Amino Acids (2006) 30: 403–408
DOI 10.1007/s00726-006-0328-8

Amino Acids
Printed in Austria

Degradation of the ACTH(4-10) analog Semax in the presence of rat basal forebrain cell cultures and plasma membranes

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Received November 1, 2005
Accepted December 29, 2005
Published online May 26, 2006;

Summary. Here a new approach of the elucidation of paths of proteolytic biodegradation of physiologically active peptides, based on the use of a peptide with isotopic label at all amino acid residues and the enrichment of HPLC samples with unlabeled peptide fragments in UV-detectable concentration, has been proposed. The method has been applied for the investigation of degradation dynamics of the neuroactive heptapeptide MEHFPGP (Semax) in the presence of plasma membranes, and cultures of glial and neuronal cells obtained from the rat basal forebrain. The splitting away of ME and GP, and formation of pentapeptides are the predominant processes in the presence of all tested objects, whereas the difference in patterns of resulting peptide products for glial and neuronal cells has been detected. In conclusion, the approach applied allows analyzing physiologically active peptide concentrations in biological tissues and degradation pathways of peptides in the presence of targets of their action.

Keywords: ACTH(4-10) analog – Melanocortins – Degradation – HPLC – Glia – Neurons

Abbreviations: ACTH, adrenocorticotropic hormone; BDNF, brain-derived neurotrophic factor; HPLC, high pressure liquid chromatography; HSCIE, high-temperature solid-state catalytic isotope exchange; NGF, nerve growth factor; NMR, nuclear magnetic resonance.

Introduction

The N-terminal fragments of the adrenocorticotropic hormone (ACTH) and their analogs (melanocortins) possess a complex of neurotrophic and behavioral properties (Hol et al., 1995; Wikberg, 2001), and may serve as a basis for the development of pharmacological remedies affecting the processes of memory formation and nervous system regeneration. The peptides ACTH(4-7) (MEHF) and ACTH(4-10) (MEHFRWG), both able to stimulate the learning of animals and showing no hormonal activity of ACTH, proved to be the most perspective melanocortins (De Wied et al., 1975; De Wied and Gispen, 1977). Elevation of the stability of the peptides to the action of peptidases, due to the substitution of L-amino acids for D-isomers, formed the principal path for the development of pharmacological preparations on the basis of these peptides (Greven and de Wied, 1973; Hock et al., 1988; Attella et al., 1992). One more approach to the increase of peptide's proteolytic stability was the introduction of a terminal oligopeptide sequence enriched with proline residues (Potaman et al., 1993). Such a substitution of the RWG sequence for PGP in ACTH(4-10) resulted in its stability increase in the presence of human blood serum and in longer duration of the modified peptide's effect as compared with natural peptide (Ashmarin et al., 1995). The peptide MEHFPGP has demonstrated the efficiency as a remedy that stimulates attention and learning (Ashmarin et al., 1995). This peptide (named Semax) is active component of the novel intranasal drugs, "SEMAX-0.1% Solution" and "SEMAX-1.0% Solution", clinically applied in Russia for the treatment of brain hypoxia and ischemia, brain strokes (Gusev et al., 1997), cranial and brain traumas and to facilitate adaptive processes to extreme situations (Ashmarin et al., 1997). Recently, it was demonstrated that Semax treatment of patients with different stages of cerebrovascular insufficiency resulted in significant clinical improvement, stabilization of the disease progress and reduced a risk of stroke and transitory ischemic attacks in the disease course (Gusev et al., 2005). Additionally, it was shown the efficiency of Semax in the treatment of glaucomatous optic neuropathy in patients with normalized ophthalmic tone (Kurysheva et al., 2001).

We have previously acquired evidence that Semax increases 5- to 7-fold the expression level of mRNA for the nerve growth factor (NGF) and the brain-derived neurotrophic factor (BDNF) in the cell culture of rat basal forebrain glial cells (Shadrina et al., 2001), and that it binds reversibly, time- and calcium-dependently to plasma membranes and increases BDNF protein level in the rat basal forebrain (Dolotov et al., 2005).

One of the principal points in the investigation of the mechanism of action of physiologically active peptides is elucidation of their formation paths in the organism's tissues by processing their precursor polypeptides as well as elucidation of paths of their proteolytic biodegradation. To study this aspect of action of regulatory peptides, a new approach has been proposed, which involves the use of uniformly tritium-labeled peptides (Zolotarev et al., 2004, 2005a). In the present study, we investigated degradation pathway of MEHFPGP (Semax) in the presence of possible targets of its action: primary cultures of rat basal forebrain glial cells and neurons, and adult rat basal forebrain plasma membranes using high pressure liquid chromatography (HPLC) analysis of degradation products from the uniformly labeled with tritium peptide.

Materials and methods

All reagents used were purchased from Sigma (USA), ICN (USA), Merck (Germany) and Serva (Germany) if not otherwise stated. All reagents were of analytical grade. HPLC was carried out using a Beckman chromatograph (USA). All peptides used were synthesized at the Institute of Molecular Genetics, Russian Academy of Sciences.

Labeling of Semax

To produce the uniformly labeled with tritium peptide Semax ([G-^{3}H]MEHFPGP), the high-temperature solid-state catalytic isotope exchange (HSCIE) reaction of organic compounds with gaseous tritium was used (Zolotarev et al., 2005b). 50 mg of alumina (Al_2O_3) was added to a solution of 2.0 mg of Semax in 1 ml of water and the mixture was evaporated in a rotor evaporator. Alumina with applied peptide was mixed with 10 mg of catalyst (5% Rh on Al_2O_3, Fluka). The produced solid mixture was placed in 10 ml ampoule, which was then vacuumed, filled with gaseous mixture of tritium (80%) and hydrogen to a pressure of 250 Torr. The ampoule was heated to 160 °C and kept at this temperature during 20 min. Then the ampoule was cooled, vacuumed hydrogen-blown and vacuumed once again. Labeled peptide was extracted from the solid reaction mixture by two 3 ml portions of 50% aqueous ethanol, the obtained mixture was united and evaporated. To remove labile hydrogen, the procedure was performed two times. Purification of [G-^{3}H]MEHFPGP was performed in a Beckman chromatograph (USA), Kromasil C18 column (8 × 150 mm) (Elsiko, Russia) with UV detection at 220 and 254 nm. HPLC eluent was 0.1% trifluoroacetic acid in methanol gradient (25–50% for 20 min), with flow rate 3 ml/min. Tritium incorporation to peptide was measured with the help of liquid scintillation counting. Tritium label distribution was conducted by tritium NMR analysis as described previously (Zolotarev et al., 2003).

Glial cultures

Basal forebrain primary glial cultures were established from 1–2 days old Sprague-Dowley rats by following a standard technique (Cole and de Vellis, 1989). Dissected tissue was dissociated gently by trituration through a plastic pipette. Cell suspension was centrifuged at 400 *g* for 5 min, and the pellet was resuspended in DMEM/F-12 culture medium supplemented with 15 mM HEPES, 6 g/l D-glucose and 15% fetal calf serum. Cells were plated into 75 cm^2 culture flasks coated with poly-D-lysine, and cultivated at 37 °C. On reaching confluency the cultured cells were trypsinized and replated. For the degradation experiments after the third passage the cells were seeded into poly-D-lysine coated 24-well cluster plates (70×10^3 cells/well, 1 ml of culture medium per well). After 3 days culture medium was changed to the serum-free DMEM/F-12 medium (1 ml/well) supplemented with 15 mM HEPES, 6 g/l D-glucose, 25 mg/l insulin, 100 mg/l transferrin, 20 nM progesterone, 100 nM putrescine and 30 nM sodium selenite, and cells were cultured for an additional 24 h. The cell cultures contained 90–95% of glial fibrillary acidic protein immunoreactive cells.

Neuronal cultures

Basal forebrain primary neuronal cultures were established from embryonic day 18 fetal Sprague-Dowley rats. Dissected tissue was dissociated gently by trituration through a plastic pipette. Cell suspension was centrifuged at 400 *g* for 5 min, and the pellet was resuspended in the serum-free DMEM/F-12 culture medium supplemented with 15 mM HEPES, 6 g/l D-glucose, 25 mg/l insulin, 100 mg/l transferrin, 20 nM progesterone, 100 nM putrescine and 30 nM sodium selenite. Cells were plated into poly-D-lysine coated 24-well cluster plates (500×10^3 cells/well, 1 ml of culture medium per well), and cultivated at 37 °C. Five days old neuronal cultures were used for the degradation assay. The cell cultures contained approximately 95% of neuron specific enolase immunoreactive cells.

Cell culture degradation assay

[G-^{3}H]MEHFPGP (specific activity, 45 Ci/mmol) in 10 µl of PBS was added to the cell culture to a final concentration of 440 nM. After the incubation for the indicated time intervals, cell culture medium samples were collected, boiled for 2 min and centrifuged at 4 °C for 15 min at 15,000 *g*. The supernatants were decanted and obtained samples were stored at −20 °C.

Plasma membrane preparation

Rat basal forebrain plasma membranes were obtained according to the method of Gray and Whittaker (1962). Sprague-Dowley male rats (weight 180–200 g) were anesthetized with CO_2, immediately killed by decapitation, and the brains were rapidly removed and dissected at 4 °C. Dissected basal forebrains were rinsed with ice-cold (4 °C) PBS, and then homogenized in 10 volumes of 10 mM Tris-HCl, pH 7.4, containing 0.32 M sucrose, 1 mM EDTA, 1 mM benzamidine, and 0.1 mM phenylmethylsulfonyl fluoride (buffer 1) in a Teflon-glass homogenizer. The homogenization and all further procedures were carried out at 4 °C. The homogenate was centrifuged for 20 min at 1,000 *g*, and the supernatant was centrifuged for 30 min at 40,000 *g*. The compact brown pellet at the bottom of the centrifuge tube enriched in mitochondria was discarded, whereas the less compact light-colored pellet was suspended in the buffer 1 and washed twice with the same buffer. The pellet was resuspended in 10 mM Tris-HCl, pH 7.4, containing 0.22 M sucrose and 1 mg/ml BSA, and stored at −196 °C. The total protein concentrations were determined according to the method of Lowry (Peterson, 1977).

Plasma membrane degradation assay

Basal forebrain plasma membrane preparation, the degradation mixture composition, and the incubation temperature corresponded to the condi-

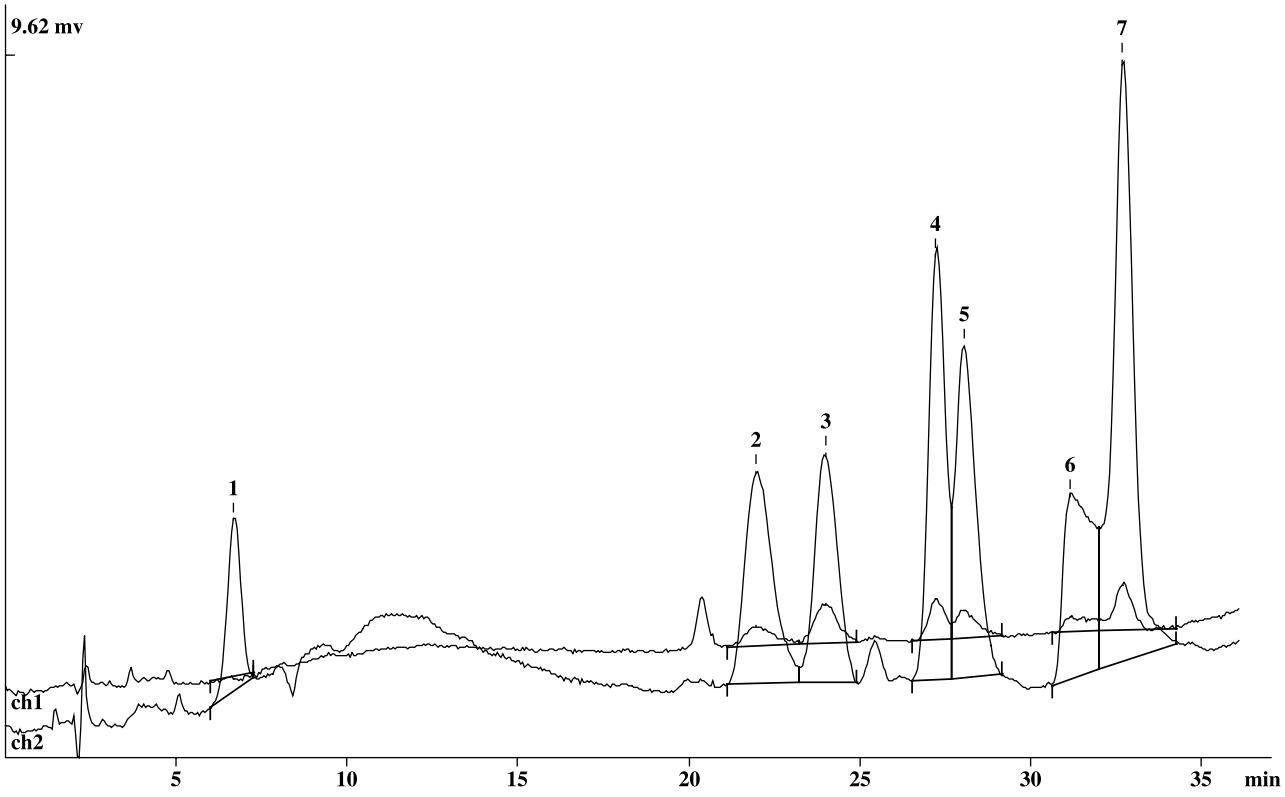


Fig. 1. HPLC of the mixture of Semax peptide fragments and the peptide fraction extracted from the sample. Detection at (ch1) 254 nm and (ch2) 220 nm. The following fractions were observed: *1*, PGP; *2*, EHFPGP; *3*, FPGP; *4*, HFPGP; *5*, MEHFPG; *6*, MEHFP; *7*, MEHFPGP

tions of radioligand analisys of [G-^{3}H]MEHFPGP binding (Dolotov et al., 2005). The degradation mixture (1 ml) contained 440 nM [G-^{3}H]MEHFPGP, basal forebrain plasma membrane preparations (0.2 mg of total protein per ml) and 1 mM $CaCl_2$ in 50 mM Tris-HCl buffer, pH 7.4. The reaction was started by the addition of membrane preparations. The tubes were transferred into a shaker incubator and kept there for a required period of time at 30 °C with permanent stirring. At the indicated periods of time 400 µl aliquots from the incubation mixture were collected, boiled for 2 min and centrifuged at 4 °C for 15 min at 15,000 *g*. The supernatants were decanted and obtained samples were stored at −20 °C.

Extraction of peptide fraction

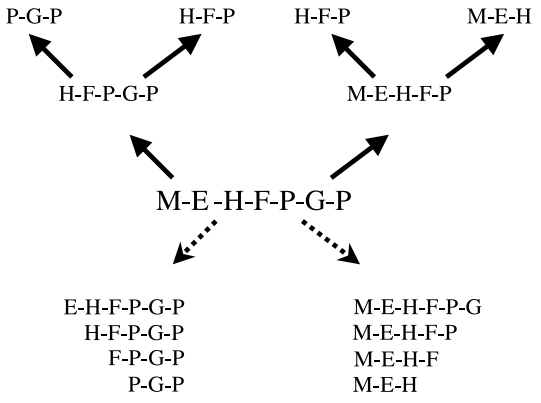
A 4-fold volume of acetonitrile was added to a 200 µl of the sample. The resulting mixture was then centrifuged at 4 °C for 15 min at 12,000 *g*, and the supernatant was dried in a rotor evaporator, and then dissolved in 1 ml of methanol and centrifuged under previous conditions. The obtained supernatant was again dried in a rotor evaporator, the dry remainder was dissolved in 100 µl of 0.1% trifluoroacetic acid.

HPLC analysis

Isolation of tritium labeled [G-^{3}H]MEHFPGP and its fragments EHFPGP, HFPGP, FPGP, MEHFPG, MEHFP from extracts was performed by HPLC. A Kromasil column, 4 × 150 mm, 5 mkm was calibrated with MEHFPGP and its fragments EHFPGP, HFPGP, FPGP, MEHFPG, MEHFP using a Beckman chromatograph with UV-detection at 220 and 254 nm. The labeled samples were mixed with Semax and its fragments (5 µg of each peptide) and subjected to gradient elution in mixtures A (0.082% trifluoroacetic acid and 0.018 heptafluorobutyiric acid) and B (80% of acetonitrile in eluent A). The peaks corresponding to the peptides MEHFPGP and its fragments were collected using UV-detection (Fig. 1). The gradient used was from 8 (0 min), 15 (8 min) to 25% of eluent B during 30 min at the flow rate of 1 ml/min. The amount of radioactivity in the peaks was measured with the help of liquid scintillation counting. The molar radioactivity of Semax peptide fragments was counted according to the tritium label distribution data in labeled MEHFPGP which were obtained previously (Zolotarev et al., 2003). The concentration of labeled peptides was counted according to the amount of radioactivity in the peptide fraction. The reproducibility of the HPLC analysis was examined using the same degradation sample in three separate HPLC assays. Differences between peaks radioactivity observed in these separate assays were within 10%.

Results and discussion

Possible paths of [G-^{3}H]MEHFPGP (Semax) degradation involve participation aminopeptidases and carboxypeptidases, dipeptidylaminopeptidases and dipeptidylcarboxypeptidases. The list of possible products is shown in Scheme 1. To identify predominant pathway of the degradation we have investigated levels of both Semax fragments EHFPGP, HFPGP, FPGP, MEHFPG, MEHFP and intact Semax (MEHFPGP) in the incubation media at different time points after administration of the peptide. Concentrations of corresponding peptides during incubation in the presence of rat basal forebrain plasma mem-



Scheme 1. Possible paths of Semax degradation by amino- and carboxypeptidases (dotted arrows), and dipeptidylaminopeptidases and dipeptidylcarboxypeptidases (black arrows)

Table 1. Concentrations of the degradation products of Semax (MEHFPGP) in the presence of rat basal forebrain plasma membranes, nM

Peptide	Time of incubation						
	0	15 min	30 min	1 h	2 h	4 h	24 h
MEHFPGP	440	370	344	277	157	78	13
EHFPGP	0	0	1	2	1	0	0
MEHFPG	0	7	13	14	13	7	2
HFPGP	0	31	52	96	114	103	44
MEHFP	0	15	29	25	32	12	2
FPGP	0	0	0	7	11	18	6

Table 2. Concentrations of the degradation products of Semax (MEHFPGP) in the presence of rat basal forebrain glial culture, nM

Peptide	Time of incubation					
	0	30 min	1 h	2 h	3 h	24 h
MEHFPGP	440	375	306	189	103	26
EHFPGP	0	1	2	4	6	1
MEHFPG	0	2	4	10	8	3
HFPGP	0	16	19	25	34	12
MEHFP	0	17	26	38	42	13
FPGP	0	0	2	7	9	6

branes, glial cells and neurons are shown in Tables 1, 2, and 3, respectively. These data indicate that the rate of MEHFPGP degradation is virtually the same in the presence of plasma membranes and glial cells, and it is markedly lower in the presence of neurons. MEHFPGP almost completely degraded at 24 h in the presence of plasma membranes and glial cells, whereas the concentration of intact MEHFPGP was about 25% of the initial concentration in the presence of neurons.

Table 3. Concentrations of the degradation products of Semax (MEHFPGP) in the presence of rat basal forebrain neuronal culture, nM

Peptide	Time of incubation					
	0	30 min	1 h	2 h	3 h	24 h
MEHFPGP	440	418	397	371	336	116
EHFPGP	0	0	0	1	2	3
MEHFPG	0	4	6	8	10	16
HFPGP	0	9	15	29	44	79
MEHFP	0	7	13	17	20	45
FPGP	0	0	0	1	4	10

In all the cases, MEHFPGP degradation proceeds both from the N-end and from the C-end of the peptide chain. The formation of 5-member fragments accompanied by the cleavage of two amino acid residues from both the N-end and the C-end considerably prevails over the formation of 6-member fragments. For each of the three objects, the concentration of fragments generated by splitting away of one amino acid residue Pro from the C-end is higher than the concentration of fragments obtained by the splitting away of the Met-residue from the N-end.

The highest concentration of the peptide EHFPGP was found 3 h post incubation in the case of glial cells and, with plasma membranes involved, the peptide was not detected at all in the incubation mixture as soon as 4 h post incubation, unlike the 5-member fragment HFPGP and the 4-member fragment FPGP. Despite that the degradation rate of Semax is virtually similar in the presence of plasma membranes and glial cells, the ratio of the N- and the C-end 5-member fragments of Semax during the initial 4 h of incubation has been found considerably different for the objects under study. Similar concentrations of the peptides MEHFP and HFPGP were registered in the presence of glial cells. In the case of plasma membranes, the concentration of HFPGP was 3–4 times as much as the concentration of MEHFP. In the case of neurons, the concentration of HFPGP is 1.5–2 times as much as the concentration of MEHFP. These differences are probably accounted for by the fact that the applied preparations of plasma membranes of adult rat basal forebrain contained a mixture of plasma membranes of both neurons and glial cells. Other possible causes might be either the presence of both intra-cellular and extra-cellular membrane bound peptidases in applied plasma membrane preparations or trace amounts of EDTA and the protease inhibitors used conventionally during the plasma membrane preparation.

Thus, our results indicate that the degradation of the heptapeptide Semax in the presence of both plasma membranes and cultures of glial and neuronal cells from rat

basal forebrain takes place from both the C-end and the N-end at comparable rates with some differences in proportions of resulting products between glial and neuronal cells. The presence in Semax of the C-terminal PGP sequence does not cause, in the presence of the studied objects, complete domination of the degradation from the N-end. The formation of pentapeptides is the predominant process, a possible proof that Semax degradation mostly involves dipeptidylpeptidases causing the removal of the ME- and -GP groups, respectively. It should be noted, in this connection, that the both heptapeptide Semax and the pentapeptide HFPGP are strong inhibitors of human serum enkephalinases (Kost et al., 2001). Based on the presented data, it is tempting to speculate that Semax can be an inhibitor of some peptidases, possibly of brain amino- and carboxypeptidases. At this point, we probably encounter an interesting situation where neuroactive peptide and its metabolite ''are regulating'' the process of their biodegradation by inhibiting a part of peptidases.

The data available concerning the effect of the peptide HFPGP on the behavior of experimental animals (Levitskaya et al., 2000) prompt that a part of Semax effects might be caused by the activity of this peptide. However, to date neuroactive properties of the peptide MEHFP were not studied. At the present time, we pursue investigation of activities of these pentapeptides with the view to explore their contribution into the neuroprotective and cognitive effects of Semax and ability to influence on the expression of the neurotrophins in the central nervous system.

Thus the approach applied allows analyzing peptides stability and their degradation pathways in the presence of targets of their action. The application of uniformly tritium labeled peptides with high molar radioactivity allows analyzing concentrations of possible products of their enzymatic hydrolysis in micro amounts of biological samples.

Acknowledgements

This study was supported by the Russian Foundation for Basic Research (grants 05-03-32411 and 03-04-48582), by Program of Molecular Cell Biology of Russian Academy of Science and INTAS YSF 2002-0336.

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