SEARCH FOR NEW DRUGS

SYNTHESIS, ANTICHOLINESTERASE, AND CALCIUM-ANTAGONIST ACTIVITY OF PHENYL ESTERS OF N-SUBSTITUTED CARBAMIC ACID

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Previously we have demonstrated that certain modification of the chemical structure of anticholinesterase compounds renders them capable of producing calcium-antagonist action in addition to the anticholinesterase effect [1 – 4]. For example, Ca^{2+} antagonism was observed in reversible cholinesterase inhibitors such as the esters of N-substituted carbamic acid with aliphatic structure of the alcohol part of the molecule [4].

The purpose of this work was to answer the question as to whether the calcium-antagonist activity is also inherent to the anticholinesterase substances interacting with cholinesterases by different mechanisms. A preliminary search showed that phenyl esters of the N-substituted carbamic acid inhibit these enzymes by a combined mechanism (simultaneously possessing reversible and irreversible features). In this connection, we have synthesized a series of compounds with the general formula $R^1NHC(O)OR^2$, where R^1 = Me (I, II), Ph (IV, V), *n*-Bu (III); R^2 = Ph (I, III, IV), C_6H_4Me-4 (II, V).

EXPERIMENTAL CHEMICAL PART

The IR spectra of synthesized compounds were measured on a Specord 75 spectrophotometer (Germany) using hexachlorobutadiene as the solvent. The data of elemental analysis (C, H, N) agree with the results of numerical calculations. Physicochemical characteristics of the synthesized compounds are given in Table 1.

General method for the synthesis of carbamates I – V. To 10 ml of dry acetonitrile was added a drop of triethylamine, followed by sequentially introduced (with stirring) corresponding isocyanate (0.5 mmole) and alcohol (0.5 mmole). The mixture was stirred for 30 min and allowed to stand for 24 h. Then the solvent was distilled off and the residue recrystallized from hexane.

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TABLE 1. Characteristics of Compounds I – V

Compound	Yield, %	M.p., °C	Empirical formula	IR spectrum ($\nu_{C=O}$, cm^{-1})
I	80	83	$C_8H_9NO_2$	1710
II	85	96	$C_9H_{11}NO_2$	1700
III	83	38	$C_{11}H_{15}NO_2$	1695
IV	89	140	$C_{13}H_{11}NO_2$	1710
V	82	111	$C_{14}H_{13}NO_2$	1700

EXPERIMENTAL BIOLOGICAL PART

Experiments were performed with acetylcholinesterase (ACE, IUPAC – IUB enzyme catalog [3.1.1.7]) from human erythrocytes and butyrylcholinesterase (BuCE, [3.1.1.8]) from horse serum with a specific activity of 2.2 and 9.6 units / mg, respectively (Perm Research Institute of Vaccines and Sera). The substrates for ACE and BuCE were represented by acetylcholine iodide at a concentration of 1 and 10 mM, respectively. The catalytic activity of enzymes was studied by a method of continuous potentiometric titration using a Radiometer RTS-822 autotitrator system (Denmark). The measurements were performed under standard conditions: 25°C; 100 mM KCl, 2 mM phosphate buffer, pH 7.5.

The irreversible component of the combined inhibition of ACE and BuCE by compounds I – V was evaluated by the bimolecular rate constant for the irreversible reaction of cholinesterases with inhibitors (k_a). The reversible component was characterized by equilibrium rate constants with respect to the inhibitor ($K_{i,r}$) and the substrate ($K_{i,s}$). The values of k_a and $K_{i,r}$ were determined graphically [5, 6] from the plot of $1/k$ versus the molar concentration of the inhibitor [I] (k is the experimental bimolecular rate constant for the enzyme interaction with inhibitor at a given [I] value. The value of $K_{i,s}$ was determined as described in [7] using the $1/v_i$ ver-

sus [I] (v_i is the initial rate of enzymatic hydrolysis of the substrate in the presence of inhibitor, which is introduced into the reaction mixture simultaneously with substrate). The anticholinesterase activity of prozerin (a well-known irreversible cholinesterase inhibitor of the carbamate type) used in three different concentrations (0.05, 0.12, and 0.25 μM for ACE and 0.3, 0.6, and 1.2 μM for BuCE) was characterized by the bimolecular rate constant (k_{II}, $M^{-1} \cdot min^{-1}$) of the enzyme inhibition [8].

The effect of carbamates on the acetylcholine sensitivity of the diaphragm was studied using an isolated phrenic-diaphragm preparation of rat [9]. The activity was evaluated by the concentration (determined before and after adding carbamate to the reaction medium) decreasing the amplitude of single diaphragm contractions (in response to indirect excitation) to half the initial level [9].

The calcium-antagonist activity of compounds I – V and the reference preparation (verapamil) was determined on isolated sections of intestinal ileum taken from male rats weighing 120 – 200 g [10, 11]. The ileum samples were initially washed for 30 min with a standard Tyrode's solution (mM: NaCl, 136.9; KCl, 2.68; $CaCl_2$, 0.9; $MgCl_2$, 1.05; $NaHCO_3$, 11.9; NaH_2PO_4, 0.42; glucose, 5.55), then for 25 min with the same solution without $CaCl_2$, and finally for 10 min with a calcium-free potassium-depolarizing solution (with the KCl content increased to 80 mM). Then calcium chloride was introduced (to a final concentration of 4 mM) into a bath with the organ and, when the curve of submaximum contraction attained a plateau, the carbamate was gradually added. Each carbamate was characterized by a concentration (EC_{50}) reducing the amplitude of $CaCl_2$-induced contraction to half of the initial value.

The contraction of ileum sections and diaphragm preparations was studied in an isometric regime using an R-612 dynograph (Beckman, USA).

Table 2 presents data on the inhibition constants determined as describe above. The k_a values are indicative of small and virtually equal rates of interaction of compounds I – V with both ACE and BuCE. Carbamates also differ but little from each other with respect to this parameter (the k_a values for ACE and BuCE in the most active compound being 3.8 and 1.5 times those of the least active). The reversible inhibition, unlike the irreversible process, exhibits a certain selectivity toward BuCE, which is most pronounced in I and II ($K_{i,r}$ for BuCE being 1/15 and 1/5 of that for ACE) and shows a stronger dependence on the chemical structure. For example, compounds IV and V with phenyl substituent at the nitrogen atom are more active than their N-methylsubstituted analogs I and II. This is reflected by the $K_{i,r}$ values, which are greater in the former pair by a factor of 65 and 20 for the BuCE inhibition, and by a factor of 73 and 51 for the ACE inhibition. The experimental plots of $1/v_i$ versus [I] (used for the $K_{i,s}$ determination), constructed at various acetylcholine concentrations, intersect at a common point situated in the upper left quadrant. This is evidence of the competing character of the ACE and BuCE inhibition by carbamates I and II (the $K_{i,s}$ values for the other carbamates were not determined).

Prozerin inhibits both enzymes in the irreversible mode, as characterized by the values $k_{II} = 2.0 \times 10^6$ and 2.1×10^5 $M^{-1} \cdot min^{-1}$ for ACE and BuCE, respectively.

It was found that prozerin exhibited pharmacological effects typical of anticholinesterases. For example, prozerin introduced at a concentration of 0.01 mM enhanced the contraction of both diaphragm and intestine (peristaltic and tonic) and produced a tenfold increase in the sensitivity of both organs toward acetylcholine. Different behavior was observed for carbamates I – V. These compounds (at a concentration of 0.5 – 2.0 mM) also increased (albeit to a lower extent) the sensitivity of the diaphragm to acetylcholine (reducing the blocking concentration to 1/2 – 1/4 of the initial level). However, the acetylcholine sensitivity of the ileum against the background of compounds I – V (0.01 – 0.2 mM) decreased (up to complete vanishing). Moreover, compounds I – V blocked the contractile response to KCl (3×10^{-2} M) and decreased the basal tension and spontaneous motor activity.

The spasmolytic action of carbamates I – V upon the intestine can be explained by their calcium-antagonist properties revealed by experiments with calcium-free potassium-depolarizing solution. Similarly to verapamil, compounds I –

TABLE 2. Anticholinesterase and Calcium-Antagonist Activity of Compounds I – V

Compound	Anti-ACE			Anti-BuCE			Ca^{2+} antagonist activity (EC_{50}, M)
	k_a for I – V, k_{II} for prozerin, $M^{-1} \cdot min^{-1}$	$K_{i,r}$, M	$K_{i,s}$, M	k_a for I – V, k_{II} for prozerin, $M^{-1} \cdot min^{-1}$	$K_{i,r}$, M	$K_{i,s}$, M	
I	3.6×10^2	1.9×10^{-2}	4.3×10^{-3}	2.5×10^2	1.3×10^{-3}	4.2×10^{-3}	5×10^{-4}
II	5.9×10^2	8.2×10^{-3}	2.2×10^{-3}	3.1×10^2	1.6×10^{-3}	1.3×10^{-3}	4×10^{-4}
III	2.0×10^2	1.3×10^{-3}	–	3.8×10^2	7.1×10^{-4}	...	9×10^{-5}
IV	7.9×10^2	2.6×10^{-4}	–	2.9×10^2	2.0×10^{-4}	...	5×10^{-5}
V	7.7×10^2	1.6×10^{-4}	–	3.3×10^2	8.0×10^{-5}	...	1×10^{-5}
Prozerin	2.0×10^6	–	–	1.6×10^{-5}	–	–	–
Verapamil	–	–	–	–	–	–	3.3×10^{-8}

inhibit the contractile effect of $CaCl_2$ (4 mM). Increasing the $CaCl_2$ concentration 1.5 – 2 times reduces the spasmolytic effect of both verapamil and compounds I – V. The calcium-antagonist activity was maximum in N-arylcarbamates (IV and V) and minimum in N-methylcarbamates (I and II). Prozerin showed no calcium-antagonist activity.

There was a reliable ($p < 0.05$) correlation between the calcium-antagonist efficiency and the degree of reversible inhibition of ACE ($r = 0.94$) and BuCE ($r = 0.92$), that is, between the EC_{50} and $K_{i,r}$ values. This fact can be explain by definite similarity of the binding sites for compounds I – V on cholinesterases and their binding sites on the potential-dependent calcium channels. As for the correlation between EC_{50} and k_a, this was characterized by negative coefficients ($r = -0.38$ and $r = -0.62$ for ACE and BuCE, respectively) and was unreliable ($p > 0.1$).

Thus, the results of our investigation complemented the previous data [1 – 4] and indicated the possibility of a directed search for compounds whose molecules combine the properties of cholinesteraze inhibitors and Ca^{2+} antagonists.

REFERENCES

1. Yu. Ya. Ivanov, V. B. Sokolov, T. A. Epishina, et al., *Dokl. Ross. Akad. Nauk.*, **328**(6), 744 – 746 (1993).
2. Yu. Ya. Ivanov, *Khim.-Farm. Zh.*, **29**(1), 23 – 25 (1995).
3. Yu. Ya. Ivanov, *Éksp. Klin. Farmakol.*, **58**(5), 6 – 8 (1995).
4. Yu. Ya. Ivanov and N. M. Smirnova, *Khim.-Farm. Zh.*, **29**(11), 19 – 20 (1995).
5. A. P. Brestkin, I. L. Brik, R. I. Volkova, et al., *Dokl. Akad. Nauk SSSR*, **200**(1), 103 – 106 (1971).
6. A. P. Brestkin and N. N. Godovikov, *Usp. Khim.*, **47**(9), 1609 – 1627 (1978).
7. M. Dixon and E. Webb, *Enzymes*, Longman, London (1979).
8. V. A. Yakovlev, *Kinetics of Enzymatic Catalysis* [in Russian], Nauka, Moscow (1965), pp. 113 – 125.
9. E. Bulbring, *Br. J. Pharmacol.*, **1**, 38 – 61 (1946).
10. I. V. Markova, I. N. Bel'gova, and L. A. Voronkova, *Farmakol. Toksikol.*, **48**(5), 72 – 75 (1985).
11. G. Ya. Shvarts, E. A. Rumyantsev, R. D. Syubaev, et al., *Farmakol. Toksikol.*, **54**(2), 40 – 43 (1991).