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Carbamoylated Enzyme Reversal as a Means of Predicting Pyridostigmine Protection Against Soman

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Abstract—The inhibition of human and mammalian red blood cell (RBC) cholinesterase (AChE) in whole blood in the presence of added pyridostigmine has been examined. After the addition of pyridostigmine to animal and human blood, red cells were separated from plasma at varying intervals and their enzyme activity measured. An apparent rate constant (k_e) was derived for the reaction sequence in which carbamate is released from AChE inhibited by pyridostigmine. The constant is a complex of the rates of decarbamoylation and reinhibition of AChE in the blood sample. Rate constants were also determined for the spontaneous reactivation (k_s) of carbamoylated AChE in the species studied. Values of k_s were greater than k_e in corresponding species but varied little between species. Pretreatment of animals with pyridostigmine is known to be an effective therapy against organophosphorus compounds, including soman. The ranking of k_e values in mammalian blood was the same as that for the protection against soman in animals: monkey > guinea-pig > rabbit > rat (k_e = 0·15, 0·07, 0·05, 0·02 h^{-1}, respectively). Since k_e for human blood (0·20 h^{-1}) was greater than that of monkey, pyridostigmine pretreatment would be expected to be an effective prophylaxis for soman in humans.

Pyridostigmine or carbamate pretreatment has been reported to protect animals from the lethal effects of soman (Berry & Davies 1970; Gordon et al 1978). Pyridostigmine protection supposedly depends on the instability of carbamoylated acetylcholinesterase enzyme (AChE, EC 3.1.1.7) which protects against phosphonylation and spontaneously degrades to form free enzyme. The decarbamoylation releases enough enzyme to maintain life (Berry & Davies 1970). The reaction, described by Aldridge & Reiner (1972), involves inhibition of AChE, owing to carbamoylation, steady state, where the rate of inhibition equals the rate of reactivation, and spontaneous decarbamoylation in which free enzyme is formed. In the present paper, work is reported where pyridostigmine is present in 5-fold excess over combined concentrations of AChE and plasma cholinesterase (ChE, EC 3.1.1.8), so there would be reinhibition of enzyme after decarbamoylation. Our aim was to study the reaction in whole blood where both enzymes are present and to determine whether the protection could be related to the reversible nature of the carbamoylated red blood cell (RBC) AChE.

Experiments were designed to follow the reaction after the addition of pyridostigmine to human and animal blood. As would occur in-vivo, there would be simultaneous reactions of pyridostigmine with both AChE and ChE enzymes; however, neither metabolic excretion nor conversion were considered. A concentration of 10^{-7} M pyridostigmine was selected as this concentration was present in human blood after the oral administration of 30–60 mg (Calvey & Chan 1977; Breyer-Pfaff et al 1985). There was a practical reason for this report. Pyridostigmine administered orally to humans is absorbed at a slower rate than it is eliminated (Breyer-Pfaff et al 1985) and would be available to react with blood enzymes for over 16 h. An apparent rate constant (k_e) was derived for the decarbamoylation reaction, a complex of the rate of decarbamoylation of AChE and the rate of reinhibition.

The efficacy of pyridostigmine prophylaxis is species selective in the ranking: monkey > guinea-pig > rabbit > rat (Gordon et al 1978; Dirnhuber et al 1979). A direct relationship exists between its protection of animals and the values of the apparent rate constant, k_e.

Materials and Methods

The analytical system consists of the following modules (Technicon Instruments Corp., Tarrytown, NY): sampler IV, proportioning pump III, water bath with #157–9223 and #157–0225 heating bath coils, colorimeter, 15 mm and 30 mm flow cells, 410 mm filters and a flatbed recorder. The analytical procedure is a modification of the automated continuous flow methods of Humiston & Wright (1967) and Groff et al (1976). The method measures the anion formed when thiocholine, the product of acetylthiocholine hydrolysis, reacts with 5,5′-dithio-bis-2-nitrobenzoic acid.

Additional equipment used was an Eppendorf, model 5414 and a Damon/IEC, model HN-SII centrifuge. A Beckman, Expandomatic SS2, pH meter.

Reagents

Buffer: 0·05 M Tris (hydroxymethyl) aminomethane (pH 8·2). Colour forming reagent: $1{\cdot}68 \times 10^{-3}$ M 5,5′-dithio-bis-2-nitrobenzoic acid. Substrate: $1{\cdot}27 \times 10^{-2}$ M acetylthiocholine iodide. Saline: 0·9% sodium chloride. Standard: 6×10^{-2} M glutathione, reduced form. Chemicals were obtained from Sigma chemical Co., St. Louis, MO.

The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Army or the Department of Defense. In conducting the research described herein, the investigators adhered to the 'Guide for the Care and Use of Laboratory Animals' of the Institute of Laboratory Animal Resources, National Research Council.

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Animal species

Blood was obtained from male Rhesus monkeys and from male and female Sprague-Dawley rats, New England white rabbits, Hartley Charles River guinea-pigs and volunteers.

Determination of RBC ChE activity

Blood was collected with EDTA as anticoagulant. Human, monkey and rabbit blood were separated from single donors while blood of the rat and guinea-pig was pooled. Red blood cells (RBC) were separated from plasma after a 2 min centrifugation of 1·5 mL blood at 15000 rev min^{-1} in a microcentrifuge. Rat, guinea-pig and rabbit RBC were diluted 1:6 with saline before assay, resulting in a final dilution of 1:38 in the manifold. Reactions took place in a #157–0225 heating bath coil and colour was detected in a 30 mm flow cell.

Human and monkey RBC samples were diluted 1:11 with saline, resulting in a final dilution of 1:69. Reactions took place in a #157–0223 heating bath coil and colour detected in a 15 mm flow cell. Both sample dilutions produced similar RBC AChE activity values. Reaction times were 3·4 and 4·5 min at 37°C for the #157–0223 and #157–0225 coils, respectively. The final concentration of substrate in the reaction mixture was 2×10^{-3} M.

Determination of RBC ChE activity after addition of pyridostigmine to whole blood

A 1% v/v solution of pyridostigmine bromide prepared in deionized water was added to 25 mL of preincubated blood to achieve 10^{-7} M in the plasma fraction. (Very little enters the red cell since pyridostigmine is a quaternary-amino carbamate.) Plasma volume was calculated as follows: blood volume (mL) × 1 − haematocrit. At various times, over 24 h periods for guinea-pig, rabbit and rat and 12 h periods for human and monkey, 1·5 mL fractions of blood were removed and RBC ChE activity determined. Control RBC ChE activities were always obtained at similar times from blood to which deionized water, instead of pyridostigmine, had been added. The control RBC sample was used to determine % activity of the pyridostigmine inhibited enzyme. Control sample variability was also determined. Time-activity data following steady state was fitted to a first order equation transformed for enzyme inhibition: $k_e t = 2{\cdot}3 \log$ (% inhibition following steady state divided by % inhibition at another time). k_e is the apparent decarbamoylation carbamate rate constant and t, the time interval selected for reversal of inhibition. Data for establishing the validity of k_e were processed by two methods: (i) conventionally, where k_e values were determined by the equation and averaged (Table 1) and (ii) where RBC AChE values determined at identical time intervals were averaged, then plotted against time (Table 2). Values for k_e and statistical data were obtained with a programmable (Texas Instruments, Ti-55-III) hand calculator. The initial data points for the determination of k_e were taken shortly after steady state: at 2 h for the human, monkey and rabbit and 4 h for the guinea-pig and rat. The selection of final time points were determined after substituting them into the above equation. The criteria of acceptance was a linear relation with a correlation coefficient of 0·950 or better (Table 1). Final time points were 12 h for the human, monkey and rabbit and 16 h for the guinea-pig and rat.

Determination of the rate of spontaneous decarbamoylation

Enough blood was pippeted into a 15 mL plastic centrifuge tube to contain 1 mL of RBC. Blood volume was calculated as follows: 1 mL RBC/haematocrit. After incubating the blood for 5 min at 37°C, pyridostigmine bromide was added to give $3\text{–}5 \times 10^{-7}$ M in the plasma fraction. The sample was mixed, kept at 37°C for 60 min and then centrifuged for 10 min at 3000 rev min^{-1}. The plasma was removed and the cells washed three times with 10 mL portions of 67 mM isotonic phosphate buffer (pH 7·4). After the third wash 5 mL of phosphate buffer at 37°C was added to the rat, rabbit and guinea-pig RBCs and 10 mL to the human and monkey RBCs. The suspension was mixed and kept at 37°C. RBC AChE activities of the cell suspensions, in the absence of plasma, were determined at 5 min intervals for the first 40 min and at 10 min intervals up to 60 min. Similar samples, without pyridostigmine, were used as controls. Procedures were based on those published by Reiner (1971).

Results

Time vs RBC AChE activity profiles for the reaction between pyridostigmine and whole blood cholinesterase enzymes are shown in Fig. 1. Enzyme activities of RBC control samples changed over the long sampling periods. Variability was greatest in the rat and least for the human, with coefficients of variation of 10·7 and 2·1%, respectively. In both human and animal blood there was rapid inhibition of AChE, followed by a steady state period, then reversal of inhibition. Guinea-pig blood ChE was most sensitive to inhibition by pyridostigmine, human blood the least. Steady state was most pronounced in the rat, rabbit and guinea-pig. Two procedures were used to determine k_e (Tables 1,2). Both show good agreement for k_e in all species. Spontaneous decarbamoylation rates, k_s, in which pyridostigmine was added to blood of animal species, the plasma removed and the red cells washed

Table 1. Apparent decarbamoylation rate constants and half-lives for the reaction between pyridostigmine and RBC AChE[a].

Species	$k_e \times 10^2$ (h^{-1})	s.d.	CV %	$t_{\frac{1}{2}}$ (h)
Human	20.0	2·7	13·6	3·5
Monkey	14·8	1·4	9·1	4·7
Guinea-pig	7·3	0·5	7·1	9·5
Rabbit	5·1	0·9	18·1	13·7
Rat	2·3	0·5	23·6	30·8

[a] Rate constants were determined for each run and then averaged to obtain the standard deviation and CV n = 6 for the human and rat, 4 for the other species.

Table 2. Apparent decarbamoylation rate constants obtained by averaging AChE inhibition and plotting against time.

Species	$k_e \times 10^2$ (h^{-1})	$t_{\frac{1}{2}}$ (h)	Correlation coefficient (r)
Human	24·5	2·8	0·988
Monkey	13·8	5·0	0·994
Guinea-pig	7·0	9·9	0·999
Rabbit	5·5	12·6	0·989
Rat	2·2	31·3	0·951

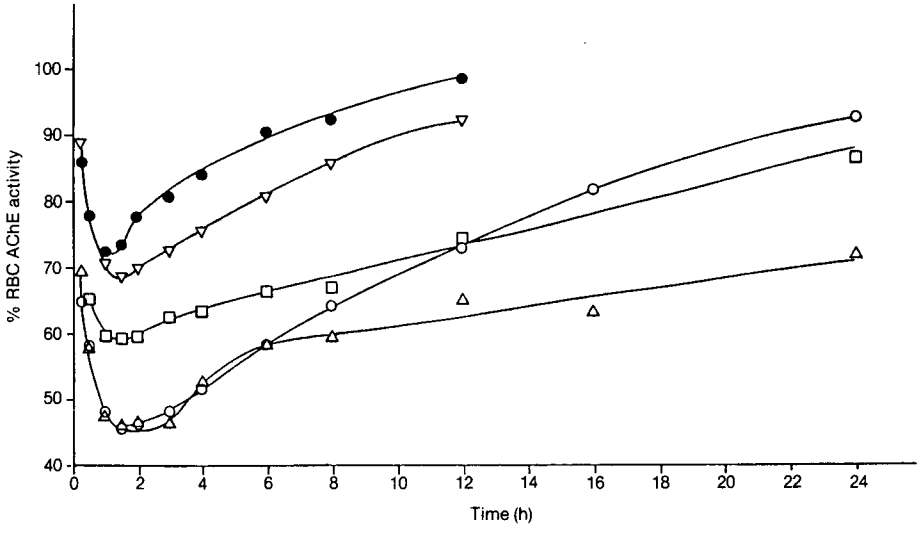

FIG. 1. Time-enzyme activity patterns for the % inhibition of mammalian RBC AChE in the presence of 10^{-7} M pyridostigmine. (● Human, ▽ monkey, ○ guinea-pig, □ rabbit, △ rat).

Table 3. Spontaneous decarbamoylation rate constants and half-lives.

Species	$k_s \times 10^2$ (min^{-1})	s.d.	CV %	$t_{\frac{1}{2}}$ (min)
Human	2·3	0·3	11·5	30·9
Monkey	2·1	0·1	6·8	33·7
Guinea-pig	1·6	0·3	17·4	45·6
Rabbit	2·2	0·2	8·6	31·7
Rat	1·6	0·2	12·0	42·2

free of carbamate, are shown in Table 3. Values of k_s were comparatively similar among the animal species. The ranking of k_e values for the reaction of pyridostigmine with RBC AChE in the species tested were: human > monkey > guinea-pig > rabbit > rat.

Discussion

The reaction of pyridostigmine with cholinesterases in whole blood was intended to simulate, to some extent, that which would occur in-vivo. However, pyridostigmine would be metabolized in the body and be eliminated from the kidney. The rate of decrease of carbamate from blood in-vivo would be different, by some constant, from that occurring here. The overall reaction, as seen in Fig. 1, proceeds as carbamoylation or inhibition of enzyme, steady state, and decarbamoylation or reactivation of enzyme. Under the conditions we used where the concentration of pyridostigmine (10^{-7} M) is at least five times in excess of the cholinesterases in blood (each about 1×10^{-8} M), the rate of decarbamoylation would occur as a result of spontaneous reactivation and reinhibition of enzyme. After measuring RBC AChE activities for up to 24 h and plotting the free AChE enzyme data resulting from decarbamoylation with time, an apparent rate constant (k_e), which follows first order kinetics, was derived.

In separate experiments spontaneous decarbamoylation constant (k_s) values were determined for the reaction between pyridostigmine and RBC AChE in human and animal species. Here excess pyridostigmine was washed free from the red cells before analysis of enzyme activity. As seen in Tables 1–3, k_s values were greater than k_e values in all species tested, ranging from 6 to 42 fold differences in human and rat, respectively. k_s values in the blood among species tested varied only by 1·5 fold, while k_e values varied by about 9. A rate approximating k_s was never observed. This may have been due to the presence of extremely low concentrations of pyridostigmine. A separate or biphasic reactivation occurred late in the decarbamoylation process.

The numerical order for values of k_e in animal blood followed identically the ranking of protection afforded by pyridostigmine in soman poisoning: monkey > guinea-pig > rabbit > rat. Since k_e for human blood is even greater than that of monkey, pyridostigmine prophylaxis would be expected to provide excellent protection against soman in humans.

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