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Effects of oximes on rate of decarbamylation of human red blood cell AChE measured with two different methods

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ABSTRACT

Treatment regimen of poisonings by organophosphorus (OP) compounds usually includes oxime therapy. The treatment options in soman poisoning are very limited due to rapid aging of the inhibited acetylcholinesterase (AChE), when the enzyme species is considered as irreversibly inhibited and resistant towards reactivation by oximes. Hence, oxime treatment probably comes too late in realistic scenarios. As an alternative, protecting part of the enzyme by reversible inhibition prior to soman exposure has been proposed. One means of protecting against soman poisoning is the prophylactic use of certain reversible inhibitors (carbamates) of AChE. The question whether there is a possibility of an interaction between pre-treating carbamates and oximes at AChE arises.

Therefore we studied the effects of the oximes obidoxime, HI 6 and MMB-4 on the rate of decarbamylation for physostigmine- and pyridostigmine-inhibited human erythrocyte AChE both in a dynamically working in vitro model and a static cuvette system.

Our results show that HI 6 increased the rate of decarbamylation for both physostigmine- and pyridostigmine-inhibited enzyme in both systems, the observed effect by HI 6 increasing with higher doses. Obidoxime had a slightly accelerating effect on the pyridostigmine-inhibited enzyme. MMB-4 applied to pyridostigmine-inhibited AChE in the static system only showed no difference to the experiments made in absence of oxime. No oxime showed a tendency to retard the rate of decarbamylation.

1. Introduction

Recently we introduced a dynamically working in vitro perfusion model for real-time determination of acetylcholinesterase (AChE) activity [1] and tested the effectiveness of carbamate pre-treatment against soman. In case of soman the inhibited enzyme rapidly becomes resistant to oxime reactivation due to spontaneous dealkylation (aging) of the alkoxy group. Our results showed that residual activity upon soman challenge was increased and a certain degree of protection could be achieved by the pre-treatment [2]. Correspondingly, in vivo data demonstrate that pre-treatment with carbamates or other reversibly blocking agents followed by atropine therapy is able to achieve significant protection against

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Abbreviations: AChE, acetylcholinesterase (E.C.3.1.1.7); AU, absorbance units; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); HI 6, 1-[[[4-(aminocarbonyl)-pyridinio]methoxy]methyl]-2-[(hydroxyimino)methyl]pyridinium dichloride; MMB-4, *N*,*N*′-monomethylenebis(4-pyridiniumaldoxime)dibromide; Obi(obidoxime), 1,1′-(oxybis-methylene)bis[4-(hydroxyimino)methyl]pyridinium dichloride; Pyr, pyridostigmine.

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soman. The effectiveness of such a pre-treatment/therapy regimen can be further increased against certain organophosphorus (OP) compounds by including therapeutic oxime [3]. Since carbamates and oximes may be used in rapid sequence, it is important to elucidate the potential effects of oximes on the carbamylated AChE.

There seems to be controversy concerning the use of oximes in carbamate intoxication, as in some cases toxicity had been reported to be exacerbated [4,5]. In most reports on decarbamylation rates the experimental conditions varied widely with respect to temperature, pH, and enzyme source. Thus erythrocytes or tissues from non-human species such as rat, rabbit, guinea pig, cow or electric eel were used [6–10]. Harris et al. reported that the rate of spontaneous decarbamylation of pyridostigmine-inhibited human erythrocyte AChE could be markedly accelerated in vitro by HI 6 [11]. As it is difficult to extrapolate animal data to humans due to substantial species differences in the pharmacokinetics, dosing of antidotes and reactivating potency of oximes we adopted human erythrocyte AChE for our determination and set the above-mentioned parameters to 37 °C at pH 7.4 simulating physiological conditions as closely as possible. To examine the influence of oximes on carbamate-inhibited enzyme we studied the effects of the oximes obidoxime and HI 6 on the rate of decarbamylation for physostigmine- and pyridostigmine-inhibited human erythrocyte AChE. We chose two different methods of analysis, our recently presented dynamically working perfusion model [1] and the well-established static cuvette system [12]. Following inhibition, excess carbamate was removed by column filtration (necessary for the static method) or discontinuation of the carbamate perfusion (in the dynamic system). Spontaneous reactivation was determined in the absence or presence of the oximes. Rate constants were calculated to assess whether the presence of oximes affects decarbamylation.

2. Materials and methods

Acetylthiocholine iodide, 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB, Ellman's reagent), (−)-physostigmine (eserine hemisulfate) and pyridostigmine bromide were purchased from Sigma (Deisenhofen, Germany), obidoxime dichloride monohydrate from Duphar (Amsterdam, Holland). HI 6 was provided by Dr. Clement (Defence Research Establishment Suffield, Ralston, Alta., Canada) and MMB-4 was made available by Prof. Fusek (Purkyne Military Medical Academy, Hradec Kralove, Czech Republic).

All other chemicals were from Merck Eurolab GmbH (Darmstadt, Germany) at the purest grade available.

Particle filters employed were Millex®-GS, 0.22 μm, Ø33 mm (Millipore; Eschborn, Germany). PD-10 columns filled with Sephadex G 25 (bed volume 9.1 mL, height 5 cm) were from Amersham Pharmacia Biotech, Germany.

2.1. Determination of enzyme activity

2.1.1. Method A: dynamic model

2.1.1.1. General experimental procedure. Experiments were conducted as recently described [1]. In short, human erythrocytes were layered onto a particle filter resulting in a stable enzyme reactor. Maximum AChE activity was determined by continuously perfusing the enzyme reactor with the medium, consisting of acetylthiocholine (0.45 mM), DTNB (0.3 mM), and sodium phosphate buffer (0.1 M, pH 7.4) containing 0.2% (w/v) gelatin. The reactor was immersed into a water-bath at 37 °C. The total flow rate through the enzyme reactor was 0.5 mL/min. The effluent passed a photometer set at 470 nm with the digitized absorbance values being collected at 1.6 s intervals. The perfusion system consisted of two HPLC pumps with integrated quaternary low-pressure gradient formers that were programmed by a computer using commercial HPLC software. As a routine, the enzyme reactor was inserted at $t = 0$ and flushed with buffer for 5 min, followed by a pulse of distilled water ($t = 5$) for 5 min to achieve hemolysis and further flushing with buffer ($t = 10$) for 5 min. At $t = 15$ DTNB and acetylthiocholine were added for the determination of the maximum enzyme activity ($t = 30$).

2.1.1.2. Inhibition of AChE with carbamates. Working solutions of physostigmine and pyridostigmine in gelatin buffer were freshly prepared for each experiment from stock solutions (89 and 222 μM, respectively) kept at −60 °C. These working solutions passed the system at 0.113 mL/min. Starting at maximum enzyme activity ($t = 30$), AChE was inhibited with 250 nM physostigmine or 5 μM pyridostigmine. After reaching steady state conditions the carbamates were discontinued to follow spontaneous decarbamylation.

2.1.1.3. Spontaneous decarbamylation in the presence of oximes. Following steady state conditions (see above) the carbamates were discontinued while oximes were added. Oxime solutions were freshly diluted from frozen stock solutions with phosphate buffer and passed the system at 0.113 mL/min.

2.1.1.4. Determination of the non-enzymatic blank value. After inhibition and reactivation of AChE, the enzyme reactor was replaced by a plain filter without enzyme. The blank values of the complete perfusion medium (acetylthiocholine, DTNB and gelatin buffer) with and without oxime and of buffer only were determined. For calculation of steady state activity the blank values were subtracted from the levels of inhibition (blank of acetylthiocholine, DTNB and gelatin buffer) and of maximum activity before inhibition (blank of acetylthiocholine, DTNB and gelatin buffer) and after reactivation (blank of oxime, acetylthiocholine, DTNB and gelatin buffer).

2.1.1.5. Determination of the decarbamylation rate constant. Direct analysis of the reaction curve following discontinuation of the carbamate allowed the calculation of the rate constants by fitting a monoexponential association function to the data points. An interval was chosen, beginning 20 min after the discontinuation of the carbamate, leaving time for carbamate wash-out and oxime wash-in to come to completion.

2.1.2. Method B: static determination

2.1.2.1. Enzyme assay. AChE activity was measured spectrophotometrically at 436 nm (Cary3Bio, Varian, Darmstadt) with a modified Ellman assay, using the molar absorption coeffi-

cient of 11.3 M^{-1} cm^{-1} [13]. The assay mixture (3.16 mL) contained 0.45 mM acetylthiocholine as substrate and 0.3 mM DTNB as chromogen in 0.1 M phosphate buffer (pH 7.4). Assays were run at 37 °C.

2.1.2.2. Determination of rate constants of spontaneous reactivation. Hemoglobin-free erythrocyte ghosts were prepared as previously described [12]. Carbamylated AChE was prepared by incubating human ghosts with 100 nM physostigmine for 30 min or with 35 μM pyridostigmine for 1 h at 37 °C to obtain an inhibition of about 80–90% of control activity.

To remove free carbamate, the inhibited enzyme solution was filled into a pre-packed PD-10 column containing Sephadex® G-25 M. The gel bed of the column had previously been equilibrated with 25 mL of phosphate buffer. When the sample had run into the column, the inhibited ghosts were eluted with 3.5 mL buffer solution. The first 10 drops of the eluate were discarded, the following 36 drops were pooled for determination of AChE activity. The eluate was then kept at 37 °C throughout the experiment. All further drops were discarded.

Starting at $t = 0$ AChE activity was measured repeatedly within 3–4 h. Aliquots (10 μL) of the eluate were removed after specified time intervals and transferred to a thermostated cuvette containing phosphate buffer and DTNB. For the determination of AChE activity acetylthiocholine was added immediately afterwards and activity measured for 1 min.

For experiments in presence of oximes, obidoxime, HI 6 or MMB-4 were added to the eluate prior to measurement of activity resulting in final oxime concentrations of 30 μM (obidoxime, HI 6) or 100 μM (HI 6, MMB-4).

The rate constant of spontaneous decarbamylation was determined by plotting AChE activity (mAU/min) versus time and fitting a one-phase exponential association function to the data points (nonlinear regression analysis).

2.2. Calculations

Data analysis was performed by curve fitting programs provided by Prism™ Version 3.0 (GraphPad Software, San Diego, CA). The reactivation rate was calculated by fitting a monoexponential association function to the data points. Means are presented along with standard deviation (S.D.) and the number of experiments.

3. Results

3.1. Method A: dynamic model

Inhibition with 250 nM physostigmine or 5 μM pyridostigmine led to about 20 and 12% residual activity at steady state, respectively. As an example of a single experiment Fig. 1 shows the time course of the enzyme activity upon administration of pyridostigmine and obidoxime.

Generally, spontaneous reactivation started upon discontinuation of the carbamate, both in presence or in absence of oximes. The decarbamylation reaction (interval starting 20 min after discontinuation of carbamate) precisely followed a monoexponential function and allowed the establishment of the rate constants. The half-lives for decarbamylation in the presence of 0.45 mM substrate are presented in Table 1.

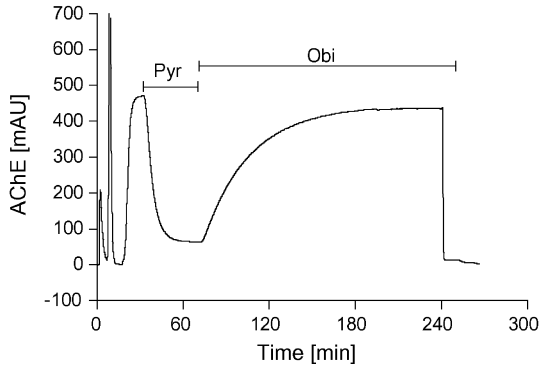

Fig. 1 – Time course of human erythrocyte AChE activity after inhibition with pyridostigmine (Pyr) and spontaneous reactivation in the presence of obidoxime (Obi, 30 μM), as measured with the dynamic system. After attaining the maximal activity, the enzyme was inhibited with pyridostigmine (5 μM) for 40 min. Then pyridostigmine was discontinued and obidoxime (30 μM) was added. After 240 min, the enzyme reactor was replaced by a plain filter and the blank values of the complete perfusion medium and gelatin buffer only were determined. Mean $k_{d,obs}$ ± S.D. was 0.0301 ± 0.0015 min^{-1} ($n = 4$). Note: the figure shows one single experiment only.

3.2. Method B: static determination

Care was taken that remaining carbamate was completely removed from the reaction mixture before AChE activity was measured. The columns used were tested for their ability to separate physostigmine and pyridostigmine from the erythrocyte membranes. For control, carbamate solutions (100 nM physostigmine or 3 μM pyridostigmine) were placed onto PD-10 columns, the eluates were collected in fractions (6 drops each). Every fraction was mixed with native ghosts and activity determined. No AChE inhibition was detected in the first 60 drops. For further experiments the first 10 drops of each eluate were discarded and 36 drops (carbamate-free) were

Table 1 – Half-lives (min) of spontaneous reactivation of human erythrocyte AChE following inhibition with carbamates, in the presence and absence of oximes, as observed in the dynamic model

Oxime	Physostigmine	Pyridostigmine
None	16.4 ± 0.7 (4)	26.9 ± 0.9 (3)
Obi, 30 μM	15.9 ± 0.6 (3)	23.1 ± 1.1 (3)
HI 6, 30 μM	14.8 ± 0.7 (5)	24.0 ± 0.3 (3)
HI 6, 100 μM	11.4 ± 0.3 (2)	22.0 ± 1.2 (2)

Mean ± S.D. (number of experiments in parentheses). Substrate concentration 0.45 mM. The goodness of fit of the individual curves was $R^2 > 0.999$. Previously, 16.9 ± 0.29 (17) and 27.0 ± 0.77 min (9) were found for physostigmine and pyridostigmine, respectively [29].

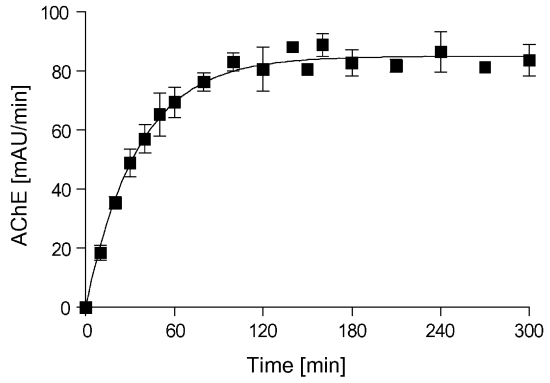

Fig. 2 – Spontaneous reactivation of pyridostigmine-inhibited (3 μM) human ghosts in presence of obidoxime (30 μM), as measured in the static cuvette system. AChE activity was measured repeatedly after spontaneous reactivation started following removal of residual carbamate from the reaction mixture. The results shown are from four different lines of determination, mean $k_{d,obs}$ ± S.D. was 0.0279 ± 0.0017 min^{-1} (n = 4).

collected for the determination of decarbamylation, all further fractions were discarded.

AChE activity of the carbamate-inhibited enzyme was determined repeatedly after excess carbamate had been removed by column filtration and decarbamylation proceeded. Activity values were then plotted against time, resulting in a curve of data points following a monoexponential association function. An example is shown for pyridostigmine and obidoxime in Fig. 2. Analysis of the individual curves led to k_d of decarbamylation, both without and in presence of oxime. Table 2 shows the observed half-lives for different combinations of carbamate and oximes.

4. Discussion

A prerequisite of our approach to determine the decarbamylation kinetics of AChE is the strict obedience of a first-order reaction. Although such a behavior may be anticipated on theoretical grounds, it has been repeatedly confirmed experimentally [9,14–16]. This behavior enables the kinetic analysis of the more advanced part of the reaction curve when excess carbamate was already effectively removed both in the dynamic and the static system; in fact, fitting procedures to the data points showed clear first-order reactions with goodness of fit, $R^2 > 0.999$ and 0.980, respectively. We restricted our studies on human erythrocyte AChE in 0.1 M sodium phosphate buffer, pH 7.4, and 37 °C to meet human in vivo conditions as closely as possible.

The half-lives for decarbamylation of physostigmine- and pyridostigmine-inhibited AChE as found with our dynamic system were indistinguishable from our earlier results and the data obtained in the static system are in good agreement with literature data (cf. Tables 1 and 2).

The oxime effects on the decarbamylation rates of physostigmine-inhibited AChE were not uniform: obidoxime (30 μM) had hardly any effect in the dynamic system and only minor effects when tested by the static method. Equimolar HI 6 was more effective in both systems, which was even more pronounced at the higher HI 6 concentration (100 μM).

With the pyridostigmine-inhibited AChE obidoxime and HI 6 had small effects at 30 μM concentration (detected in both systems). At 100 μM HI 6 the decarbamylation rate was more increased which was particularly pronounced in the static system. MMB-4 (100 μM) was without effect (static system only). It appears that the oxime-induced decarbamylation was generally more effective with the physostigmine-inhibited enzyme (Fig. 3). Conceivably, the bulkier dimethylcarbamyl residue may impede the action of oximes.

As shown in Fig. 3 the linear line drawn through the ratio of log k values of pyridostigmine and physostigmine in the

Table 2 – Half-lives (min) of spontaneous reactivation of human erythrocyte AChE following inhibition with carbamates, in the presence and absence of oximes, as observed in the static system

Oxime	Physostigmine	Pyridostigmine
None	17.6 ± 0.8 (4)	29.0 ± 0.7 (4)
Obi, 30 μM	14.6 ± 0.6 (4)	25.0 ± 1.5 (4)
HI 6, 30 μM	13.3 ± 1.0 (4)	25.0 ± 0.8 (4)
HI 6, 100 μM	8.3 ± 1.0 (4)	18.2 ± 0.9 (4)
MMB-4, 100 μM	n.d.	29.0 ± 0.03 (2)

Mean ± S.D. (number of experiments in parentheses). The goodness of fit of the individual curves was $R^2 > 0.980$. n.d. = not determined. Reported half-lives of spontaneous reactivation following physostigmine were 14.8 ± 0.9 (S.E.M.) [15] and 15.8 min [30]. For pyridostigmine 30.9 [31] and 28 min [30] were reported.

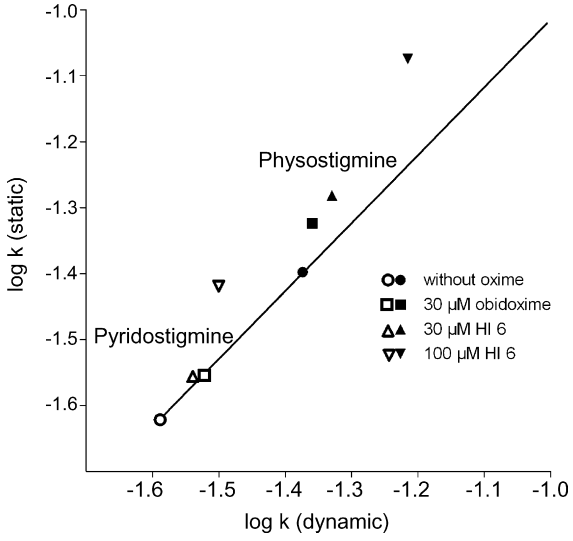

Fig. 3 – Double-logarithmic plot of the decarbamylation rate constants (min^{-1}) of human red blood cell AChE obtained in the dynamic and the static system. Open symbols refer to the pyridostigmine-inhibited enzyme, closed symbols refer to the physostigmine pretreated AChE. The line was drawn through the data obtained without oxime added (slope 1.03). Oxime effectiveness was more pronounced in the static system.

dynamic and the static system without oximes gave a slope not different from unity (1.03), while the enhancement of decarbamylation by oximes was somewhat more extensive when tested by the conventional static assay, which is suggestive of some artificial effects. There are three possible interfering reactions:

(i) Substrate was present during decarbamylation in the dynamic system, which could influence reactions at the active site (decarbamylation) by binding of acetylthiocholine to the peripheral anionic site (PAS) at the rim of the gorge [17]. This reaction could compete with electrostatic interactions of the quaternary bispyridinium oximes and may hence change the positioning of the reactivators.
(ii) Oximes are known to inhibit reversibly AChE in a competitive and a non-competitive manner [18]. Such an effect may be more marked in the dynamic system where the oxime concentration is higher than in the static one, where the sample is extensively diluted for activity determination. A constant inhibition of the enzyme, however, should not affect half-life calculation for decarbamylation.
(iii) Oxime-induced esterolysis of acetylthiocholine could mimic AChE activity [19,20]. Such a constant increment in AChE activity can distort the spontaneous reactivation kinetics and shortens the apparent half-life. This effect will play no role when the reaction mixture is markedly diluted in the test assay, as in the static system. The contribution of the latter effect was tested and it turned out that the small increase in thiocholine formation was not responsible for the observed effect.

We thus consider the substrate-induced retardation of the oxime-induced decarbamylation the most plausible explanation for the difference in de-inhibition kinetics in the two assay systems.

There was a long-lasting debate whether oximes influence the decarbamylation of AChE at all. Wilson et al. reported [21] that 2-PAM (up to 1 mM) did not increase the rate of enzyme activity from dimethylcarbamylated AChE of electric eel at pH 7.0 and 25 °C. Similarly, O'Brien found that 2-PAM (100 μM) was not able to reactivate monomethylcarbamylated bovine RBC-AChE [22]. When looking at the methods employed to come to the above conclusion, several problems may be encountered. Because 2-PAM interfers with the Ellman assay (see above), concentrated AChE was incubated with carbaryl (5 μM) and then diluted (1:1000) into a 100 μM 2-PAM solution. Aliquots were withdrawn and allowed to react with the same volume of acetylcholine solution, whose decrease was determined by the Hestrin method [23]. The half-life of spontaneous reactivation at 38 °C was 23 min with and without 2-PAM. It cannot be ruled out, however, that the 2-PAM-induced reactivation of the carbamylated AChE was obscured by the 2-PAM-dependent (50 μM) inhibition of AChE upon reaction with acetylcholine (4 mM).

Harris et al., who observed increased human RBC-AChE activity when oximes were present along with pyridostigmine compared to the absence of oximes, argued that this effect may be partly due to inhibition of re-carbamylation and partly due to enhanced decarbamylation, elicited by the oximes 2-PAM and HI 6 [11].

A more differentiated picture arose from the experiments of Dawson and Poretski in that neither 2-PAM nor HI 6 (0.1 mM each) did accelerate the decarbamylation of dimethylcarbamyl-AChE from electric eel at pH 7.0 and 25 °C. In contrast, bovine and human RBC-AChE was 2.0 and 1.84 fold faster decarbamylated by 0.1 mM HI 6, respectively [8]. Interestingly, increased rates in decarbamylation of dimethylcarbamyl-AChE of bovine RBCs were also observed with the non-oxime SAD-128 and the non-oxime analogue of TMB-4 [8]. Conceivably, quaternary pyridinium compounds exert allosteric effects [24] upon binding to the peripheral anionic site of AChE (see above). Such an allosteric effect upon binding to PAS of AChE has also been supposed to explain the stimulating action of edrophonium on the spontaneous reactivation of *O*-ethyl methylphosphonyl-AChE [25].

It thus appears that divergent results on the action of oximes on carbamylated AChE may markedly depend on the experimental conditions and the enzyme source used [26].

Here, we were able to confirm that HI 6 is effective in accelerating the rate of decarbamylation of human erythrocyte AChE inhibited by physostigmine or pyridostigmine under physiological conditions. While therapeutic obidoxime concentrations (10–30 μM) [27] have a slight effect on the decarbamylation reaction, HI 6 that may be required at higher concentrations [28] will shift the equilibrium of carbamylated AChE when administered during carbamate pretreatment. It is calculated that 100 μM HI 6 will reduce the fraction from, e.g. 30% dimethylcarbamylated enzyme (pyridostigmine prophylaxis) to 26 and 21%, using the data from the dynamic or static model, respectively.

In conclusion, it can be expected that the administration of an oxime to a carbamate pre-treated subject before exposure to an OP will diminish the fraction of protected enzyme. If the oxime is given to a carbamate-pretreated subject after exposure to a short-lasting OP, oxime-induced decarbamylation may increase the rate of functionally active AChE.

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