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Semax and Selank Inhibit the Enkephalin-Degrading Enzymes of Human Serum

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Abstract—Dose-dependent effect of synthetic heptapeptides Semax (Met-Glu-His-Phe-Pro-Gly-Pro) and Selank (Thr-Lys-Pro-Arg-Pro-Gly-Pro) on the enkephalin-degrading enzymes of human serum was demonstrated. The inhibitory effects of Semax (IC_{50} 10 μM) and Selank (IC_{50} 20 μM) are more pronounced than that of puromycin (IC_{50} 10 mM), bacitracin, and some other inhibitors of peptidases. Beside the heptapeptides, their pentapeptide fragments also possessed an inhibitory effect; tri-, tetra- and hexapeptide fragments did not display such an effect. As the above enzymes take part in degradation of not only enkephalins but also other regulatory peptides, it can be assumed that one of the mechanisms of biological activity of Semax and Selank is related to this inhibitory activity of theirs.

Key words: *Semax, Selank, enkephalinases, inhibitors; regulatory peptides; opioid system*

INTRODUCTION

This study deals with two heptapeptides, Semax (Met-Glu-His-Phe-Pro-Gly-Pro) and Selank (Thr-Lys-Pro-Arg-Pro-Gly-Pro), previously synthesized by us on the basis of the biologically active fragment of adrenocorticotropic hormone (4–7) and tetrapeptide taftsin, respectively, with addition of the Pro-Gly-Pro sequence to the *C*-terminus of the peptides to stabilize them [1, 2].

Both heptapeptides can positively affect the CNS state. Thus, Selank exhibits a pronounced antistressor, anxiolytic effect and optimizes the processes of learning and memory formation [2, 3]. Semax displays a nootropic effect, enhances the selective attention at the moment of receiving information, improves the consolidation of memory, augments the learning ability, and widens the brain adaptability by increasing its stability to stressor damage, hypobaric and vascular hypoxia [4, 5]. Clinical studies revealed a high efficiency of Semax in the treatment of intellectual-mnestic disorders and asthenic states of various etiologies [4], as well as in the treatment of brain insult [6]. *In vivo* studies demonstrated the ability of Semax to increase the viability of nervous cells in rat brain embryonal cultures [7]. The radioreceptor method revealed the specific binding sites for Semax on the membrane fraction of the rat brain cells [7].

It should be noted that some biological effects of Semax and Selank are similar to the effects mediated by endogenous opioids. Thus, Semax, like opioid peptides [8–10], exhibits the growth factor properties in the culture of neural and other tissues, stimulates learning and memory processes whereas Selank, similarly to opioids [11], possesses an anxiolytic effect.

We therefore assumed that one of the mechanisms of action of Semax and Selank is related to their effect on the endogenous opioid system, which can be due to both direct interaction of the peptides with the opioid receptors and their influence on the activity of the enzymes of the endogenous opioids processing or degradation. The high activity of enzymes degrading endogenous opioid peptides determines their rapid digestion, for example, the half-life time of enkephalins in blood is only 1–2 min [12].

In this context, the present work was aimed to studying the ability of Semax and Selank and their peptide fragments to affect the rate of [Leu]enkephalin degradation by human serum enzymes.

RESULTS AND DISCUSSION

It is known that at least seven enzymes hydrolyzing enkephalins at different peptide bonds are present in the human serum [13]. Previously we showed [14] that kinetics of degradation of [Leu]enkephalin in this multienzyme system under the conditions employed in this study satisfies the pseudosteady-state criteria: the reaction rate depends linearly on the serum dilution and

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remains constant during 30-min incubation. Therefore, the influence of studied peptides on the human serum enzymes activity was assessed by the change in the accumulation of the products of 0.15 μM [[^{3}H]Leu]enkephalin cleavage during 15-min incubation. The digestion rate for [Leu]enkephalin in inhibitors-free incubation medium was 5.0 ± 0.2 nM/min. The inhibition extent was determined relative to this control. As references, commercial inhibitors of proteolytic enzymes were used: *N*-carboxymethyl-Phe-Leu (CMPL), *D*-phenylalanine methyl ester (*D*-PAM), bacitracin, leupeptin, and puromycin.

It was found that both Semax and Selank considerably decelerate the [Leu]enkephalin degradation by the human serum enzymes (Fig. 1). The dose-dependence curves of this effect for both heptapeptides are practically equal, and the concentration of peptides at which 50% inhibition of the enzymes is observed (IC_{50}) is about 10 μM for Semax and 15–20 μM for Selank. In the same conditions, all inhibitors used for comparison proved considerably less active (Fig. 1).

The serum enkephalin-degrading enzymes include aminopeptidases, providing about 70% of the overall enkephalinase activity, and more enkephalin-specific diamino- and dicarboxypeptidases, such as endopeptidase 24-11 (enkephalinase B), angiotensin-converting enzyme, and enkephalinase A [13]. The inhibitors used in the present work for comparison differ in specificity toward various types of the enkephalin-degrading enzymes. Thus, a specific inhibitor of enkephalinases CMPL at the maximum concentration attainable with its low solubility in aqueous solutions (50 μM) reduced the degradation rate of enkephalin by not more than 25% (Fig. 1). *D*-PAM and puromycin within the entire concentration range studied are also less active than Semax and Selank. Leupeptin, an inhibitor of serine and leucine proteinases, which also possesses a peptide nature, is the most similar in efficiency to the heptapeptides under study and has IC_{50} about 50 μM (Fig. 1). A peptidase inhibitor conventionally used in the radioreceptor and other biochemical studies is bacitracin, whose content in its preparations, like in the case of antibiotics, is evaluated in activity units. This study showed that bacitracin in the most usable concentration of 50 μg/ml (3 int. units/ml) reduces the enkephalinase activity of serum by 50%. In the same weight concentration, which corresponds to approximately 50 μM, the heptapeptides cause 70% inhibition of the enzymes.

Puromycin and bacitracin preferentially interact with aminopeptidases, providing, as noted above, for a major portion of the overall enkephalinase activity. The relatively low extent of inhibition of enkephalin hydrolysis by puromycin and bacitracin may be due to both the presence of puromycin-nonsensitive aminopeptidase in serum and a hypothetical increase in the rate of enkephalin hydrolysis by dipeptidylpeptidases through the aminopeptidase inhibition at low substrate concentrations.

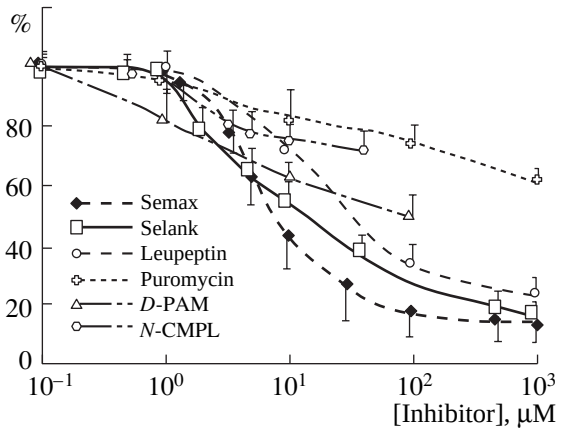

Fig. 1. Effect of Semax, Selank, and standard inhibitors of proteolytic enzymes on the rate of degradation of [Leu]enkephalin by the human serum enzymes. *x* axis, concentrations of the inhibitors in the logarithmic scale; *y* axis, residual activity.

It can be assumed that Semax and Selank interact with the serum enkephalin-degrading enzymes and are, like enkephalins, their substrates. This is supported by the data that the half-life time of Semax and Selank in blood is, like in the case of enkephalins, only several minutes. The processes of enzymatic hydrolysis of these peptides are similar: degradation of Semax, like that of enkephalins, begins with the splitting of the *N*-terminal amino acid [15].

The next step of the study included a comparison of the inhibitory capacities of the peptide fragments of the heptapeptides studied: tripeptide Pro-Gly-Pro, common for Semax and Selank, and their *C*-terminal tetra-, penta-, and hexapeptides:

Semax	Selank	aa number
Met-Glu-His-Phe-Pro-Gly-Pro	Thr-Lys-Pro-Arg-Pro-Gly-Pro	7
Glu-His-Phe-Pro-Gly-Pro	Lys-Pro-Arg-Pro-Gly-Pro	6
His-Phe-Pro-Gly-Pro	Pro-Arg-Pro-Gly-Pro	5
Phe-Pro-Gly-Pro	–	4
Pro-Gly-Pro	Pro-Gly-Pro	3

It was thus shown (Fig. 2) that the pentapeptide fragments of Semax and Selank reduce the rate of enzymatic hydrolysis of [Leu]enkephalin to 60% of the control at a concentration of 0.5 mM whereas for the native heptapeptides the same effect was observed at micromolar concentrations (see Fig. 1). The tri-, tetra-, and hexapeptide fragments at 0.5 mM concentrations do not exert any substantial effect on the serum enkephalinases. The fact that the Semax hexapeptide fragment, relatively stable in the organism, does not practically reduce the serum enkephalinase activity (Fig. 2) evidences that the inhibition of enkephalin degradation by the heptapeptides proceeds through their competition

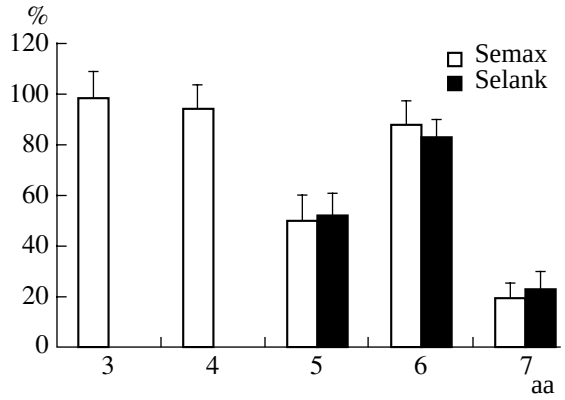

Fig. 2. Inhibitory effect of Semax, Selank, and their peptide fragments in 0.5 mM concentrations on the enkephalinase activity of human serum. *y* axis, residual activity.

with the substrate for the active sites of enzymes, in particular, aminopeptidases.

It can therefore be assumed that one of the mechanisms of biological action of Semax and Selank is related to their ability to inhibit enzymes degrading biologically active peptides, in particular, enkephalins. Enzymatic degradation of enkephalins, determining their very short life-time, is supposed to largely limit both their peripheral and central biological effects. The inhibition of enkephalin-degrading enzymes by Semax and Selank may be the reason for the fact that a number of their central effects [3, 4] are similar to the behavioral effects of opioids [10, 11]. A component of the nootropic and antiischemic action of Semax [5] and Selank [16] may also be related to an increase in the opioid peptides level in the brain tissues, because a possibility of the repairing action of opioids was shown with dalargin, a synthetic analogue of [Leu]enkephalin, as an example [17, 18].

The additional importance of these results is due to the fact that the enzymes hydrolyzing the endogenous opioid peptides, in particular, enkephalinases, take part to an extent in the degradation of other regulatory peptides—substance P, bradykinin, angiotensin, and others [12]. Thus, it can be assumed that some biological effects of Semax and Selank are related to the regulation of the content of regulatory peptides through the inhibition of their degradation enzymes.

EXPERIMENTAL

Serum was obtained from five healthy volunteers by a standard procedure including 30-min incubation of venous blood at 37°C followed by outlining the clot with a glass rod and centrifuging (1000 *g*, 10 min, 4°C). The resulting sera were pooled and, as 0.2-ml aliquots, frozen and stored at –20°C.

Enkephalinase activity was determined by the rate of accumulation of the radioactive products of the enzymatic degradation of [[^{3}H]Leu]enkephalin using the modified method [14]. The incubation mixture (final volume 50 μl) contained tenfold diluted serum, 10 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 1 μCi (150 nM) of [[^{3}H]Leu]enkephalin with a specific radioactivity of 120 Ci/mmol, obtained as described in [19], and the peptides to be tested at the corresponding concentrations. Incubation was carried out for 15 min at 37°C and stopped by the addition of 5 μl of 0.2 M HCl.

Radioactive products of [Leu]enkephalin digestion were separated by TLC on silica gel plates (Merck, Germany) in a 40 : 40 : 1 : 19 ethyl acetate– isopropanol–water–acetic acid system. As markers corresponding to the reaction products, peptides Tyr-Gly, Tyr-Gly-Gly-Phe (Serva), and Tyr-Gly-Gly, as well as tyrosine and cold [Leu]enkephalin (Sigma) served. The R_f values were 0.54 for [Leu]enkephalin and 0.25–0.42 for digestion products. The zones corresponding to the marker positions were cut out, placed into scintillation flasks, treated with 0.4 ml of water (30-min stirring) to extract the peptide fragments from silica gel, mixed with 5 ml of the scintillation cocktail, and counted on a liquid scintillation spectrometer. Each point was determined as an average of 3–5 independent experiments.

Semax, Selank, and their peptide fragments were synthesized by the classic methods of peptide chemistry as described in [1, 20].

[Leu]enkephalin, puromycin, leupeptin, *N*-carboxymethyl-Phe-Leu, *D*-phenylalanine methyl ester, and bacitracin (59400 int. units/g) were obtained from Sigma.

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