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Effect of Cholinergic Drugs on the Activity of Basic Carboxypeptidases in Rat Nervous Tissue

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Abstract—Effects of a single administration of cholinergic drugs (arecoline, atropine, nicotine, mecamylamine) on the activity of carboxypeptidase H and of phenylmethylsulfonyl fluoride-inhibited carboxypeptidase, which are involved in metabolism of neuropeptides, were studied in brain parts and the adrenal glands of rats. The enzyme activities were determined fluorimetrically using specific inhibitors and substrates. In the majority of cases the enzyme activities decreased, and this decrease was retained for at least 72 h. Changes in the activities of the studied enzymes depended on the type of cholinergic action, the nervous system part, and the time after the injection. The changes in activities of the studied carboxypeptidases are supposed to be a possible mechanism responsible for changes in the levels of neuropeptides under the influence of high doses of the drugs.

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Key words: nervous system, carboxypeptidases, cholinergic drugs

The primary pharmacological reaction of central cholinergic drugs is generally thought to be associated with activation or inhibition of cholinoreceptors [1]. However, it is difficult to explain the diversity of pharmacological effects (in particular, tranquilization, analgesia, etc.) of such drugs only from this standpoint. Data on effects of these drugs on metabolism of mediators (noradrenaline, dopamine, serotonin), phospholipids, and protein synthesis [1] do not sufficiently explain the mechanism of their action.

The contribution of the peptidergic system to the action mechanisms of cholinergic drugs is now widely discussed [1-4]. Injections of these drugs are accompanied by significant changes in the levels of various neuropeptides [1, 5-11], many of which play important roles in etiology and pathogenesis of alcoholism, narcotics addition, Parkinson's disease, Alzheimer's disease, i.e. diseases in which the cholinergic system is involved [2, 12, 13]. It seems that many physiological effects caused by changes in cholinergic system functioning are mediated through the peptidergic system [1, 14]. Molecular mechanisms of action of cholinergic drugs at the levels of biologically active peptides are unclear. The active forms of regulatory peptides are produced as a result of post-translational modification of propeptides [15-17]. Basic carboxypeptidases, exopeptidases, in particular carboxypeptidase H (CPH) and phenylmethylsulfonyl fluoride-inhibited carboxypeptidase (PMSF-CP), which catalyze the cleavage of arginine and lysine residues from the C-terminus of propeptides, play the leading role in the final stages of this process [18, 19].

The purpose of this work was to study the effects of arecoline, atropine, nicotine, and mecamylamine on the activities of CPH and PMSF-CP in the brain and adrenals of rats.

MATERIALS AND METHODS

The experiments were performed on randomly bred white male rats with body weight of 200-300 g and age of 140-160 days. The animals were kept under standard conditions of the vivarium. The drugs dissolved in saline were injected intraperitoneally. The injected volume was 2.5 ml per kg body weight. Doses of the drugs were as follows

Abbreviations: CPH, carboxypeptidase H; FSH, follicle-stimulating hormone; GABA, γ-aminobutyric acid; GEMSA, guanidine ethylmercaptosuccinic acid; GnRF, gonadotropin-releasing factor; LH, luteinizing hormone; PMSF-CP, phenylmethylsulfonyl fluoride-inhibited carboxypeptidase.

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(per kg body weight): arecoline (2 mg), atropine (2.5 mg), nicotine and mecamylamine (1 mg). Animals of the control group were injected with the same amount of saline. The animals were subjected to anesthesia with chloroform [20] and decapitated 0.5, 4, 24, and 72 h after the injection; the adrenals and the brain were isolated and the brain parts were separated. Tissue samples were homogenized in 50 mM sodium acetate buffer containing 50 mM NaCl (pH 5.6) at the ratio of 1 : 50 (w/v). All procedures with the brain parts were performed at 4°C. Activities of the studied enzymes were determined in the resulting homogenates by fluorimetry [21].

The CPH activity was assessed by hydrolysis of dansyl-Phe-Ala-Arg at pH 5.6 using the highly specific inhibitor of the enzyme guanidine ethylmercaptosuccinic acid (GEMSA) [21]; PMSF-CP activity was determined by hydrolysis of dansyl-Phe-Ala-Arg at pH 5.6 with PMSF as an inhibitor of the enzyme [18]. The experimental samples contained 50 μl of homogenate and 150 μl of 50 mM sodium acetate buffer supplemented with 50 mM NaCl. The control sample was supplemented with GEMSA (in the case of CPH) or with PMSF (in the case of PMSF-CP) in the final concentration of 1 μM and 1 mM, respectively. The samples were preincubated for 8 min at 37°C, then 50 μl of preheated to 37°C substrate solution (concentration in the sample 42 μM) prepared in the same buffer were added and incubated for 60 min at 37°C. The reaction was stopped by addition of 50 μl of 1 M HCl. To extract the reaction product (dansyl-Phe-Ala in the case of CPH and dansyl-Phe-Leu in the case of PMSF-CP), the samples were supplemented with 1.5 ml of chloroform and intensively shaken for 60 sec. The chloroform and aqueous phases were separated by centrifugation for 10 min at 1000 rpm.

Fluorescence of the chloroform phase was measured using a Fuorat ABLF-2 fluorimeter at λ_{ex} = 360 nm and λ_{em} = 530 nm in a 1-cm cuvette. Solution of dansyl-Phe-Ala in chloroform was used as a standard.

The enzyme activity was determined as the difference in the fluorescence increase in the samples in the presence and in the absence of the inhibitor and expressed in nmol product per minute of incubation calculated per mg protein. The protein amount in the samples was determined by the Lowry method [22].

The normal distribution of the data was monitored using the χ^2-test [23]. The significance of differences between mean values was determined using Student's *t*-test [23].

RESULTS AND DISCUSSION

Data on the enzyme activities under the influence of cholinergic drugs are presented in the table. In most cases, arecoline and atropine caused a pronounced decrease in the activities of both studied enzymes, and in some parts of the brain the decrease was retained for 72 h.

Injections of nicotine and mecamylamine in the dose of 1 mg per kg body weight caused biphasic changes in activities of CPH and PMSF-CP in the majority of the brain parts: the enzyme activities were decreased during the first hours and then they increased.

Most often, arecoline, atropine, nicotine, and mecamylamine caused a decrease in activities of both CPH and PMSF-CP in the majority of parts of the nervous system of rats. These results are in good agreement with data on the inhibition of functioning of the central nervous system (suppression of conditioned and unconditioned reflexes, disorders in attention and all types of memory) under the influence of high doses of choline activators and choline inhibitors [1, 2, 5, 6] and also with data on a decrease in the levels of various neuropeptides in the brain and blood serum in response to injection of cholinergic drugs [1, 5-11]. Thus, high concentrations of m-choline activators induced a decrease in the level of the atrial natriuretic peptide in the brain and a decrease in the concentration of neuropeptide Y in the rat hypothalamus [8, 10]. Injections of atropine were accompanied by a pronounced decrease in the levels of Met- and Leu-enkephalins and of β-endorphin in rat brain and blood serum [1]. Moreover, inhibition of the m-choline-reactive system observed under the influence of atropine was accompanied by a decrease in the somatostatin level in the rat hippocampus [8]. High doses of nicotine caused changes in blood serum concentrations of various peptides: ACTH, somatostatin, prolactin, luteinizing and follicle-stimulating hormones (LH and FSH, respectively), vasopressin, β-endorphin, and gonadotropin-releasing factor (GnRF) [16]. Injections of inhibitors of n-cholinoreceptors led to a decrease in the level of atrial natriuretic peptide in the brain [5, 6].

It is known that CPH is involved in the processing of ACTH precursors, enkephalins, vasopressin, atrial natriuretic peptide, and of many other precursor forms of biologically active peptides [18, 19]. Based on the amino acid sequence of these peptides and on the PMSF-CP substrate specificity, it was suggested that PMSF-inhibited carboxypeptidase, in addition to CPH, could be involved in the processing of precursors of these regulatory peptides [18, 24, 25]. Thus, a decrease in the levels of biologically active peptides by cholinergic drugs seems, in particular, to be caused by a decrease in activities of the enzymes CPH and PMSF-CP that are responsible for their metabolism.

A decrease in the levels of neuropeptides in the brain and blood serum of animals seems to be a mechanism through which suppressor effects of cholinomimetics and cholinolytics on the functioning of the central nervous system are mediated. This is confirmed by data that the psychomotor excitement, tachycardia, and memory disorders caused by high doses of cholinolytics can be abol-

Effects of a single injection of cholinergic drugs on the activity of carboxypeptidase H (CPH) and of phenylmethylsulfonyl fluoride-inhibited carboxypeptidase (PMSF-CP) in brain parts and in adrenals of rats (control is taken for 100%, mean ± S.E.M., $n = 6$)

Tissue	CPH activity, %				PMSF-CP activity, %			
	time after injection, h				time after injection, h			
	0.5	4	24	72	0.5	4	24	72
	Arecoline							
Hypophysis	96 ± 6	66 ± 8*	54 ± 6**	86 ± 8	44 ± 7**	45 ± 2***	30 ± 2***	98 ± 7
Olfactory lobe	106 ± 9	65 ± 6*	51 ± 9*	70 ± 7*	93 ± 6	65 ± 6**	78 ± 5*	60 ± 2***
Mesencephalon	96 ± 9	100 ± 7	49 ± 6*	60 ± 4**	91 ± 9	40 ± 5*	65 ± 7*	104 ± 6
Medulla oblongata	84 ± 7*	41 ± 4**	93 ± 8	52 ± 7**	96 ± 9	60 ± 3**	92 ± 8	106 ± 7
Hypothalamus	96 ± 10	74 ± 12	60 ± 5**	45 ± 4***	95 ± 8	55 ± 6*	96 ± 9	55 ± 6*
Hippocampus	72 ± 9*	65 ± 3***	106 ± 11	46 ± 7**	90 ± 9	81 ± 9	115 ± 12	110 ± 12
Striatum	70 ± 6**	67 ± 9*	76 ± 9	40 ± 8**	96 ± 10	55 ± 3***	117 ± 11	85 ± 8
Adrenals	46 ± 4**	90 ± 9	104 ± 9	90 ± 5	52 ± 2***	40 ± 3***	110 ± 10	80 ± 11
	Atropine							
Hypophysis	65 ± 7*	75 ± 4**	96 ± 9	87 ± 8	25 ± 15*	5 ± 1***	97 ± 12	120 ± 18
Olfactory lobe	106 ± 7	94 ± 7	45 ± 5**	89 ± 2*	75 ± 19	60 ± 6***	70 ± 9*	105 ± 7
Mesencephalon	86 ± 4*	87 ± 8	48 ± 6*	39 ± 4**	99 ± 5	54 ± 9*	110 ± 7	103 ± 15
Medulla oblongata	100 ± 8	54 ± 5**	87 ± 7	53 ± 4**	92 ± 8	84 ± 11	88 ± 8	111 ± 8
Hypothalamus	56 ± 5**	65 ± 8*	60 ± 7*	65 ± 5**	80 ± 15	90 ± 9	85 ± 11	57 ± 15*
Hippocampus	52 ± 10*	56 ± 6**	79 ± 14	70 ± 6*	51 ± 6**	115 ± 13	50 ± 15*	87 ± 13
Striatum	76 ± 3**	65 ± 9*	94 ± 5	66 ± 8*	101 ± 11	60 ± 4***	112 ± 8	105 ± 8
Adrenals	80 ± 14	110 ± 15	113 ± 14	45 ± 14*	62 ± 5**	30 ± 2***	88 ± 7	77 ± 12
	Nicotine							
Hypophysis	96 ± 5	54 ± 4***	66 ± 20	89 ± 9	5 ± 1.5**	8 ± 1***	14 ± 5**	191 ± 21**
Olfactory lobe	96 ± 11	86 ± 8	60 ± 9*	97 ± 7	85 ± 12	58 ± 4***	96 ± 5	129 ± 21
Mesencephalon	90 ± 6	89 ± 6	76 ± 16	65 ± 9*	103 ± 9	86 ± 9	91 ± 1	103 ± 12
Medulla oblongata	95 ± 8	57 ± 5**	86 ± 8	70 ± 9*	92 ± 9	80 ± 6*	94 ± 6	95 ± 7
Hypothalamus	96 ± 5	94 ± 7	59 ± 7**	90 ± 8	94 ± 10	115 ± 11	110 ± 10	64 ± 5**
Hippocampus	57 ± 15*	80 ± 3**	99 ± 5	105 ± 9	52 ± 15*	95 ± 10	125 ± 18	112 ± 10
Striatum	53 ± 7**	73 ± 13	86 ± 10	84 ± 15	53 ± 3***	61 ± 7**	127 ± 11*	136 ± 24
Adrenals	46 ± 8**	9 ± 1***	90 ± 9	156 ± 13*	27 ± 4***	32 ± 2***	106 ± 9	98 ± 8
	Mecamylamine							
Hypophysis	116 ± 4*	28 ± 3***	88 ± 9	119 ± 13	24 ± 12**	21 ± 1***	50 ± 18*	139 ± 28
Olfactory lobe	110 ± 13	83 ± 5*	104 ± 9	94 ± 9	109 ± 10	45 ± 4***	54 ± 9**	103 ± 7
Mesencephalon	99 ± 9	106 ± 8	77 ± 15	70 ± 9*	87 ± 9	75 ± 20	70 ± 22	94 ± 8
Medulla oblongata	80 ± 58	74 ± 8	95 ± 8	90 ± 10	70 ± 19	75 ± 7*	106 ± 10	92 ± 9
Hypothalamus	88 ± 9	90 ± 10	90 ± 9	85 ± 4*	118 ± 12	80 ± 14	89 ± 10	104 ± 9
Hippocampus	88 ± 11	90 ± 12	107 ± 4	90 ± 6	80 ± 15	80 ± 14	119 ± 18	52 ± 10**
Striatum	87 ± 1*	110 ± 10	113 ± 11	56 ± 8**	56 ± 8**	99 ± 9	114 ± 12	124 ± 17
Adrenals	45 ± 18*	95 ± 9	224 ± 27**	177 ± 19*	70 ± 11*	40 ± 3***	107 ± 11	91 ± 10

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ relative to control.

ished by injections of Met- and Leu-enkephalins, β-endorphin, arginyl-vasopressin, and also of the *C*-terminal oxytocin tripeptide prolyl-leucyl-glycinamide. Repeated injections of cholinolytics in psychotoxic doses decreased manifestations of psychopathological symptoms [1], which suggested the development of tolerance. Blood plasma of tolerant animals displayed a protective effect in recipients injected once with a high dose of cholinolytics. The appearance of an excess of enkephalins and β-endorphin during the development of the tolerance suggests that these opioid peptides can act as a tolerance factor [1].

These hypotheses are in agreement with our findings. The effects of cholinergic drugs on the activities of CPH and PMSF-CP in male rats were most pronounced in the hypothesis, striatum, and adrenals – the parts where opioid peptides are synthesized and are present in high concentrations [26]. Both basic carboxypeptidases can be involved in the response of hypophysis, striatum, and adrenals to changes in the state of the cholinergic system. Considering the distribution of the studied enzymes in rat nervous tissue, it was suggested that in the hypophysis and adrenals this function is mainly performed by CPH and PMSF-CP, respectively. However, injection of nicotine had the strongest effect on the CPH activity in the adrenals, whereas changes in the PMSF-CP activity were the most pronounced in the hypophysis. This seems to indicate that such separation of functions is not absolute, and both carboxypeptidases can be involved in metabolism of regulatory peptides in all parts of the nervous system.

Injection of nicotine induced an increase in the CPH activity in the adrenals after 72 h, and the PMSF-CP activity in the hypophysis increased after 72 h and in the striatum after 24 h. The increase in activities of the enzymes responsible for the processing of neuropeptides in the hypophysis, striatum, and adrenals 24 and 72 h after the injection of nicotine is in agreement with the data on a biphasic character of the action of nicotine on the nervous system: an initial suppression of functions changes to a subsequent excitement [5, 6]. This is accompanied by two phases in changes in the levels of such regulatory peptides as ACTH, somatotropin, prolactin, vasopressin, and β-endorphin [5]. A significant decrease in the activities of the carboxypeptidases observed 0.5 and 4 h after the injection of nicotine and a subsequent increase in their activities after 24 h in the striatum and after 72 h in the hypophysis and adrenals is likely to be a mechanism involved in manifestation of the biphasic action of nicotine.

The increase in the CPH activity observed in the hypophysis 0.5 h after the injection of an n-cholinoreceptor antagonist mecamylamine seemed to indicate a contribution of this enzyme to a transient increase in the concentrations of LH and FSH recorded on the injection of mecamylamine in the dose of 1 mg/kg [5, 14], which could also mediate an increase in the CPH activity in the adrenals 24 and 72 h after the injection of mecamylamine.

The strongest effect of nicotine on activities of both enzymes was also recorded in the hypophysis. Hypophysis is the place of synthesis of LH and FSH [26]. It seems that an increase in the concentration of n-cholinoreceptor agonists in the brain on the parenteral injection of nicotine affects, first of all, the hypophysis and adrenals. Some data indicate an involvement of the n-cholinergic system in the control of tonic secretion of gonadotropins in females [16]. Thus, changes in activities of the studied carboxypeptidases in the rat hypophysis correlate with changes in the FSH secretion in the presence of nicotine [5]. And changes in the activities of these enzymes, which are involved in the processing of precursors of pituitary gonadotropic hormones, can be a mechanism of the nicotine-induced decrease in the synthesis and secretion of LH and FSH.

In rat hypothalamus a decrease in PMSF-CP and CPH activities was found, respectively, 72 and 24 h after the injection of nicotine. Nicotine is known to slightly inhibit secretion of arginine-vasopressin from the paraventricular and supraoptic nuclei of the hypothalamus [5]. Considering the substrate specificity of the two carboxypeptidases, they are supposed to be involved in the processing of preproGnRF. Because nicotine decreases the synthesis and secretion of GnRF [5, 7], the decrease in the activities of CPH and PMSF-inhibited CP in the rat hypothalamus can be a mode of regulation of GnRF by n-cholinoreactive neurons.

Cholinergic drugs are likely to influence the level of oxytocin mRNA in the paraventricular nucleus of the hypothalamus (and also various other physiological processes) by modulating receptors of γ-aminobutyric acid (GABA). GABA is involved in the regulation of release of many hormones secreted by the adenohypophysis [10]. GABA and its receptors can contribute to secretion of LH [27, 28]. The CPH activity is decreased in all parts of the brain on injection of γ-hydroxybutyric acid [29]. A presynaptic action of cholinolytics leads to an increase in the concentration of endogenous GABA and, as a result, to a decrease in the enzyme activity.

In addition to the GABAergic system, the effect of m-cholinergic drugs on the CPH and PMSF-CP activities can be mediated through the adrenergic, dopaminergic, and serotoninergic systems. The release of these neuromediators from presynaptic terminals is controlled by presynaptic m-cholinoreceptors [1]. Their binding with arecoline inhibits the release of dopamine, adrenaline, and serotonin in the synapses, whereas binding with atropine increases the release of these neuromediators. The systems of these neuromediators control synthesis and secretion of such neuropeptides as somatostatin, GnRF, prolactin, LH, and FSH [8, 28], which are processed with involvement of the studied enzymes.

The action of m-cholinomimetics on m-cholinoreceptors triggers the system of secondary messengers represented by G-proteins, 3′,5′-AMP, and GMP cyclase (in the case of m_2 and m_4 receptors) [1, 14]. As a result, secondary messengers cyclic guanosine monophosphate (cGMP) and cyclic adenosine monophosphate (cAMP) appear in neurons in increased amounts. Both cAMP and cGMP promote the release of contents of secretory vesicles [17, 30]. Therefore, cAMP- and cGMP-dependent mechanisms also can be involved in the regulation of carboxypeptidase activities under the influence of cholinergic drugs.

n-Cholinomimetics are known to promote the opening of ion channels in the plasma membrane and to stimulate phosphorylation of tyrosine residues in proteins [1]. Arecoline, atropine, and nicotine are involved in the regulation of intracellular concentration of Ca^{2+} [1]. Calcium ions influence the release of the contents of secretory vesicles by facilitating the fusion of synaptic vesicles with presynaptic membranes [17, 31]. Although Ca^{2+} fails to directly influence the activity of CPH [31], the enzyme has binding site for these ions. Calcium ions promote the aggregation of CPH and its binding with the membrane [31]. The biological roles of soluble and membrane-bound CPH seem to be different [18, 31]. Both these forms are supposed to be involved in processing, but the membrane-bound form can also contribute to sorting peptides [18]. Moreover, the activity of prohormone convertase (an enzyme involved in the processing of proCPN) is shown to be regulated by Ca^{2+} [30, 31]. Therefore, it is suggested that the action of cholinergic drugs on the CPH activity can be mediated through the intracellular concentration of Ca^{2+}. This can lead to changes in the enzyme activity and also in the ratio between the soluble and membrane-bound forms, which in turn influences the processing and sorting of biologically active peptides.

Steroids, e.g. glucocorticoids, influence the gene expression level [15]. Glucocorticoids influence the expression of CPH mRNA [31], in particular, dexamethasone decreases the level of CPH mRNA in AtT-20 cells [31]. Cholinergic drugs are known to change levels of cortisone and aldosterone [1]. Therefore, the action of cholinergic drugs on the activities of CPH and PMSF-CP is supposed to be realized through changes in the expression of the corresponding genes. Moreover, we found that changes in CPH and PMSF-CP activities in the nervous system of rats were the most pronounced 24 and 72 h after injections of the drugs. This indirectly confirms the hypothetical action mechanism of cholinergic drugs on the activities of the studied carboxypeptidases.

Thus, both PMSF-CP and CPH seem to play an important role in the regulation of levels of active forms of neuropeptides on injection of cholinergic drugs. These enzymes seem to be involved in the regulation of levels of Met- and Leu-enkephalins, GnRF, LH, FSH, oxytocin, vasopressin, prolactin, and other biologically active peptides responsible for the control of functioning of many regulatory systems involved in processes of memory, attention, learning, and other function of the central nervous system that are influenced on injections of cholinomimetics and cholinolytics. Both CPH and PMSF-CP are likely to be involved in the processing not only of various biological peptides but also in the processing of the same peptides in different sections of the nervous system.

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