In vitro Inhibition of Cholinesterases by Carbamates – A Kinetic Study

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Kinetics and mechanism of *in vitro* hydrolyses of acetylcholine and acetylthiocholine by carbamates were studied in a batch reactor at 25 °C, pH 8, and ionic strength of 0.11 M. Every hydrolysis was monitored by 3–4 independent methods. All studied hydrolyses can be described by the model of competitive inhibition with an irreversible step (k_3). A table of obtained average values of rate constants and discussion of the resultes are given.

Key words: Acetylcholine, Hydrolysis, Kinetics, Cholinesterases

Introduction

Cholinesterases belong to the group of serine hydrolases. In vertebrates, they are present in two forms: acetylcholinesterase (ACHE, EC 3.1.1.7) and butyrylcholinesterase (BCHE, EC 3.1.1.8). ACHE hydrolyses the neurotransmitter acetylcholine and therefore high activity of this enzyme causes disturbance of nervous impulse transmission at cholinergic synapses. The cholinergic insufficiency is associated with Alzheimer's disease. Beyond extensive inhibition of ACHE leads to accumulation of acetylcholine and enhanced stimulation of the postsynaptic cholinergic receptor (Pope *et al*., 2005).

Administration of ACHE inhibitors (ACHEI) is currently the approved approach for the treatment of Alzheimer's disease (Giacobini, 2000). Three types of ACHEI have been employed (Bar-On *et al*., 2002): 1.) classical reversible inhibitors, which are generally tertiary amines; 2.) irreversible inhibitors, such as organophosphates, which covalently phosphorylate or phosphonylate the esteratic site of the enzyme; and 3.) slow substrates, typified by the carbamates, that also react covalently with the enzyme. The inhibition process by carbamates is determined by two distinct reaction steps: formation of the transient enzyme-inhibitor complex (Michaelis complex) and carbamoylation of the enzyme (Barak *et al*., 2005). The transient Michaelis complex and the carbamoylated enzyme are two species determining the rate of carbamoylation and decarbamoylation, respectively, and therefore contribute to the efficacy of carbamate as drug (Barak *et al*., 2005).

The present paper deals with the kinetic parameters of cholinesterases inhibition by 3-*N,N*-diethylaminophenyl-*N′,N′*-dibutylcarbamate (DBK), 3-*N,N*-diethylaminophenyl-*N′,N′*-diethylcarbamate (DEK), 3-*N,N*-diethylaminophenyl-*N′,N′*-diisopropylcarbamate (DIK), and 3-*N,N*-dimethylaminophenyl-*N′,N′*-dibutylcarbamate (DMK).

Materials and Methods

Chemicals

Enzymes: Acetylcholinesterase (EC 3.1.1.7, ACHE) lyophilizate from electric eel and butyrylcholinesterase (EC 3.1.1.8, BCHE) from horse serum, both from Sigma-Aldrich, kept at −20 °C.

Substrates: Acetylthiocholine iodide (ATCH) and acetylcholine chloride (ACH), both from Sigma-Aldrich, kept at 5 °C.

Inhibitors: 3-*N,N*-Diethylaminophenyl-*N′,N′*-dibutylcarbamate (DBK), 3-*N,N*-diethylaminophenyl-*N′,N′*-diethylcarbamate (DEK), 3-*N,N*-diethylaminophenyl-*N′,N′*-diisopropylcarbamate (DIK) and 3-*N,N*-dimethylaminophenyl-*N′,N′*-dibutylcarbamate (DMK) were synthesized at the Department of Organic Chemistry, Faculty of Chemical Technology, University of Pardubice. The melting point of all inhibitors agrees with literature data.

Others: 5,5′-Dithiobis-2-nitrobenzoic acid (Ellman's reagent, DTNB), 1,4-dioxane p. a., $Fe(NO_3)_3 \cdot 9H_2O$, hydroxylamine hydrochloride p. a., all from Sigma-Aldrich. HCl and KOH p. a. both from Lachema Brno, CZ. Phosphate buffer, pH 8, ionic strength I = 0.11 M.

Analytical solutions

Detailed description of analytical solutions and enzyme preparation are given in Zdražilová *et al.* (2006a).

Measuring procedures

All experiments were done at 25 °C with enzyme preparation activity (EPA) 3.5 U in the initial reaction mixture. The determination of EPA is described in Komersová *et al.* (2007). The measuring procedures (ELM, PHS, HXE, HPLC) are described in detail in our previous paper (Zdražilová *et al.*, 2006a).

Calculations

From the original experimental data (noninhibited and inhibited hydrolyses), the dependences of the actual product or substrate concentrations vs. t were obtained. According to Zdražilová *et al.* (2006a), the noninhibited hydrolysis of ACH (ATCH) by cholinesterases can be described by the reaction course

$$\mathrm{E} + \mathrm{S} \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} \mathrm{ES} \xrightarrow{k_2} \mathrm{P} + \mathrm{HA} + \mathrm{E}, \qquad (1)$$

where E is the enzyme (BCHE or ACHE), S the substrate (ATCH or ACH), and ES the complex substate-enzyme which decomposes into the product of hydrolysis P [thiocholine (TCH) or choline (CH)] and acetic acid (HA). Therefore original dependences concentration-time were tested for the validity of this reaction scheme (using the PC program GEPASI which solves the corresponding differential kinetic equations). In positive cases, the rate constants k_i and optimal values of $[S]_0$ and $[E]_0$ were obtained. For noninhibited hydrolyses which fulfil reaction scheme (1) and the Michaelis-Menten equation, the optimal values of K_M and V_m were calculated from the rate konstants k_i. Irreversibly inhibited hydrolysis with reversible binding step and irrevesible catalytic step follows reaction scheme (2) where I is carbamate as inhibitor and EI is the complex enzyme-inhibitor (Komersová *et al.*, 2006; Zdražilová *et al.*, 2006b).

$$\begin{array}{ccccc} & & \overset{k_1}{\rightleftarrows} & \overset{k_2}{\rightarrow} & \\ \mathrm{E} + \mathrm{S} & & \mathrm{ES} & \rightarrow \mathrm{P} + \mathrm{HA} + \mathrm{E}. & (2) \\ +\mathrm{I} \downarrow k_3 & & k_{-1} & & \\ \mathrm{EI} & & & & \end{array}$$

The hydrolyses inhibited by carbamates were tested for the validity of this kinetic model and in positive cases, the rate constants k_i and optimal values of $[S]_0$ and $[E]_0$ were obtained using GEPASI.

Results and Discussion

For all measured hydrolyses, standard deviation (in GEPASI) and the graphical comparison of the theoretical and experimental dependences were applied to the appreciation of corresponding reaction scheme validity. In positive cases, for every combination S + E + I and every used analytical method average values of rate constants (with standard deviation) and the initial concentration of enzyme $[E]_0$ were obtained. Results are given in Table I and Table II.

From the performed experiments following conlusions can be drawn:

Table I. Mean optimal values and deviations of rate constants k_i and initial enzyme concentrations $[E]_0$ according to (1) calculated by GEPASI.

S + E	$k_1 \times 10^{-4}$ [M^{-1} s^{-1}]	k_{-1} [s^{-1}]	k_2 [s^{-1}]	$[E_0]$ [nM]
ACH + ACHE	16.6 ± 1.8	310.0 ± 27.2	24.3 ± 3.7	110.8 ± 8.3
ACH + BCHE	3.5 ± 0.6	337.0 ± 28.0	71.4 ± 9.6	124.7 ± 10.6
ATCH + ACHE	0.045 ± 0.02	1.1 ± 0.3	33.7 ± 5.8	145.2 ± 13.1
ATCH + BCHE	0.24 ± 0.06	3.6 ± 0.7	55.1 ± 7.6	100.9 ± 12.1

Table II. Mean values and deviations of rate constants k_3 for every combination S + E + I according to (2) calculated by GEPASI.

Inhibitor I	k_3 [M^{-1} s^{-1}]			
	ACH + ACHE	ACH + BCHE	ATCH +ACHE	ATCH + BCHE
DBK	6.7 ± 0.8	72.9 ± 8.8	71.6 ± 9.7	180.6 ± 22.3
DEK	5.7 ± 0.8	364.5 ± 43.2	–	–
DIK	2.7 ± 0.4	11.04 ± 13.0	–	132.6 ± 15.7
DMK	135.7 ± 15.1	741.7 ± 90.2	–	68.8 ± 8.9

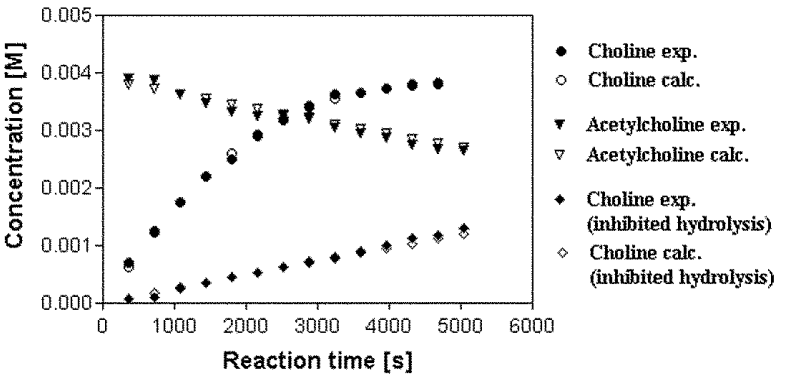

Fig. 1. Noninhibited and inhibited (by 3-*N,N*-dimethylaminophenyl-*N′,N′*-dibutylcarbamate) hydrolysis of ACH by AChE (experimental and calculated data) determined by HPLC, evaluated by GEPASI. Initial concentration: $[ACH]_0 = 4$ mM; ACHE activity = 0.14 U; phosphate buffer, pH 8; ionic strength: $I = 0.11$ M; 25 °C; inhibited hydrolysis: initial concentration of inhibitor $[DMK]_0 = 120\ \mu$M.

1.) All noninhibited total hydrolyses of ACH and ATCH fulfil the reaction course (1) and the rate of the hydrolyses can be described by the Michaelis-Menten equation up to substrate exhaustion. Mean values of rate constants k_2 (see Table I) show that both substrates (ACH and ATCH) are hydrolyzed faster by BCHE than AChE.

2.) Inhibition of ATCH and ACH hydrolyses by the tested carbamates can be described by the model of competitive inhibition (2) with an irreversible step (k_3). Mean values of rate constants k_3 for every combination S + E + I are given in Table II. As can be seen from the presented results, the hydrolysis of ACH by both enzymes (AChE or BCHE) is inhibited by all tested carbamates. The hydrolysis of ATCH by AChE is inhibited only by DBK and for the inhibiton of ATCH hydrolysis by BCHE, DEK as inhibitor cannot be used (see Table II).

3.) Average values of the kinetic konstants k_i determined for the same combination S + E or S + E + I by the different analytical methods correspond well and all used experimental methods are usable for monitoring of both substrate hydrolyses. But only the HPLC method enables the parallel monitoring of the substrate concentration (ATCH or ACH) and both products (CH or TCH and HA), see Fig. 1.

4.) Mean values of constants k_3 (Table II) show that DMK is the most effective inhibitor of AChE (from electric eel) and BCHE (from horse serum) from tested carbamates.

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Barak D., Ordentlich A., Kaplan D., Kronman C., Velan B., and Schafferman A. (2005), Lessons from functional analysis of AChE covalent and noncovalent inhibitors for design of AD therapeutic agents. Chem.-Biol. Interact. **157–158**, 219–226.

Bar-On P., Millard C. B., Harel M., Dvir H., Enz A., Sussman J. L., and Silman I. (2002), Kinetic and structural studies on the interaction of cholinesterases with the anti-Alzheimer drug rivastigmine. Biochemistry **41**, 3555–3564.

Giacobini E. (ed.) (2000), Cholinesterases and Cholinesterase Inhibitors. Martin Dunitz Ltd., London.

http://gepasi.dbs.aber.ac.uk/softw/gepasi.html

Komersová A., Komers K., and Čegan A. (2007), New findings about Ellman's method to determine cholinesterase activity. Z. Naturforsch. **62c**, 150–154.

Pope C., Karanth S., and Liu J. (2005), Pharmacology and toxicology of cholinesterase inhibitors: uses and misuses of a common mechanism of action. Environ. Toxicol. Pharm. **19**, 433–446.

Zdražilová P., Štěpánková Š., Vránová M., Komers K., Komersová A., and Čegan A. (2006a), Kinetics of total enzymatic hydrolysis of acetylcholine and acetylthiocholine. Z. Naturforsch. **61c**, 289–294.

Zdražilová P., Štěpánková Š., Komersová A., Vránová M., Komers K., and Čegan A. (2006b), Kinetics of 13 new cholinesterase inhibitors. Z. Naturforsch. **61c**, 611–617.