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REACTIONS OF USUAL AND ATYPICAL HUMAN SERUM CHOLINESTERASE PHENOTYPES WITH PROGRESSIVE AND REVERSIBLE INHIBITORS

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The reaction of usual (U) and atypical (A) cholinesterase phenotypes was studied with six organophosphorus compounds, two pyridinium oximes (HI-6 and PAM-2) and with 4-4'-bipyridine (4,4'-BP). No difference in the inhibition rate constants for the two phenotypes was found with the progressive inhibitors tabun, sarin, paraoxon and soman. The other two progressive inhibitors, VX and the positively charged phosphostigmine, inhibited the U phenotype more strongly than the A phenotype.

The positively charged reversible inhibitor HI-6 showed a higher affinity for the U than for the A phenotype, while PAM-2 and the non-charged 4,4'-BP did not show a significant difference in their affinity towards the two enzymes.

Both phenotypes phosphylated by VX or sarin were reactivatable by HI-6 and PAM-2, and the A phenotype was always reactivated more slowly than the U phenotype. The paraoxon-inhibited phenotypes were reactivated at equal rates with PAM-2 but were not reactivated with HI-6. The phosphylated phenotypes did not reactivate spontaneously during one hour.

The effect of reversible inhibitors upon the rate of phosphylation (protection) was tested with HI-6 (for inhibition by soman, tabun and paraoxon) and with 4,4'-BP (for inhibition by soman). By applying the concentrations of the protectors equal to their enzyme/inhibitor dissociation constants, a better protection of the U than of the A phenotype was achieved by HI-6, but equal protection was given by 4,4'-BP.

KEY WORDS: Cholinesterase phenotypes, organophosphorus compounds, pyridine inhibition, reversible inhibition, protection, reactivation.

INTRODUCTION

The physiological function of human serum cholinesterase (EC 3.1.1.8) is still unknown, but the measurement of its activity is widely used as an indicator for the degree of exposure to anticholinesterases and for following the recovery during the antidotal treatment. In comparison to the usual enzyme, some genetic variants of serum cholinesterase reveal different properties in their reactions with some compounds.[1-4] The most frequently found cholinesterase variant in persons who display unusual reactions to common clinical drugs is the atypical cholinesterase. The phenotype differs from the usual enzyme by a single substitution at nucleotide 290 which changes

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Asp-70 to Gly-70.[2] Numerous substrates and inhibitors of different structure were tested for their interaction with the usual and atypical enzymes, and it was found that the atypical phenotype shows a lower affinity for positively charged compounds.[1-4] Such individual genetic variations may cause different responses in different persons to the same medication or poison.

In the therapy of organophosphorus poisoning oximes serve as antidotes. The most effective are positively charged pyridinium compounds. Literature data on the interaction of various cholinesterase phenotypes with this important group of compounds are lacking. The main objective of the present work was therefore, to examine differences between the usual and atypical phenotypes in their inhibition by organophosphates and organophosphonates, and in reactivation and protection with two clinically used pyridinium oxime antidotes PAM-2 and HI-6. The uncharged non-oxime compound 4,4′-bipyridine was used for comparison. Information was also gained on phosphylation of phenotypes by highly toxic warfare poisons.

MATERIALS AND METHODS

Enzymes

The cholinesterase (EC 3.1.1.8) phenotypes were determined by the dibucaine number, fluoride number and inhibition with Ro 02-0683.[1,3,5] The source of enzyme were usual (U) and atypical (A) native human sera.

Tested Compounds

The structural formulae of organophosphorus inhibitors are given in Table 1. The purity of organophosphorus compounds was >97%. A stock solution of phosphostigmine (10 mM) was prepared in water and of the other inhibitors (2.0 or 4.0 mg/mL) in propylene glycol; all further dilutions were made with water shortly before use. The pyridinium compounds HI-6: [1-(2-hydroxyiminomehtyl-1-pyridino)-3-(4-carbamoyl-1-pyridino)-2-oxapropane dichloride] and PAM-2: [(2-hydroxy-iminomethyl)-1-methyl-pyridinium chloride] were supplied by the Laboratory of Organic Chemistry, Faculty of Science and Mathematics University of Zagreb, Zagreb, Croatia. 4,4′-bipyridine (4,4′-BP) was a commercial product (Riedel-de Haen A.G., D-Seelze-Hannover). The 10 mM stock solutions and all subsequent dilutions of oximes and 4,4′-BP were prepared in water immediately before use.

Enzyme Assays

All assays were performed in 0.1 M phosphate buffer pH 7.4 at 25 °C. The final serum concentration during the activity measurements was 0.7% for the U serum and 1.4% for the A serum. The enzyme activity was measured with acetylthiocholine iodide (ATCh) (final concentration 1.0 mM if not stated otherwise) by the spectrophotometric method of Ellman *et al.*[6] with the thiol reagent 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) (final concentration 0.33 mM). Measurements were performed on a Unicam SP 500 spectrophotometer in cuvettes (1.0 cm) at 412 nm over a 2 min period.

Progressive Inhibition

100 μL of inhibitor was added to the assay medium which contained 200 μL of the enzyme-buffer suspension, 100 μL DTNB and 500 μL buffer. After a given time of inhibition (up to 6 min), 100 μL ATCh was added and the enzyme activity was measured.

Reactivation of the Phosphylated Enzyme

Serum was incubated with paraoxon, sarin or VX. The concentrations during incubation were 0.60 μM for paraoxon and 10.0 μM for sarin; for inhibition of phenotypes by VX, equi-inhibitory concentrations were used: 0.80 μM for the U phenotype and 17.0 μM for the A phenotype. At the end of the incubation period (10 min for paraoxon and VX, and 1 min for sarin) approximately 90% of the enzyme activity was inhibited. The reaction mixture was then diluted with buffer (150- and 75-fold for the U and A serum respectively) containing oxime, and at different time intervals (starting 1 min after dilution), 1.0 mL aliquots were withdrawn and the activity was measured for up to 60 min reactivation; during this time phosphylated enzyme did not reactivate spontaneously.

Reversible Inhibition

Reversible inhibition with pyridine compounds was assayed in a reaction medium containing 200 μL enzyme-buffer suspension, 100 μL DTNB, 200 μL reversible inhibitor, 400 μL buffer and 100 μL ATCh. Final concentrations of the reversible inhibitor were 0.50 mM and 1.0 mM for HI-6, 1.0 and 2.0 mM for PAM-2, and 1.0, 2.5 and 5.0 mM for 4,4′-BP. Substrate concentrations ranged from 0.02 to 10.0 mM. Corrections were made for the non-enzymic (oxime-catalysed) substrate hydrolysis.[7]

Progressive Inhibition in the Presence of a Reversible Inhibitor (Protection)

The procedure was the same as for progressive enzyme inhibition, except that the reaction medium contained also 100 μL of the pyridine compound. The concentrations of organophosphorus compounds were: 0.01 μM soman, 2.0 μM tabun and 0.15 μM paraoxon. The time course of inhibition was measured over a period from zero to 5 min.

RESULTS AND DISCUSSION

Progressive enzyme inhibition was measured with two to four diffrerent concentrations of the organophosphorus compound and the second-order rate constants for the inhibition, k_a, given in Table 1 were calculated from equation:

$$\ln (v_o/v_{op}) = k_a.t_i.op \quad (1)$$

where v_o and v_{op} are the enzyme activities without and with the organophosphorus compound present at its concentration op and at the time of inhibition t_i. Our constants obtained for sarin and tabun were lower than those reported in the literature[8,9] and we cannot explain this.

Table 1 The second-order rate constants (k_a) for progressive inhibition of the usual (U) and atypical (A) human serum cholinesterase phenotypes

Inhibitor	$k_a 10^{-5}$/L mol^{-1} min^{-1}		
	U	A	k_a(U)/k_a(A)
Tabun	2.9	2.8	1.0
Sarin	6.5	5.3	1.2
Paraoxon	30	31	0.97
Soman	685	695	0.99
VX	21	1.0	21
Phosphostigmine	380	8.1	47

The values are means of 2–8 experiments.
Tabun: $[(CH_3)_2N](C_2H_5O)P(O)(CN)$; Sarin: $(i\text{-}C_3H_7O)(CH_3)P(O)(F)$; Paraoxon: $(C_2H_5O)_2P(O)$-$[O(C_6H_4\text{-}4\text{-}NO_2)]$; Soman: $[(CH_3)_3C.CH(CH_3)O](CH_3)P(O)(F)$; VX: $(C_2H_5O)(CH_3)P(O)[S(CH_2)_2N(i$-$C_3H_7)_2]$; Phosphostigmine: $(C_2H_5O)_2P(O)[O(C_6H_4)\overset{+}{N}(CH_3)].CH_3SO_4^-$.

With tabun, sarin, paraoxon and soman the inhibition rates were equal for both phenotypes. This agrees with the literature data for the U and A phenotypes in their reaction with other progressive inhibitors.[1-3,10] VX and the positively charged phosphostigmine were less potent progressive inhibitors of the A phenotype than of the U phenotype and the ratios of their rate constants were 47 and 21 respectively.

The reactivation of phospylated phenotypes was studied with paraoxon and sarin, both of which inhibited the phenotypes equally, and with VX, which inhibited the phenotypes differently (Table 1). The second-order reactivation constants, k_r, (Table 2), were calculated from equation (2):

$$\ln[(v_r - v_1)/(v_r - v_t)] = k.t_r = k_r.[\text{oxime}].t_r \quad (2)$$

where v_r is the activity of the non-phosphylated enzyme in the presence of the oxime, v_1 and v_t are activities of the phosphylated enzyme after one and t minutes of reactivation, k is the first-order rate constant of reactivation and t_r is the time of reactivation. The calculation was based on the total reactivator concentration, without distinction between the protonated and the non-protonated oxime.

Table 2 The second-order rate constants for reactivation (k_r) by oximes of the phosphylated usual (U) and atypical (A) cholinesterase phenotypes

OP	Phenotype	HI-6 $10^{-2}.(k_r \pm SD)$ L mol^{-1} min^{-1}	PAM-2 $10^{-2}.(k_r \pm SD)$ L mol^{-1} min^{-1}
Paraoxon	U	0	0.48*
	A	0	0.48*
Sarin	U	2.0±0.3	2.1±0.3
	A	1.2±0.3	1.5±0.4
VX	U	0.80*	1.8±0.4
	A	0.40*	0.57±0.03

Reactivation was performed with 0.25 mM and 0.50 mM oximes. Results marked * were obtained with 0.25 mM oxime only. The results are means of 2–4 experiments.

In all studies reactions the reactivation was not complete; it followed equation (2) up to about 10–20 min, reaching thereafter values (20–90% reactivation) which stayed unchanged until the end of measurement (up to 60 min). The first-order reactivation constants were evaluated from the first, linear part of the reactivation curve. With the exception of the paraoxon-inhibited phenotypes, in all cases the phosphylated U phenotype was reactivated somewhat more easily than the A phenotype.

Reversible inhibition of the phenotypes by HI-6, PAM-2 and 4,4′-BP was measured in the presence of substrate; the degree of enzyme inhibition ranged between 15 and 80%. From the degree of inhibition, the apparent enzyme/inhibitor dissociation constant K_{app} was calculated.[11–14] For all inhibitors studied, K_{app} was a non-linear function of the substrate concentration as reported previously for the same and other compounds.[12–14] The enzyme-inhibitor dissociation constant K_i was obtained by graphical extrapolation of K_{app} to zero substrate concentration (Table 3). For HI-6, the K_i value was slightly lower for the U phenotype than for the A phenotype while K_i values for PAM-2 and 4,4′-BP were similar for both phenotypes. Consequently, no difference in the affinity for the U and A sera was revealed between the charged oximes and the uncharged compound 4,4′-BP (Table 3).

The protective effect of reversible inhibitors in phosphylation of phenotypes was investigated with HI-6 as protector against inhibition by soman, tabun and paraoxon, and with 4,4′-BP to inhibition by soman. The protective effect was tested by measurement of the phosphylation rate in the absence and in the presence of the protector.

Results of protection measurements are presented (Table 3) as a ratio k_a/k'_a which defines the protective effect produced by binding of the reversible inhibitor to the catalytic site[12–14] (equation (3))

$$k_a/k'_a = 1 + i/K_i \tag{3}$$

Table 3 The enzyme inhibitor dissociation constants (K_i) for the usual (U) and atypical (A) cholinesterase phenotypes and protective effect of pyridine compounds in phosphylation of the phenotypes

	Phenotype	
Pyridine compoun	(U)	(A)
Reversible inhibitio	($K_i \pm$SD) 1 mM	
HI-6	0.23 ± 0.02 (4)	0.47 ± 0.16 (4)
PAM-2	0.88 ± 0.14 (2)	1.1 ± 0.2 (2)
4,4′-BP	1.6 ± 0.6 (3)	1.8 ± 0.6 (2)
Protection	k_a/k'_a	
HI-6: Soman	4.2 (4)	2.8 (3)
Tabun	2.5 (3)	1.4 (2)
Paraoxon	2.2 (3)	1.0 (2)
4,4′-BP: Soman	1.9 (3)	1.9 (2)

Protection is expressed a the ratio k_a/k'_a. The number of experiments is given in parentheses. The concentr ions of the reversible inhibitor in protection were: 0.25 mM HI-6 (for U serum) and 50 mM (for A serum) and 1.6 mM for 4,4′-BP (for both phenotypes).

where k_a and k'_a are the second-order rate constants for inhibition in the absence and presence of the reversible inhibitor (protector) *i*. If the concentration of a reversible inhibitor is equal to its dissociation constant of the enzyme/inhibitor complex, the ratio of the rate constants is expected to be 2.0 (equation (3)). The rate of progressive inhibition was reduced in the presence of both protectors in all studied reactions but one, i.e., in the case of inhibition of A phenotype by paraoxon (cf. Table 3). The protection by 4,4′-BP (1.6 mM) was as expected according to the equation (3) and was the same for both phenotypes having the same dissociation constant. With all studied inhibitors, HI-6 protected the U better than the A phenotype. However, the protection with HI-6 in inhibition by soman was higher and protection of A in inhibition by tabun and paraoxon lower than theoretically expected. The reason for these deviations was not further investigated.

The best differential effect between the U and A phenotypes was observed in progressive inhibition, whereas it was much less expressed in reversible inhibition and also in reactivation and protection. The differences in the reactivating and protective effects of HI-6 and PAM-2 between the phenotypes are too small to have some practical impact on their antidotal property in persons with cholinesterase variants. Considering the lower affinity of the A phenotype towards the positively charged compound, persons with this cholinesterase variant are expected to be less sensitive to the inhibition by charged organophosphorus compounds, but slightly more refractant to the therapy with pyridinium antidotes.

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