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SHORT COMMUNICATIONS

Influence of atropine upon reactivation and ageing of rat and human erythrocyte acetylcholinesterase inhibited by soman

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The current therapy of intoxication by the majority of organophosphates is administration of atropine and an oxime. Atropine acts as a blocker at the muscarinic receptor and thus protects the receptor site from acetylcholine accumulating after inhibition of acetylcholinesterase (AChE; acetylcholine hydrolase; EC 3.1.1.7) by the organophosphates. The therapeutic effect of the oximes is based on their ability to reactivate the inhibited AChE. The therapy may be hampered by a transformation of the inhibited enzyme into a non-reactivatible form. This process, called ageing, is based on dealkylation and proceeds rapidly when AChE is inhibited by the nerve agent soman (1,2,2-trimethyl-propyl methylphosphonofluoridate).

The efficacy of reactivation of soman-inhibited AChE by oximes has often been tested in *in vitro* studies [1, 2]. It has been shown that within one species soman-inhibited AChEs from various sources hardly differ with regard to their ability to be reactivated and their tendency to age [3–5]. Therefore, erythrocyte AChE, which is easily accessible, has been used as an attractive enzyme preparation to test the reactivating potencies of oximes. In a recent study, however, Kuhnen *et al.* [6] reported differences between soman-inhibited membrane-bound and solubilized AChE from human erythrocytes with respect to ageing, reactivation by the oxime HI-6 and the influence of atropine upon these processes. These results are disconcerting because reactivation of inhibited solubilized AChE rather than that of membrane-bound AChE from erythrocytes is often used in screening programs to decide whether oximes should be rejected or not for further investigation.

However, as already stated by Kuhnen *et al.* [6], the results of their reactivation and ageing experiments were obtained in an experimental design that was quite different from *in vivo* conditions. The membrane-bound AChE might be more or less similar to its native state, but the conditions (pH, buffer and temperature) used by these authors were quite artificial.

In the present investigation we repeated the study of Kuhnen *et al.* [6] by measurement of the reactivation and ageing under conditions more similar to the *in vivo* situation. In addition to AChE derived from human erythrocytes, AChE obtained from rat erythrocytes was used. Membrane-bound and solubilized AChE were studied with respect to reactivation by HI-6 and ageing in the absence and presence of atropine after inhibition with soman. The results suggest that the conclusions of Kuhnen *et al.* [6] may not be valid under *in vivo* conditions.

Materials and methods

Materials. Rat blood was obtained by aorta puncture under hexobarbital anaesthesia from male Wistar (WAG/Rij) rats with a body weight of 180–200 g, bred in the Medical Biological Laboratory TNO under SPF conditions. The blood was collected in a syringe containing heparin (75 IE/rat). Human blood was obtained from volunteers of the Medical Biological Laboratory TNO by venapuncture and was collected in heparinized tubes. Blood pooled from at least three animals or individuals was used.

Racemic soman was obtained from the Prins Maurits Laboratory TNO and was at least 99% pure (GLC).

The oxime HI-6 [1-(4-aminocarbonylpyridinio)methoxymethyl-2-hydroxyiminomethylpyridinium dichloride] was a gift of Dr P. A. Lockwood, Defence Research Establishment, Suffield, Canada.

All other reagents were commercial products of analytical grade.

Preparation of red cell ghosts and solubilization of AChE. Erythyrocyte ghosts were prepared according to the modified procedure of Dodge *et al.* [7] as described by Hanahan and Ekholm [8] except that sodium phosphate buffers, pH 7.4, were used instead of Tris buffers. Finally, the suspension with ghost-bound (GB)-AChE was diluted with 20 ideal milliosmolar (imOsM) sodium phosphate buffer [7] containing 0.1% sodium azide (final protein concentration for rat ghosts was approx. 6 μg protein/μl and for human ghosts approx. 10 μg protein/μl).

The solubilization of AChE was carried out as described by De Jong and Wolring [9]. The obtained solution with AChE solubilized from ghosts (GS-AChE; final concentration for rat solution was approx. 4 μg protein/μl and for human solution approx. 15 μg protein/μl) was stored in 5 imOsM sodium phosphate buffer, pH 7.4, in the presence of 1% Triton X-100 and 0.1% sodium azide.

GB- and GS-AChE were stored for up to 2 weeks at 4° without significant loss of AChE activity.

Reactivation and ageing. Reactivation and ageing experiments were carried out according to De Jong and Wolring [9] with some minor modifications.

Inhibited rat and human erythrocyte AChE were obtained by incubating the suspensions of GB- and GS-AChE (1500 μg for rat and 500 μg for human) with soman (final concentrations 30 and 10 nM, respectively) in 100 imOsM sodium phosphate buffer, pH 10.0, containing 0.1% (w/v) bovine serum albumin (BSA) for 30 min at room temperature (final vol. 1 ml). The incubation medium with the solubilized enzyme contained also Triton X-100 [final concentration 0.1% (w/v)]. During the incubation with soman the pH was controlled every 5 min and adjusted to pH 10.0 with 100 imOsM trisodium phosphate. Removal of the excess inhibitor was achieved by alkaline hydrolysis, and by extraction of the mixture with either the adsorbent XAD-2 (100 mg/ml) or an equal volume of hexane. The completeness of the removal of the excess soman was checked by the absence of an inhibitory effect of a 10-fold dilution of the inhibited enzyme solution on non-inhibited AChE during 90-min incubation at pH 7.4 and at room temperature.

Ageing and reactivation were determined at room temperature and were started by diluting the inhibited enzyme solutions 10 times with 310 imOsM sodium phosphate buffer, pH 7.4, containing 0.1% (w/v) BSA (buffer I)

without or with added HI-6 (30 μM), respectively (final vol. 1 ml). The incubation medium with the solubilized AChE contained also 0.1% (w/v) Triton X-100. Various concentrations of atropine sulphate were added. In reactivation experiments two samples of 200 μl were taken after 90 min of incubation and diluted 5 times with ice-cold 67 mM sodium phosphate buffer, pH 7.4 (buffer II). To determine the rate of ageing of rat and human erythrocyte AChE, six samples of 190 μl were taken in duplicate during 60 and 5 min, respectively, and added to 10 μl HI-6 (final concentration 30 μM) in buffer I. After 90 min the incubate was diluted 5 times with ice-cold buffer II.

The restored enzyme activity was determined by incubating the 1-ml samples with 100 μl of a solution of 10 mM acetylthiocholine iodide and 2 mM 5,5-dithiobis(2-nitrobenzoic acid) in buffer II for 20 min at 37°, according to the method described by Ellman *et al.* [10]. The reaction was terminated by the addition of 10 μl 3.1 mM eserine in H_2O. Subsequently, the extinction at 412 nm was measured. Enzyme activities were corrected for spontaneous hydrolysis and for oxime-induced hydrolysis of the substrate.

Blanks for the activity of the enzyme, for the activity of the enzyme incubated with oxime and for the activity of the inhibited enzyme were run in a similar manner.

The percentages of reactivation and rate constants of ageing were calculated as described by De Jong and Wolring [9].

Determination of protein. Amounts of protein were determined by the method of Lowry *et al.* [11].

RESULTS

GB- and GS-AChE from rat and human erythrocytes were inhibited completely after the incubation with soman for 30 min at pH 10.0. The concentration of soman used was at about a 2-fold molar excess as was determined in separate experiments by incubating various concentrations of the inhibitor with the enzyme. Hardly any enzyme aged at pH 10.0. This was concluded from the slight decrease of percentages of reactivation of the inhibited enzyme when treated by 30 μM HI-6 30 and 60 min after the start of the inhibition. Incubation of AChE at pH 10.0 for 30 min and treatment of the enzyme solution with XAD-2 or hexane diminished the enzyme activity by 6–10%; this decrease was corrected for.

Results of reactivation experiments are summarized in Table 1. Reactivation data represent percentages of maximal reactivation by 30 μM HI-6. About 50% of the soman-inhibited AChE from rat and human erythrocyte ghosts could be reactivated. Solubilization of the enzyme did not affect the percentages of reactivation, neither did the presence of 0.01–1 mM atropine. In addition, the reactivation by 1 mM HI-6 was studied in a limited number of experiments. GS-AChE from rat could be reactivated completely in 90 min, whereas 76% of GB-AChE from human erythrocytes was restored in 15 min. Again, 1 mM atropine did not affect the reactivation of the human erythrocyte enzyme.

The rate constant of ageing of human erythrocyte AChE (0.24 min^{-1}) was about 10 times higher than the rate constant of ageing of rat erythrocyte AChE (0.021 min^{-1}; see Table 2). The rate constants of GB- and GS-AChE were similar. A high concentration of atropine (1 mM) decreased the ageing rate constant by 50–60% for rat and 28–30% for human enzyme. No effects were found using 0.1 and 0.01 mM atropine.

DISCUSSION

In this study it has been demonstrated that after inhibition with soman, membrane-bound and solubilized AChE from rat or human erythrocyte ghosts behave similarly with respect to reactivation by HI-6 and ageing. The percentage of reactivation and the ageing rate constant of solubilized AChE from both origins are consistent with the values found by De Jong and Wolring [9]. The ageing rate constants of GB- and GS-AChE from human erythrocytes are smaller than those found by Kuhnen *et al.* ([6]; 0.035 and 0.013 min^{-1}, respectively), which is probably due to differences in pH, buffer and temperature [12]. The effects of atropine on the ageing rate constant are consistent with the effects described by Kuhnen *et al.* [6], and may be caused by the allosteric effect of atropine on the enzyme, which has been reported before for quaternary ammonium compounds [13, 14].

In our study atropine did not influence reactivation of human GB-AChE in contrast to the results of Kuhnen *et al.* [6]. In their study, it was found that only 39% of human GB-AChE could be reactivated by 1 mM HI-6 in 15 min, which seems to be extremely low in comparison with our results and results obtained by others [15, 16]. The stimulating effect of atropine on reactivation of GB-AChE found by these authors, may be an artefact due to invalid measures to remove excess soman.

In conclusion, reactivation by 30 μM HI-6 and the rate constant of ageing of soman-inhibited membrane-bound and solubilized AChE from rat or human erythrocyte ghosts were found to be similar. Atropine affects equally the rate constant of ageing of the inhibited enzyme from both preparations.

These results highly suggest that the conclusions of Kuhnen *et al.* [6], namely that soman-inhibited membrane-bound and solubilized AChE from erythrocytes behave differently with regard to reactivation by HI-6 and ageing in absence and presence of atropine, are not valid due to inappropiate experimental conditions.

Table 1. Percentages of maximum reactivation of soman-inhibited membrane-bound (GB-) and solubilized (GS-) AChE from rat and human erythtrocytes by 30 μM HI-6 in the absence and presence of various concentrations of atropine at pH 7.4 and room temperature*

Concn of atropine (mM)	Percentage of reactivation (%)			
	Rat		Human	
	GB-AChE	GS-AChE	GB-AChE	GS-AChE
0	54 ± 3 (5)	58 ± 2 (5)	49 ± 2 (5)	55 ± 3 (4)
0.01	55 ± 2 (6)	53 (1)	49 ± 1 (4)	55 ± 2 (4)
0.1	52 ± 4 (5)	63 ± 5 (4)	50 ± 2 (4)	54 ± 2 (4)
1	51 ± 2 (5)	63 ± 5 (4)	47 ± 1 (4)	53 ± 1 (4)

* All values represent the mean ±SEM; number of experiments is given in parentheses.

Table 2. Rate constant of ageing of soman-inhibited membrane-bound (GB-) and solubilized (GS-) AChE from rat and human erythrocytes in the absence and presence of various concentrations of atropine at pH 7.4 and room temperature*

Concn of atropine (mM)	Rate constant (10^{-3} min^{-1}) Rat GB-AChE	Rat GS-AChE	Human GB-AChE	Human GS-AChE
0	20.5 ± 2.6 (3)	21.5 ± 2.7 (4)	256 ± 17 (3)	231 ± 16 (3)
0.01	16.9 ± 2.4 (3)	21.0 (1)	259 ± 9 (3)	216 ± 20 (3)
0.1	14.9 ± 2.9 (3)	13.9 ± 3.9 (3)	252 ± 14 (3)	236 ± 9 (3)
1	8.0 ± 0.6 (3)†	10.5 ± 3.2 (3)	184 ± 9 (3)†	161 ± 10 (3)†

* All values represent the mean ±SEM: number of experiments is given in parentheses.
† Significantly different from control (no addition); $P < 0.05$ (Student's t-test, two-tailed).

Medical Biological Laboratory TNO
Lange Kleiweg 139
2288 GJ Rijswijk
The Netherlands
**Prins Maurits Laboratory TNO*
Lange Kleiweg 137
2288 GJ Rijswijk
The Netherlands

CORNELIA J. VAN DONGEN,
RONALD M. ELSKAMP
LEO P. A. DE JONG*

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Acute effects of oltipraz on adult *Schistosoma mansoni* and its antagonism *in vitro*

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Oltipraz is an effective antischistosomal compound whose mode of action is yet to be determined. Bueding *et al.* [1] observed a depression of parasite glutathione (GSH) content as one of the earliest biochemical changes after administration of oltipraz *in vivo*. To further define the relationship between the antischistosomal activity of oltipraz and parasite GSH levels, the acute effects of oltipraz on various biochemical and physiological parameters of *Schistosoma mansoni in vivo* and *in vitro* are presented.

Methods

Parasite preparation and incubation media. Adult *Schistosoma mansoni*, 45–55 days post-infection, were dissected from Swiss Webster mice by the method of Fetterer *et al.* [2]. Where male parasites were used, worms were dissected into media containing 0.05% sodium pentobarbital, mechanically separated and the males placed into fresh media and maintained at 37° until assay.

For *in vivo* experiments, mice were dosed by gavage at either 125 mg/kg or 250 mg/kg oltipraz in peanut oil and killed at the specified times after dosing.

Glutathione assay. After incubation, worms were filtered, weighed and homogenized (1:10, w/v) in 6% trichloroacetic acid (TCA). Aliquots were removed for protein determination by the method of Albro [3] or centrifuged at 12,000 *g* for 10 min and then derivatized and analyzed for GSH by HPLC as described by Reed *et al.* [4].

Characterization of ^{35}S-labeled metabolites. Male schistosomes were transferred to wells (multi-well tissue culture plates, Flow Laboratories, Inc., McLean, VA) containing