compounds react with a free hydroxyl in aqueous solution the rates of hydrolysis do not differ appreciably[10].

The *in vitro* experiments reported here indicate that these inhibitors which only differ in their alkoxy group do not exhibit marked differences in their affinity for the enzyme but they do differ in the rate at which they inhibit the enzyme. The kinetic parameters of the reactions established here indicate that plasma cholinesterase can act *in vivo* as an important detoxicating factor for this group of organophosphates, as had been found in the case of cocaine[11].

REFERENCES

1. Myers D. K.: Biochem. J. *51*, 303 (1952).
2. Hoff L., Losse H.: Dtsch. Med. Wochenschr. *80*, 529 (1955).
3. Ornst F.: *Ph.D. Thesis*. Military Academy, Brno 1966.
4. Ellman G. L.: Arch. Biochem. Biophys. *82*, 70 (1959).
5. *Programmes for Scientific and Technical Calculations.* Dept. Appl. Mathematics, Agricultural Cooperative Slusovice, Slusovice 1984.
6. Adams D. H., Whithaker V. P.: Biochim. Biophys. Acta *4*, 543 (1950).

7. Rosenthal R. W., Proper R., Epstein J.: J. Phys. Chem. *60*, 1596 (1956).
8. Patocka J.: Sb. Ved. Pr. Lek. Fak. Karlovy Univ. Hradci Kralove *13*, 351 (1970).
9. Kotyk A., Horak J.: *Enzymova kinetika*, p. 124. Academia, Praha 1977.
10. Cabal J.: Collect. Czech. Chem. Commun. *60*, 1162 (1995).
11. Mattes C., Bradley R., Slaughter E., Browne S.: Pharm. Lett. *58*, 257 (1996).