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KINETICS OF THE INHIBITION OF HUMAN SERUM CHOLINESTERASE PHENOTYPES WITH THE DIMETHYLCARBAMATE OF (2-HYDROXY-5-PHENYLBENZYL)-TRIMETHYLAMMONIUM BROMIDE (Ro 02-0683)

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Abstract—The inhibition of the human serum cholinesterase phenotypes, usual (U), atypical (A) and heterozygous (UA), by the dimethylcarbamate of (2-hydroxy-5-phenylbenzyl)-trimethylammonium bromide (Ro 02-0683), was followed with benzoylcholine, acetyl-, butyryl- and propionyl-thiocholine as substrates. The first-order rate constants were calculated from the linear part of the inhibition curves and were independent of the substrate used for measuring the enzyme activity. The second-order rate constants for the U, UA and A phenotypes were 8.3×10^6, 6.1×10^6 and $0.05 \times 10^6\ M^{-1}\ min^{-1}$, respectively. The constant of the enzyme–inhibitor complex for the atypical serum was 7.7 μM, and the rate of carbamylation of the enzyme was $0.386\ min^{-1}$. The rate of reactivation of carbamylated usual and atypical enzyme was found to be same; the half-time of reactivation was about 3.5 hr. The deviation from the linearity of the inhibition course was explained by spontaneous reactivation of the inhibited enzyme; the theoretical inhibition curves were in good agreement with the experimentally obtained values. The three phenotypes could be distinguished by the rate of inhibition by the dimethylcarbamate, Ro 02-0683, in the progressive phase of inhibition or by the degree of inhibition in the apparent steady-state.

Usual (U†) and atypical (A) human serum cholinesterase (EC 3.1.1.8) differ in only one amino acid in their primary structure. This difference accounts for the difference in catalytic properties of the two phenotypes. Usual enzyme can hydrolyse the pharmacologically important ester, succinylcholine, while atypical enzyme cannot. The two cholinesterase phenotypes differ also in the reaction with many other compounds, mainly positively charged. However, the time course of interaction and the intermediate steps for the two enzymes have been studied collaterally only for a few compounds [1]. This paper deals with the reaction of U, A and heterozygous UA human serum cholinesterase with positively charged compound Ro 02-0683. The use of Ro 02-0683 provides supportive information for the purpose of phenotyping some of the variants [2, 3].

MATERIALS AND METHODS

Serum samples. Samples of human serum were chosen from the laboratory stock (5 U, 3 UA and 2 A sera). The samples were assigned to cholinesterase phenotypes according to selective inhibition by dibucain, fluoride and Ro 02-0683 by means of standard phenotyping methods [2, 3].

Activity measurements. All experiments were performed in 67 mM phosphate buffer pH 7.4 at 25°. Stock solutions of DTNB reagent (0.01 M) in buffer and the inhibitor Ro 02-0683 (0.01 M) in water were stored at 4°. Dilutions of the reagents were made in buffer immediately before the experiments. Cholinesterase activity was measured with ASCh, PSCh, BSCh or BzCh; the final concentrations were 1.0, 4.0, 1.0 mM and 50 μM, respectively [4, 5].

Inhibition measurements. The procedure for measuring cholinesterase inhibition was as follows: into 2.0 mL of serum (100 times diluted with buffer), 1.0 mL Ro 02-0683 was added. After incubation, a mixture of 100 μL DTNB and 50 μL of substrate was added and the remaining activity measured.

For atypical serum an additional set of assays was performed: after the incubation of serum with inhibitor, the reaction mixture was diluted three times with 4.0 or 10 mM PSCh and the activity measured.

Spontaneous reactivation. Undiluted serum (1.1 mL) was incubated with 0.1 mL inhibitor for 30 min at 25°. The final inhibitor concentrations were 40 nM and 3.0 μM for the usual and atypical enzyme, respectively. Separation of free inhibitor was achieved by applying the serum on a Sephadex G-25 column. Elution was carried out with 10 mM phosphate buffer. The 2-mL fraction which contained most of the serum proteins was collected and diluted 60 times with buffer. At suitable time intervals, 3-mL samples were withdrawn and the activity was

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† Abbreviations: U, A and UA, usual, atypical and heterozygous human serum cholinesterase phenotypes; Ro 02-683, dimethylcarbamate of (2-hydroxy-5-phenylbenzyl)-trimethylammonium bromide; DTNB, 5,5′-dithio-bis-nitrobenzoic acid; ASCh, acetylthiocholine; PSCh, propionylthiocholine; BSCh, butyrylthiocholine; BzCh, benzoylcholine.

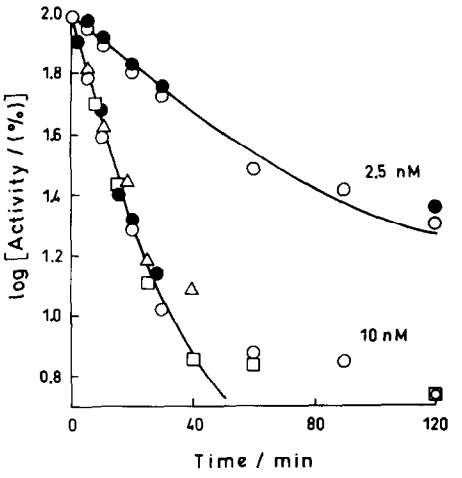

Fig. 1. Progressive inhibition of usual serum cholinesterase by Ro 02-0683. The experimental points are means of the activities measured with PSCh (○), BzCh (●), ASCh (△) and BSCh (□). The inhibitor concentrations are indicated. Solid lines represent the theoretical calculated values.

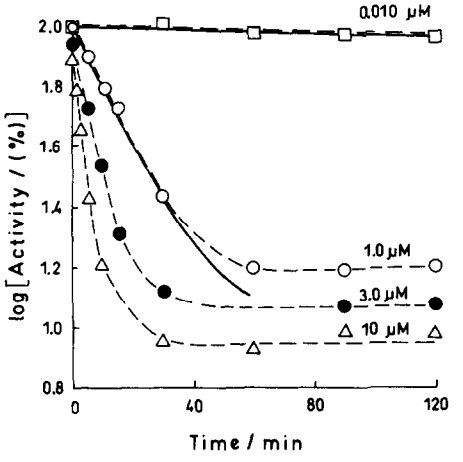

Fig. 3. Progressive inhibition of atypical serum cholinesterase by Ro 02-0683. The experimental points (dashed lines) are means of the remaining activity measured with 4 mM PSCh. Solid lines represent the theoretical calculated values.

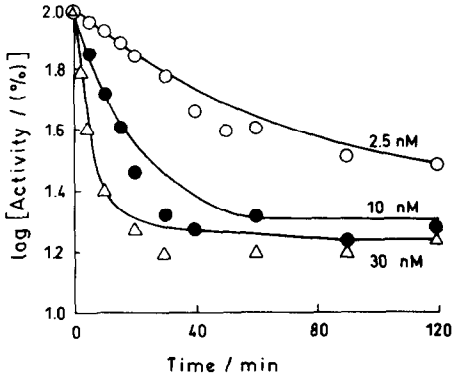

Fig. 2. Progressive inhibition of UA serum cholinesterase by Ro 02-0683; 2.5 nM (○), 10 nM (●) and 30 nM (△). The experimental points are means of the activities measured with 4 mM PSCh. Solid lines represent the theoretical calculated values.

Table 1. Inhibition of usual, atypical and UA cholinesterase phenotypes by Ro 02-0683 (*ab*)

ab (μM)	Substrate	100 ($k \pm$ SD) (min^{-1})	N
U phenotype			
0.030	PSCh	25 ± 1	5
0.010	PSCh	8.1 ± 0.3	11
0.010	ASCh	7.9 ± 0.1	2
0.010	BSCh	8.3 ± 0.2	2
0.010	BzCh	8.9 ± 0.8	5
0.0025	PSCh	2.1 ± 0.2	5
0.0025	BzCh	2.0 ± 0.3	5
UA phenotype			
0.030	PSCh	18 ± 1	2
0.010	PSCh	6.0 ± 0.3	6
0.010	BSCh	5.5	1
0.010	BzCh	5.8	1
0.0025	PSCh	1.6 ± 0.1	3
A phenotype			
10	PSCh	18.1 ± 0.9	3
10*	PSCh	21.9 ± 0.1	2
10†	PSCh	23.9 ± 1.9	2
3	PSCh	10.6 ± 0.5	2
3†	PSCh	11.8 ± 0.6	2
1	PSCh	4.9	1
0.330	PSCh	1.4	1
0.010	PSCh	ND	2

The mean values of the first-order rate constants ($k \pm$ SD) at a given inhibitor concentration were calculated according to Eqn 1.

* Activity measured after three times dilution with 4 mM PSCh and † with 10 mM PSCh. N is number of experiments. ND, not determined.

measured as described above. The spontaneous reactivation was followed for 2 hr.

Kinetic equations. The reaction scheme for the inhibition of cholinesterase with carbamates, analogous to organophosphates, includes a Michaelis-type enzyme–inhibitor complex and an acylated (carbamylated) enzyme. To calculate the rate constants of the reaction the following equations were used (cf. Ref. 6).

$$\ln (v_0/v_t) = kt. \quad (1)$$

v_0 and v_t stand for the enzyme activities of the control sample and of the inhibited sample at time t. The first-order rate constant of inhibition (k) at a given inhibitor concentration (ab) and its relation to the other constants are:

$$ab/k = K_a/k_{+2} + ab/k_{+2} \quad (2)$$

$$K_a = k_{+2}/K_a. \quad (3)$$

K_a is an enzyme–inhibitor constant analogous to the

Table 2. Inhibition of serum cholinesterase phenotypes by Ro 02-0683 and reactivation of the carbamylated enzyme

Phenotype	$10^{-6} k_a$ ($M^{-1} min^{-1}$)	% Activity		10^3 ($k_{+3} \pm$ SD) (min^{-1})
		60 min	120 min	
U	8.3	7	7	3.0 ± 0.9
UA	6.1	22	21	—
A	0.050	94	92	3.5 ± 0.4

The second-order rate constants k_a were calculated from the values given in Table 1 (Eqns 3 and 4).

% Activity, the activity which remained after 60 or 120 min inhibition by 10 nM inhibitor.

The rate constants of reactivation ($k_{+3} \pm$ SD) were calculated from three experiments (Eqn 5).

Michaelis constant, k_{+2} is the first-order rate constant of carbamylation and k_a is the overall second-order rate constant of inhibition.

When K_a is much greater than ab, Eqn 2 reduces into:

$$k_a = k/ab. \quad (4)$$

The carbamylated enzyme is prone to spontaneous reactivation so the following equation is valid to calculate the first-order rate constant of reactivation k_{+3}:

$$\ln [(v_0 - v)/(v_0 - v_t)] = k_{+3} t. \quad (5)$$

v_0 stands for the activity of the control sample; v and v_t stand for the activities of inhibited sample at the beginning of reactivation and at time t.

The time course of enzyme interaction with an inhibitor which includes both progressive inhibition and spontaneous reactivation is defined by the equation:

$$v_0 - v_t = [(k_a v_0 ab)/(k_a ab + k_{+3})] \times \{1 - \exp[-(k_a ab + k_{+3})t]\}. \quad (6)$$

$v_0 - v_t$ represents the concentration of carbamylated enzyme at time t.

The dissociation constant of the enzyme–inhibitor complex is defined by:

$$K_i = v_i ab/(v_0 - v_i). \quad (7)$$

v_0 and v_i are the enzyme activities in the absence and in the presence of inhibitor.

RESULTS AND DISCUSSION

The time course of inhibition of the cholinesterase phenotypes U, UA and A by Ro 02-0683 is shown in Figs 1–3. From the slope of the linear part of the inhibition curves the first-order rate constants were calculated (Table 1). For any given inhibitor concentration the constants were the same irrespective of the substrate used for measuring activity (Fig. 1 and Table 1). For U and UA cholinesterases the first-order rate constants were linearly dependent on the inhibitor concentrations and the second-order rate constants were calculated according to Eqn 4 (Table 2). The non-linear relationship between constants and inhibitor concentrations (Table 1) for atypical enzyme indicated the presence of the reversible enzyme–inhibitor complex and the respective constants (Eqn 2) were: $K_a = 7.7\,\mu M$, $k_{+2} = 0.386\ min^{-1}$. The reversible complex was revealed also as a "zero-time" inhibition (Fig. 3); with 10 μM inhibitor and at 4 mM PSCh, 24% activity was inhibited and the apparent dissociation constant was $K_i = 32\,\mu M$ (Eqn 7). This value was about four times higher than the value of the K_a constant. This pointed to the competitive nature of the complex. By increasing the substrate concentration during the assay and by dilution of the reaction mixture, the reversible inhibition at "zero-time" was abolished, while the progressive inhibition remained the same (Table 1).

The rates of spontaneous reactivation of the two dimethylcarbamylated phenotypes were the same (Table 2). Supposing that k_{+2} for the usual enzyme was also the same as that measured for the atypical enzyme (cf. Ref. 1), the K_a constant estimated for the usual enzyme by using Eqn 3 should have had the value of 47 nM. However, in our experiments the maximum inhibitor concentration (30 nM) remained below the K_a value and, therefore, the enzyme–inhibitor complex for the usual enzyme was not observed.

The curved inhibition course can be explained by the spontaneous reactivation of the inhibited enzyme which occurred simultaneously with inhibition [7]. The theoretical inhibition curves were calculated (Eqn 6) for the inhibition of the enzymes (Figs 1–3) taking into account the rate constants of inhibition and reactivation determined separately (Table 2). For the inhibition of UA enzyme the activity of 20% remained almost constant. Since UA phenotype has been considered as a mixture of U and A molecules [2], we assumed that 20% activity should have been the atypical enzyme contribution to the whole activity, which at that inhibitor concentration was inhibited very slowly. The U phenotype was presented by the major part of the activity (80%). The theoretical course of inhibition was calcualted by applying Eqn 6 to each of U and A activities using the constants obtained for these two phenotypes (Table 2); by summarizing both activities the theoretical values were obtained (Fig. 2).

A good agreement was obtained between the theoretical values and experimental points (Figs 1–3). The theoretical calculated constant of reactivation for the usual enzyme (calculated from the steady-state of inhibition, cf. Ref. 7) was found to be within two standard deviations of the experimentally obtained reactivation constant.

In phenotyping serum cholinesterases, the inhibition by 10 nM Ro 02-0683 after 120 min has been suggested as a standardized procedure [3]. In our experiments the degree of inhibition at 60 and 120 min was shown to be the same for each of the three phenotypes (Table 2) and therefore the inhibition time could be reduced from 2 to 1 hr. The three phenotpyes differed also in the initial phase of inhibition. The k_a value for the atypical enzyme was 166 times lower than for the usual enzyme and 122 times lower than for the UA enzyme. Therefore, these three phenotypes can be differentiated on the basis of their k_a values even in a few minutes.

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