Current Topics in
Peptide & Protein
Research
Vol. 14, 2013

Original Communication

A comparative study of biological activity of peptides, the derivatives of the third intracellular loop of rat luteinizing hormone receptor, modified by hydrophobic radicals at the N- and C-termini

Alexander O. Shpakov[1,*], Elena A. Shpakova[2] and Kira V. Derkach[1]

[1]Sechenov Institute of Evolutionary Physiology and Biochemistry, Russian Academy of Sciences, Thorez av. 44, 194223 St. Petersburg, [2]Institute of Macromolecular Compounds, Russian Academy of Sciences, Bol'shoj av. 31, 199004 St. Petersburg, Russia

ABSTRACT

Peptides, the derivatives of intracellular regions of G protein-coupled receptors (GPCRs), are able to regulate the activity of hormonal signaling systems, acting as agonists and antagonists of the cognate receptor. The modification of GPCR-peptides by hydrophobic radicals significantly increases their biological activity. However, despite a significant progress in the development of GPCR-peptides modified by hydrophobic radical, referred to as pepducins, the impact of the localization, the hydrophobicity and the number of hydrophobic radicals involved in peptide activity has been scarcely studied. The aim of this work is a comparative study of the influence of peptide 562–572 corresponding to the C-terminal region of the third intracellular loop of rat luteinizing hormone (LH) receptor and its acylated analogs modified by palmitate and decanoate at the N- or C-terminus, or at both termini, on the functional activity of gonadotropin-sensitive adenylate cyclase (AC) system in the rat testicular membranes. We showed that peptides modified by acyl radicals at the C-terminus, where in full-size LH receptor the sixth transmembrane region is located, stimulated in a dose-dependent manner the basal AC activity and GppNHp binding capacity of G_s protein and reduced their activation by human chorionic gonadotropin. The C-palmitoylated peptide was much more active than its decanoyl counterpart, and the action exerted on AC system was tissue and receptor specific. The N-acylated peptides were not active as regulators of the AC system, but slightly affected the gonadotropin-induced signal transduction via AC system. Along with this, peptides modified by decanoyl at the C- and N-termini non-selectively decreased, but not very much, the AC inhibitory effects of different hormones. The data obtained support the hypothesis that the hydrophobic radical in GPCR-peptide mimics the transmembrane region and must be comparable with it in the size and hydrophobicity. The establishment of the criteria of modification of GPCR-peptides with hydrophobic radicals is one of the most promising ways to create drugs capable of controlling the biochemical and physiological processes *in vivo*.

KEYWORDS: adenylyl cyclase, decanoate, G protein-coupled receptor, heterotrimeric G protein, hydrophobic radical, luteinizing hormone, palmitate, peptide, testes, third intracellular loop

ABBREVIATIONS

AC, adenylyl cyclase; CTX, cholera toxin; GPCR, G protein-coupled receptor; GppNHp, β,γ-imidoguanosine-5'-triphosphate; hCG, human chorionic gonadotropin; ICL, intracellular loop;

*Corresponding author: alex_shpakov@list.ru

LH, luteinizing hormone; PACAP-38, pituitary adenylyl cyclase-activating polypeptide-38; Pal, palmitoyl; PAR, protease-activated receptor; PTX, pertussis toxin; TM, transmembrane region; TSH, thyroid-stimulating hormone

INTRODUCTION

Peptides, the derivatives of functionally important intracellular regions of G protein-coupled receptors (GPCRs), are able to specifically interact with the components of hormonal signaling systems, preferably the receptors homologous to them. They can act as intracellular full agonists and in the absence of hormonal stimulus trigger the intracellular signaling cascades, and as intracellular inverse agonists and antagonists that modulate or block the hormonal signaling [1-4]. The selectivity and efficiency of GPCR-peptides depend on the coincidence of their primary structures with those of intracellular regions participating in the formation of biologically active conformation of GPCRs and responsible for their specific interaction with heterotrimeric G proteins, β-arrestins and other signal proteins. The modification of GPCR-peptides by hydrophobic radicals and membrane-permeable sequences, as well as the design of intracellular loop-like cyclic and dimeric structures also contribute significantly to the biological activity of the peptides.

Of special interest is the modification of GPCR-peptides by hydrophobic radicals, preferably fatty acid residues. The lipophilic derivatives of GPCR-peptides, generally referred to as pepducins, were discovered in 2002 by Kuliopulos *et al.* [5, 6]. In the recent years they have been intensively studied and used as selective regulators of hormonal signaling systems *in vitro*, and to control and regulate the physiological and biochemical processes in norm and in pathological conditions *in vivo*. As it follows from our and the other authors' data, pepducins are more active than their unmodified analogs [6-11]. This should be ascribed to the ability of pepducins to penetrate the cell membrane and to anchor on the inner side of the membrane near the targets of their regulatory action, and is based on the similarity between 3D structure of pepducins and homologous region in an intact receptor [4, 12, 13]. However, despite a significant progress in the development of hydrophobic radical-modified GPCR-peptides, the impact of localization of hydrophobic radicals, their chemical nature and the number of these radicals into functional activity of the peptides has been hardly studied. The hydrophobic radical is assumed to mimic the transmembrane region (TM) which is adjacent to the intracellular region corresponding to the peptide [3, 5, 12, 14]. Consequently, the hydrophobic radical must be localized in the membrane-proximal segment of peptide and be comparable in size and hydrophobicity with the adjacent TM of receptor.

To test this assumption, a comparative study was carried out focusing on the influence of peptide 562–572 corresponding to the C-terminal portion of the third intracellular loop (ICL3) of rat luteinizing hormone (LH) receptor and its acylated derivatives on the functional activity of gonadotropin-sensitive adenylate cyclase (AC) system in the testes of rats. Special attention was given to the analogs of peptide 562–572 which differ in the acyl radicals (palmitate and decanoate), localization and the number of the radicals in peptide molecule. We showed that lipophylic peptides modified by both acyl radicals at the C-terminus, where in full-size receptor TM is located, stimulated in a dose-dependent manner the basal activity of the enzyme AC and heterotrimeric G_s protein, and reduced their activation by gonadotropin in the testicular membranes of rats. The N-acylated peptides were not active as regulators of the AC system, but affected, though a little, the gonadotropin-induced signal transduction via the AC system. Thus, we confirmed the hypothesis that the hydrophobic acyl radical mimics TM and in all probability is located in the locus next to TM.

MATERIALS AND METHODS

Animals

In the experiments 5 month-old male Wistar rats (230–260 g) obtained from the Rappolovo nursery (St. Petersburg) were used. The animals were housed in plastic sawdust-covered cages with a normal light-dark cycle and received a standard laboratory chow and water *ad libitum*. Their decapitation was performed between 10.00 and 11.00 a.m. All procedures were carried out under the Institutional Guidelines (the Bioethics Committee, December 23, 2010) and "Guidelines for the treatment of animals in behavior research and teaching" [15].

Chemicals and radiochemicals

Human chorionic gonadropin (hCG), pituitary AC-activating polypeptide-38 (PACAP-38), isoproterenol, somatostatin, forskolin, cAMP, ATP, GTP, β,γ-imidoguanosine-5'-triphosphate (GppNHp), were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Calbiochem (San Diego, CA, USA). [α-^{32}P]-ATP (4 Ci/mmol) was purchased from Institute of Reactor Materials (Moscow, Russia), β,γ-imido [8-^{3}H]-guanosine-5'-triphosphate ([8-^{3}H]-GppNHp) (5 Ci/mmol) from Amersham (UK); the type HA 0.45 μm nitrocellulose filters were from Sigma-Aldrich Chemie GmbH (Germany).

The synthesis of peptide 562–572 and its lipophilic derivatives

The peptide Asn^{562}-Lys-Asp-Thr-Lys-Ile-Ala-Lys-Lys-Nle-Ala^{572}-amide with the residue Met^{571} replaced by norleucine and its derivatives modified by hydrophobic radicals were synthesized by the standard solid phase reaction procedures using a semiautomatic synthesizer NPS-400 (Neosystem Laboratories, France) (Table 1). The synthesis was performed using *p*-methylbenzhydrylamine resin (capacity 1.16 mmols per g) and amino acid derivatives protected by *tert*-butyloxycarbonyl (Boc) groups. The formation of peptide bond was performed by the activated ester method. The 1-oxybenzotriazolyl esters of Boc-protected amino acids, prepared using *N,N*-diisopropylcarbodiimide, were added to the peptidyl-polimer for conjugation with free amino groups. For the acylation to be complete, three-fold excess of acylating agent was used, and the reaction continued for 24 h. The cleavage from resin and deprotection were performed using a mixture of 1 ml trifluoromethanesulfonic acid, 10 ml TFA, 1 ml thioanisole and 0.5 ml ethandithiol for 1 h at 4 °C and for 1.5 h at room temperature (the quantities of reagents were calculated per 1 g of peptidyl-polymer). Then 100 ml of diethyl ether was added to the reaction mixture. The precipitate that was formed was separated by filtration, dissolved in 5 ml of TFA, filtered to remove the resin and precipitated with 100 ml of dry diethyl ether. To introduce the acyl radicals of palmitic and decanoic acids into the peptide chain, the lipophilic derivatives of lysine prepared by condensing *N*α-*t*-BOC-lysine and pentafluorophenyl ester of palmitic or decanoic acid in the presence of triethylamine, were used. Peptide 562–572 and its lipophilic derivatives were purified by RP-HPLC on a Vydac C18 218TP column using water-acetonitrile linear gradient containing 0.1% TFA. The identity and purification degree of peptides were assessed using RP-HPLC and electrospray ionization mass spectrometry (Table 1). Note that the highly hydrophobic peptide N[K(Pal)]DTKIAKK-Nle-A$^{562–572}$-[K(Pal)]A-amide modified with palmitic acid residues at the N- and C-termini did not dissolve in aqueous solvents and could not be used in biological experiments.

Preparation of the plasma membranes

The isolation of the plasma membranes from the rat testes was carried out as described previously [10]. For each fraction 7-8 animals were taken. The crushed testes were homogenized with a Polytron in 10 volumes of ice-cold 40 mM Tris-HCl buffer, pH 7.4, containing 5 mM $MgCl_2$, 320 mM sucrose and a cocktail of protease inhibitors 500 μM *O*-fenantrolin, 2 μM pepstatin and 1 mM phenylmethylsulphonyl fluoride. The resulting homogenate was centrifuged at 1500 × *g* for 10 min at 4 °C. Then the supernatant was centrifuged at

Table 1. The structure of peptides and their mass-spectra data.

No.	Structure	M_{exp}	M_{calc}
I	NKDTKIAKK-Nle-A$^{562–572}$-amide	1227.67	1227.64
II	N[K(Dec)]DTKIAKK-Nle-A$^{562–572}$-KA-amide	1381.92	1382.02
III	NKDTKIAKK-Nle-A$^{562–572}$-[K(Dec)]A-amide	1381.89	1382.02
IV	N[K(Dec)]DTKIAKK-Nle-A$^{562–572}$-[K(Dec)]A-amide	1735.39	1735.41
V	N[K(Pal)]DTKIAKK-Nle-A$^{562–572}$-KA-amide	1665.16	1665.13
VI	NKDTKIAKK-Nle-A$^{562–572}$-[K(Pal)]A-amide	1665.14	1665.13

Pal – palmitoyl; Dec – decanoyl.

20 000 × *g* for 30 min at 4 °C. The pellets were washed by 10 volumes of the same buffer without sucrose and centrifuged again at 20 000 × *g* for 30 min.

Preparation of the plasma membranes from the brain and the heart ventricles of rats was performed as described previously [9, 16]. The final pellets were suspended in 50 mM Tris-HCl buffer, pH 7.4, to produce the membrane fraction with a protein concentration range of 1-4 mg/ml and stored at -70 °C. The protein concentration was measured by the method of Lowry *et al.* [17] using BSA as a standard.

Adenylyl cyclase assay

The determination of adenylyl cyclase (EC 4.6.1.1) activity in the plasma membranes was performed according to Salomon *et al.* [18], with our modifications [9]. The incubation mixture (final volume 50 μl) contained 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM ATP, 0.1 mM cAMP, 20 mM creatine phosphate, 0.2 mg/ml creatine phosphokinase, 1 μCi [α-^{32}P]-ATP, and 20-60 μg of membrane protein. The enzyme reaction was carried out at 37 °C for 12 min. Each assay was carried out in triplicate at least three times, and the results were expressed as pmol cAMP/min per mg of membrane protein. The basal AC activity was measured in the absence of forskolin, bacterial toxins, hormones, and peptides. To estimate AC inhibitory effects of peptides or hormones, the enzyme was pre-activated by forskolin (10^{-5} M). To study the influence of peptide 562-572 and its lipophilic derivatives on AC activity, the membrane fractions were incubated with them at 4 °C for 10 min.

[8-^{3}H]-GppNHp binding capacity assay

[8-^{3}H]-GppNHp binding of G proteins was performed as described earlier [9]. The incubation mixture (final volume 50 μl) contained 25 mM HEPES-Na buffer, pH 7.4, 1 mM EDTA, 5 mM $MgCl_2$, 100 mM NaCl, 1 mM dithiothreitol, 1 μM GppNHp, 0.1% BSA, 0.5-1 μCi [8-^{3}H]-GppNHp, and 50-100 μg of membrane protein. The GppNHp binding was carried out at 30 °C for 45 min. The reaction was terminated by dilution of 100 μl of the washing buffer (20 mM K^+/Na^+ phosphate buffer, pH 8.0) containing 0.1% Lubrol-PX and the samples were filtered through 0.45 μm nitrocellulose filters. Each filter was washed out three times with 2 ml of the washing buffer, dried and placed in a toluene scintillator. The filter-bound radioactivity was measured using a LKB 1209/1215 RackBeta scintillation counter. The specific GppNHp binding was estimated as the difference between a total binding and non-specific binding measured in the presence of 10 mM GppNHp. Each assay was carried out in triplicate at least three times and the results were expressed as pmol [8-^{3}H]-GppNHp per mg of membrane protein. The basal level of GppNHp binding was measured in the absence of hormonal agents and peptides. To study the influence of peptide 562–572 and its lipophilic derivatives on GppNHp binding capacity, the membrane fractions were incubated with them at 4 °C for 10 min.

ADP ribosylation of G protein by the bacterial toxins

ADP ribosylation of the testicular membranes was carried out as follows, using bacterial toxins [9]. The membranes (the concentration of proteins was about 1.0-1.5 mg/ml) were incubated at 37 °C for 45 min with 100 μg/ml of cholera toxin (CTX) or 10 μg/ml of pertussis toxin (PTX) in 400 μl of 50 mM Tris-HCl buffer, pH 7.8, containing 2 mM $MgCl_2$, 1 mM EDTA, 10 mM dithiothreitol, 0.1 mM NAD, 1 mM NADP, 0.1 mM GppNHp (for CTX) or GTP (for PTX), 1 mM ATP, 10 mM thymidine, and a cocktail of protease inhibitors (500 μM *O*-fenantrolin, 2 μM pepstatin, 100 μM phenylmethylsulphonyl fluoride). The toxins were pre-activated in the presence of 20 mM dithiothreitol and 0.1% SDS (for CTX) or 1 mM ATP and 0.1% Lubrol-PX (for PTX) at 37 °C for 15 min. To terminate the reaction of ADP-ribosylation, the incubation mixture was diluted to a final volume of 5 ml with ice-cold 50 mM Tris-HCl buffer, pH 7.5, containing 5 mM $MgCl_2$, and then centrifuged at 37 000 × *g* for 15 min. The pellet was resuspended in the same buffer and used to study the activity of AC and G proteins. The control testicular membranes were treated in the same manner, but without toxins.

Statistical analysis

The data is presented as the mean ± SEM. The difference in AC activity and GppNHp binding capacity in the control and the peptide-, hormone- and bacterial toxin-treated membranes in each

case was statistically assessed using one-way analysis of variance (ANOVA) and considered significant at $P < 0.05$.

RESULTS

The comparative study of the influence of peptide 562–572 and its acylated derivatives on the basal AC activity and GppNHp binding capacity in the testicular membranes showed the peptide VI, NKDTKIAKK-Nle-$A^{562–572}$-[K(Pal)]A-amide, as the most active (Fig. 1, Table 2). At the concentration of 10^{-5} M it stimulated the basal AC activity two times, and its stimulating effect at higher concentrations, up to 10^{-3} M, did not change significantly. Peptide VI at the concentration of 10^{-4} M increased GppNHp binding by 48%, which is much higher than the corresponding effects of the other peptides investigated by us. Peptides III and IV modified by decanoate at the C terminus were less active compared with peptide VI, and their AC and GppNHp binding stimulating effects slightly exceeded those of unmodified peptide I. The AC stimulating effect of peptides I and III increased at the concentrations 10^{-5} M to 10^{-3} M, whereas the effect of peptide IV modified with decanoyl radicals at both the N- and C-termini reached maximum at the concentration of 10^{-5} M. Peptides II and V acylated by decanoate and palmitate residues at the N-terminus had very little, if at all, effect on the basal AC activity and GppNHp binding capacity (Fig. 1, Table 2).

To identify heterotrimeric G proteins, the targets of peptide 562–572 and its acylated analogs, we used the ADP-ribosylation by CXT that selectively blocks GTPase activity of G_s proteins and converts them into a permanently active GTP-bound, hormone-insensitive state, and PTX that selectively blocks the functional interaction between hormone-activated GPCR and $G_{i/o}$ proteins [19-21]. In the CTX-treated testicular membranes the activity of AC was increased by 175%, and GppNHp binding was decreased by 34%, as compared with control. In the PTX-treated membranes neither AC activity, nor GppNHp binding differed significantly from those in the control membranes. The treatment with CTX blocked the stimulating effects of peptides on AC activity, while PTX treatment had no effect (data not shown). In the case of GppNHp binding, CTX treatment almost completely inhibited the increase of GppNHp binding capacity induced by the C-acylated peptides IV and VI, but only partially decreased the corresponding effects of peptides I and III (Table 2). In the PTX-treated membranes GppNHp binding stimulating effects

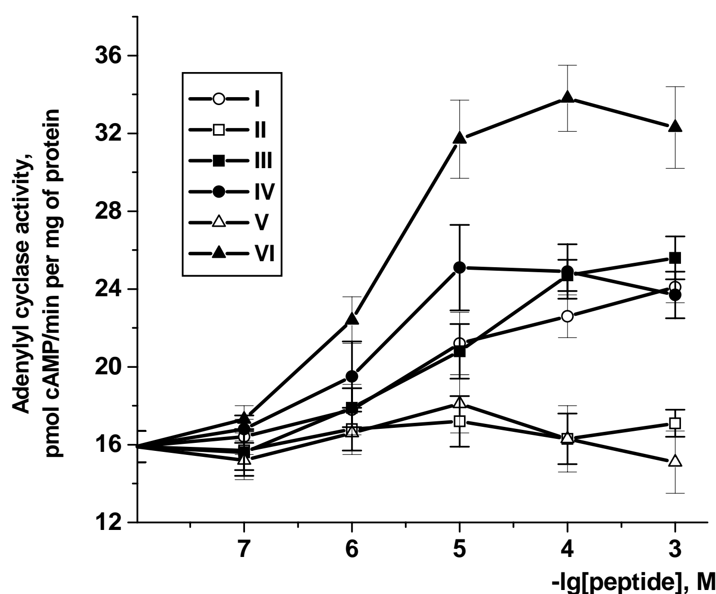

Fig. 1. The AC stimulating effect of peptide 562–572 and its acylated derivatives in the testicular membranes. The values are the mean ± SEM of three individual experiments, each performed in triplicate.

Table 2. The influence of peptides on GppNHp binding capacity in the testicular membranes treated by pertussis (PTX) and cholera toxins (CTX) (pmol [8-^{3}H] GppNHp/mg of membrane protein).

	[8-^{3}H]-GppNHp binding, pmol [8-^{3}H]-GppNHp/mg of membrane protein		
	Control membranes	PTX-treated membranes	CTX-treated membranes
Without peptide	2.42 ± 0.17	2.23 ± 0.20	1.59 ± 0.11
Peptide I, 10^{-4} M	2.93 ± 0.22 (21)	2.60 ± 0.09 (15)	1.78 ± 0.05 (8)
Peptide II, 10^{-4} M	2.65 ± 0.15 (10)	2.29 ± 0.14 (2)	1.76 ± 0.12 (7)
Peptide III, 10^{-4} M	3.06 ± 0.08* (26)	2.70 ± 0.11 (19)	1.84 ± 0.14 (10)
Peptide IV, 10^{-4} M	3.29 ± 0.12* (36)	3.03 ± 0.06 (33)	1.70 ± 0.13 (5)
Peptide V, 10^{-4} M	2.39 ± 0.16 (-)	2.33 ± 0.10 (4)	1.57 ± 0.06 (-)
Peptide VI, 10^{-4} M	3.58 ± 0.14* (48)	3.30 ± 0.22 (44)	1.72 ± 0.12 (5)

Figures in parentheses designate the stimulating effects of peptides on GppNHp binding in % over the basal level of GppNHp binding (the effects are calculated relative to the basal level of GppNHp binding in the control membranes, taken as 100%). The values are expressed as the mean ± SEM of three individual experiments. *–$P < 0.05$.

Table 3. The influence of peptide 562–572 and its acylated derivatives on the basal AC activity in the plasma membranes isolated from the brain and the heart ventricles.

	Brain	Heart
Without peptide	24.1 ± 1.3	26.9 ± 1.2
Peptide I, 10^{-4} M	26.5 ± 1.0 (10)	28.0 ± 1.1 (4)
Peptide II, 10^{-4} M	23.1 ± 1.5 (-)	26.3 ± 0.7 (-)
Peptide III, 10^{-4} M	28.3 ± 0.5 (17)*	29.8 ± 1.6 (11)
Peptide IV, 10^{-4} M	27.1 ± 1.4 (12)	27.5 ± 1.6 (2)
Peptide V, 10^{-4} M	25.7 ± 0.6 (7)	28.4 ± 1.3 (6)
Peptide VI, 10^{-4} M	29.2 ± 1.2 (21)*	31.5 ± 1.4 (17)*

Figures in parentheses represent the AC stimulating effects of peptides in % over the basal AC activity taken as 100%. The values are expressed as the mean ± SEM of three individual experiments. *–$P < 0.05$.

of peptide IV and VI were not changed, and the corresponding effects of peptides I and III were decreased, although not very much.

Thus, the derivatives of peptide 562–572, modified by fatty acid radicals at the C-terminus, mimicked hormone-activated LH receptor and increased the activity of the components of AC system, whereas the acylation of the peptide at the N-terminus was ineffective. The elongation of acyl chain from C_{10} in the case of decanoate to C_{16} in the case of palmitate increased AC and GppNHp binding stimulating effects of the acylated peptide.

The peptides under study had a little effect on the basal AC activity in the plasma membrane fractions isolated from the brain and the heart ventricles, with the exception of peptides III and VI, which at the concentration of 10^{-4} M increased the basal activity of the enzyme in both tissues (Table 3). However, their AC effects were much weaker than those in the testis. These data speak in favor of tissue specificity of the acylated derivatives of peptide 562–572 activating AC predominantly in the testis, where LH receptors homologous to them are present.

In the testicular membranes hCG (10^{-8} M) that specifically interacts with the G_s-coupled LH receptor increased the basal AC activity by 658% and GppNHp-binding by 146%, while the stimulating effects of PACAP-38 (10^{-6} M) and β-agonist isoproterenol (10^{-5} M) were less pronounced. PACAP-38 and isoproterenol increased AC activity by 279 and 136% and GppNHp-binding by 58 and 43%, respectively. The treatment of the testicular membranes with somatostatin (10^{-6} M) that specifically binds with the G_i-coupled somatostatin receptors led to the decrease of forskolin-stimulated AC activity by 33% (AC stimulating effect of 10^{-5} M forskolin without somatostatin was taken as 100%) and the increase of GppNHp binding by 44%. To study the influence of the acylated peptides on AC and GppNHp-binding effects of the hormones, the testicular membranes were pre-incubated with peptides (5 min, 4 ºC) followed by the addition of hormone.

The C-palmitoylated peptide VI significantly reduced the stimulating effect of hCG. In the testicular membranes pre-treated with peptide VI (10^{-4} M), the AC stimulating effect of hCG was 33% of that in the control, and hCG-induced increase of GTP binding was decreased almost two times compared with that in the untreated membranes (Fig. 2). Peptides III and IV having the C-terminal decanoate also significantly reduced gonadotropin-stimulated AC activity and GTP binding, and the inhibitory influence of peptide IV modified by decanoyl radicals at the N- and C-termini was comparable to that of peptide VI. Peptides II and V with N-terminal acyl radical decreased hCG-induced stimulation of AC activity and GppNHp binding less than the peptides modified by C-terminal acyl radical, but by their inhibitory action they were comparable to unmodified peptide I (Fig. 2). Note that peptides II and V did not affect the basal activity of AC system. This indicates that the ability of acylated derivatives of peptide 562–572 to reduce hCG-induced stimulation of AC system depends on the location of fatty acid radicals not so much as when they stimulate the basal activity of the system.

The most active peptides I, III, IV and VI at the concentration of 10^{-4} M, effective in the case of hCG-sensitive AC system, had little influence on the stimulating effects of PACAP-38 and isoproterenol on AC activity and GppNHp-binding in the testicular membranes. Nor did they influence the corresponding effects of isoproterenol and relaxin in the plasma membranes isolated from the heart ventricles (data not shown). This is the evidence for receptor specificity of peptide 562–572 and its C-acylated derivatives, which in the rat testes specifically inhibited hCG-induced stimulation of AC and G_s-proteins, but had no effect on stimulation of the AC system by the other hormones in the testes and in the heart.

As far as unmodified peptide I and peptides II and III modified by decanoate are concerned, they weakened, but not much, the inhibitory effect of somatostatin and noradrenaline, the agonist of G_i-coupled α2-adrenergic receptor, on forskolin-stimulated AC activity and their stimulatory effect on GppNHp binding in the testicular (somatostatin) and myocardial (somatostatin, noradrenaline) membranes (Fig. 3). In the testicular membranes pre-incubation with peptide III (10^{-4} M) led to the decrease of AC inhibitory effects of somatostatin by 21% and of its stimulating effect on GppNHp binding by 25%. Furthermore, in the myocardial membranes the regulatory effects of somatostatin and noradrenaline were weakened in the presence of peptide IV (Fig. 3). These findings indicate that peptide 562-572 and some of its acylated derivatives are able to exert undesirable action on the signal transduction via G_i-coupled AC system.

DISCUSSION

We showed that peptide 562–572, the derivative of the C-terminal region of rat LH receptor ICL3, modified by the palmitoyl radical at the C-terminus was a more potent regulator and modulator of AC system in the testicular membranes, compared with its unmodified counterpart. Substitution of C-terminal palmitate by decanoate led to a decrease of biological activity of the acylated peptide. The modification of peptide 562–572 by decanoyl radicals at each end led to an increase, but not large, of its AC and GppNHp binding stimulating effects and its inhibitory influence on hCG-induced stimulation of AC system. However, this peptide was less potent than the C-palmitoylated peptide. The length of palmitoyl residue is closer to the width of the membrane monolayer, while decanoyl residue is much shorter. This gives

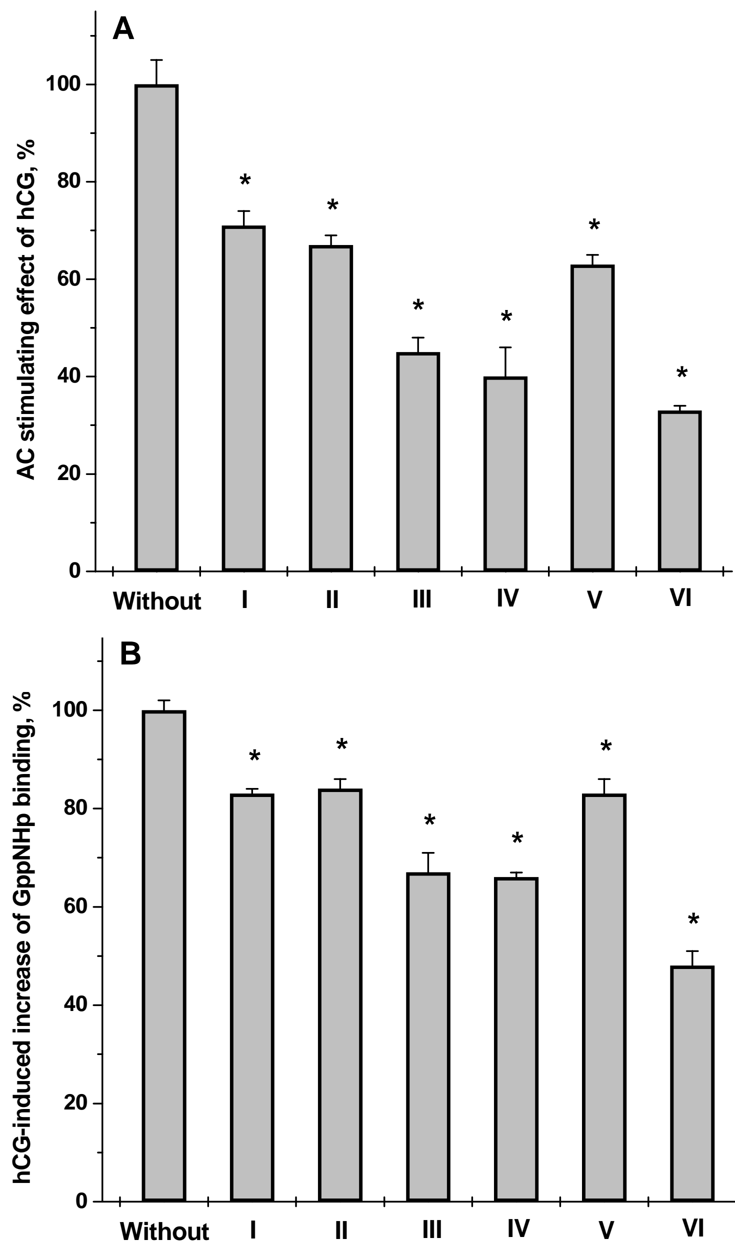

Fig. 2. The influence of peptide 562–572 and its acylated derivatives on hCG-induced stimulation of AC activity (A) and GppNHp-binding capacity (B) in the testicular membranes. Peptides were taken at the concentration of 10^{-4} M. Stimulating effects of hCG (10^{-8} M) on AC activity (A) and GppNHp-binding (B) in the absence of peptides are taken as 100%. The values are expressed as the mean ± SEM of three individual experiments, each performed in triplicate. *–$P < 0.05$.

grounds to say that the length of the acyl radical in pepducin must be comparable with the width of a monolayer of the plasma membrane. This view is supported by the data obtained in the study of peptides, the derivatives of ICLs of protease-activated receptors (PARs), i.e. the modification of GPCR-peptide by palmitoyl residue resulted in an increase of its functional activity to the same degree as its elongation by addition of hydrophobic segment corresponding to about 2/3 of the entire TM [5, 6]. Earlier it was shown that palmitoyl radical is responsible for the transport of palmitoylated

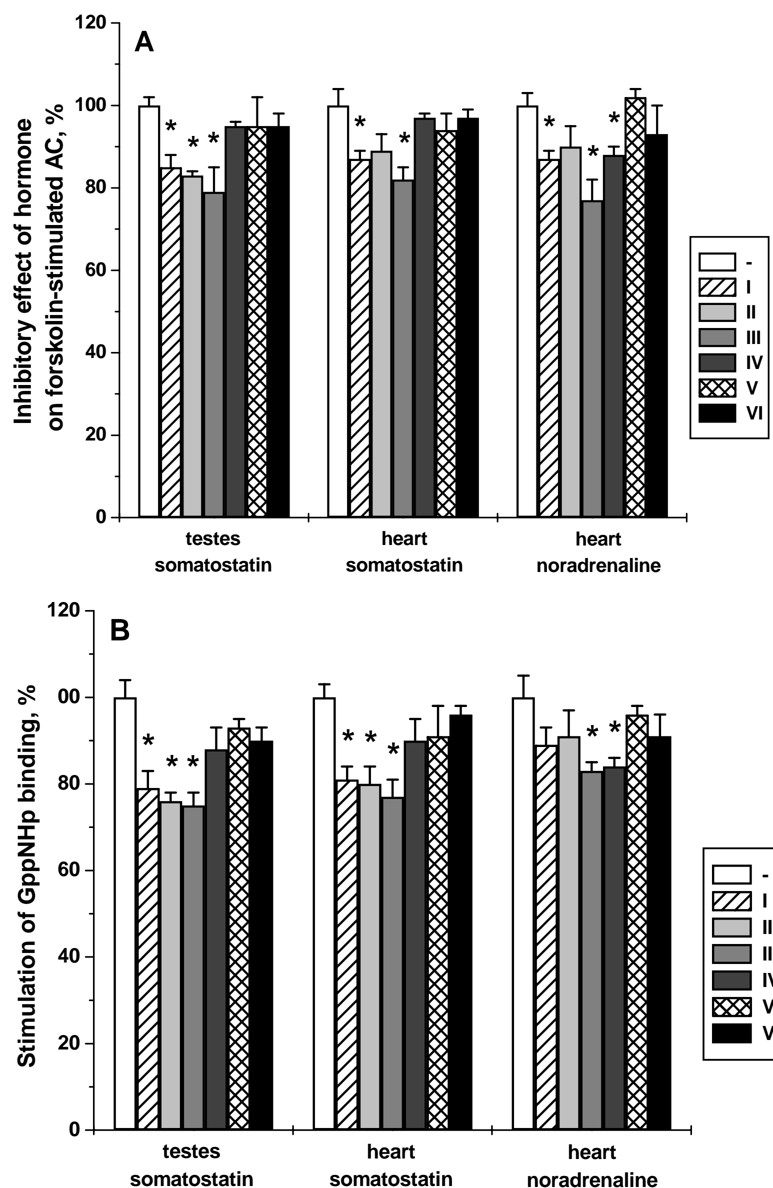

Fig. 3. The influence of peptide 562–572 and its acylated derivatives on AC inhibitory effect (A) and GppNHp binding stimulating effect (B) induced by somatostatin and noradrenaline in the testicular and cardiac membranes. Peptides were taken at the concentration of 10^{-4} M. The inhibitory effects of somatostatin (10^{-6} M) and noradrenaline (10^{-5} M) on forskolin-stimulated AC activity and their stimulating effects on GppNHp-binding capacity in the absence of peptides are taken as 100%. The values are expressed as the mean (SEM of three individual experiments, each performed in triplicate. *–$P < 0.05$.

peptides across the membrane and their attachment to the inner membrane surface, which is very important for the biological activity of GPCR-peptides in the *in vitro* and *in vivo* conditions [2-6, 12]. As for the hydrophobic radical, it is involved in stabilization of the α-helical structure of peptide, increasing the content of helical conformation, as shown in the case of palmitoylated peptides [22, 23]. There is data available suggesting that in a majority of GPCRs the membrane-proximal regions of ICLs have helical conformation which is stabilized by the adjacent helical TM. This allows them to effectively interact with G-protein α-subunit and with complementary intracellular regions of GPCR [24].

We put forward a suggestion that the hydrophobic radical in GPCR-peptides is likely to be located in the same position as TM in the native receptor. Since peptide 562-572 that we studied corresponds to the C-terminal portion of ICL3, hydrophobic radical simulating TM6 located immediately after ICL3 should be placed at the C-terminus of peptide. The N-terminus of peptide corresponds to the middle of ICL3 and is located at a rather far distance, 12 amino acid residues, from TM5 preceding this loop. The modification of peptide by acyl radical at the N-terminus leads to its unsuitable anchoring in the membrane and hinders the effective interaction between GPCR-peptide and the regions, complementary to it, of the cognate receptor or other signal proteins. It was confirmed by our data that the peptides acylated by palmitate or decanoate at the N-terminus did not affect the basal activity of AC system and had a little effect on its stimulation by gonadotropin, while the peptides acylated at the C-terminus were, on the contrary, highly active.

That hydrophobic radical must be localized in the position of peptide, where in the full-size receptor TM is located, is supported by the data obtained earlier. The C-palmitoylated peptides corresponding to the C-terminal portion of ICL3 of 5-hydroxytryptamine receptor of the type 6, relaxin receptor RXFP1 and thyroid-stimulating hormone (TSH) receptor with high selectivity and efficiency stimulated the activity of the components of AC system and modulated the signal transduction via GPCR homologous to them, and these peptides in the specific activity greatly exceeded their analogs lacking the hydrophobic radical [8, 9, 11]. The C-palmitoylated peptide 612–627, the derivative of TSH receptor ICL3, *in vitro* increased the basal activity of AC system in the thyroidal membranes and inhibited TSH-induced AC stimulation [11], and *in vivo*, when administered intranasally, in a dose-dependent manner increased the level of thyroid hormones in rats, while the unmodified counterpart was inactive [25].

In GPCR-peptides containing the entire ICL or its N-terminal portion, the modification of peptide N-terminus by appropriate acyl radical was effective. The N-palmitoylated peptides corresponding to the entire ICL3 of PAR2 selectively inhibited the agonist-induced activation of PAR2 in neutrophils, pancreatic acinar cells, primary cholangiocarcinoma cells, and SW620 colorectal cancer cells [26-28]. The N-palmitoylated peptides, the derivatives of the entire ICL1 and ICL3 of PAR4, selectively inhibited the transduction of thrombin signal via PAR4, and *in vivo* prevented PAR4-mediated arterial thrombosis in animals with the carotid artery occlusion [12, 29]. Peptide, the derivative of the ICL1 of structurally related chemokine receptors CXCR1 and CPCR2, modified at the N-terminus by lithocholic acid, which is a steroid acid present in the mammalian bile, and N-palmitoylated peptide corresponding to the ICL3 of the same receptors both selectively inhibited the proliferation of endothelial cells and blocked tube formation induced by CXCR1/2-agonists interleukin-8 and chemokine CXCL1/GRO-α, *in vivo* they decreased angiogenesis and tumor progression in ovarian cancer xenografts [30].

The regulatory influence of the most active peptides I, III, IV and VI on G_s-coupled AC system was receptor and tissue specific. These peptides were active preferably in the testes, where there are receptors homologous to them, and affected signal transduction triggered by hCG that specifically binds to LH receptor. Earlier we and other authors showed that GPCR-peptides and their lipophilic analogs are able to effectively modulate the hormonal signalling only in the tissues and the cell cultures where the cognate receptors are expressed [6, 9, 11, 31, 32]. Contrary to the cell cultures where PAR1 was expressed, in the cells lacking the cognate receptor or having the mutant PAR1, peptide corresponding to the N-terminal portion of the ICL3 had no effect on the stimulation of phospholipase Cβ activity induced by PAR1-agonists [6]. The N-palmitoylated peptide 374–386 corresponding to membrane-proximal region of the intracellular C-terminal domain of PAR1 stimulated phosphoinositide turnover only in the cell cultures with expressed PAR1 [31]. These results coincide with the molecular model of the action of GPCR-peptides that is based on the highly specific interaction between the peptide and complementary regions of homologous receptor. This model gives, in addition, a good explanation of the receptor specificity of GPCR-peptides. We showed that none of the investigated peptides, the most active peptide VI in particular, affected the stimulation of AC system induced by different

hormones (β-agonist isoproterenol, polypeptide hormones PACAP-38 and relaxin) in the testicular and myocardial membranes. Other works also presented evidence for receptor specificity of GPCR-peptides derived from intracellular regions of PARs, chemokine receptors, 5-hydroxytryptamine receptors, sphingosine 1-phosphate receptor, relaxin receptor, TSH receptor, and others [6-11, 26, 33, 34].

At the same time, we revealed that unmodified peptide 562–572 and its analogs modified by decanoyl radicals reduced AC inhibitory effects of somatostatin and noradrenaline and their stimulating effect on GppNHp binding capacity both in the testicular and myocardial membranes. These effects were not receptor and tissue specific, which demonstrates a non-specific interaction of these peptides with the heterotrimeric G_i-proteins responsible for functional coupling between the enzyme AC and the G_i-coupled somatostatin and α_2-adrenergic receptors. The suggestion that GPCR-peptides can activate G_i-proteins is supported by the fact that the AC stimulating effect of the peptide was detected in the PTX-treated testicular membranes, although it was weakly expressed. Earlier it was shown that polycationic peptides, including the artificial peptides and natural toxins, non-homologous to GPCR, were able to interact directly with G_i-proteins [35, 36]. They increased their GTP-binding capacity and GTPase activity and weakened the signal transduction via G_i-coupled receptors. It seems likely that positively charged peptides interact with the negatively charged C-terminal segment of $G\alpha_i$-subunit [37].

CONCLUSION

Thus, we showed that to increase the functional activity of peptide corresponding to the ICL3 of LH receptor, it must be modified with palmitoyl radical at the C-terminus, where in the full-size LH receptor TM6 is located. Substitution of palmitoyl radical with decanoyl reduced the activity and specificity of the C-acylated peptide. The modification of peptide 562–572 at the N-terminal site corresponding to the middle of ICL3 gave inactive analogs. These data support the hypothesis that the hydrophobic radical in GPCR-peptide mimics the TM and must be comparable with it in size and hydrophobicity. The establishment of the criteria for the modification of GPCR-peptides with hydrophobic radicals is one of the most reliable ways to create, on the basis of peptides, both selective functional probes to study the hormonal signalling *in vitro* and drugs to control the biochemical and physiological processes in norm and pathology.

ACKNOWLEDGMENTS

This work was supported by Grants from the Russian Foundation of Basic Research (Nos. 12-04-00351 and 12-03-31716). We express our thanks to Inga Menina for linguistic assistance.

CONFLICT OF INTEREST STATEMENT

The authors report no conflict of interest in this research.

REFERENCES

1. Shpakov, A. O. and Pertseva, M. N. 2007, Signal Transduction Research Trends, N. O. Grachevsky (Ed.), Nova Science Publishers, Inc., New York, 45.
2. Miller, J., Agarwal, A., Devi, L. A., Fontanini, K., Hamilton, J. A., Pin, J. P., Shields, D. C., Spek, C. A., Sakmar, T. P., Kuliopulos, A. and Hunt, S. W. 2009, Ann. N.Y. Acad. Sci., 1180, E1.
3. Shpakov, A. O. 2011, Global J. Biochem., 2, 96.
4. Tressel, S. L., Koukos, G., Tchernychev, B., Jacques, S. L., Covic, L. and Kuliopulos, A. 2011, Meth. Mol. Biol., 683, 259.
5. Covic, L., Gresser, A. L., Talavera, J., Swift, S. and Kuliopulos, A. 2002, Proc. Natl. Acad. Sci. USA, 99, 643.
6. Covic, L., Misra, M., Badar, J., Singh, C. and Kuliopulos, A. 2002, Nat. Med., 8, 1161.
7. Kubo, S., Ishiki, T., Doe, I., Sekiguchi, F., Nishikawa, H., Kawai, K., Matsui, H. and Kawabata, A. 2006, Ann. N.Y. Acad. Sci., 1091, 445.
8. Shpakov, A. O., Gur'yanov, I. A., Kuznetsova, L. A., Plesneva, S. A., Shpakova, E. A., Vlasov, G. P. and Pertseva, M. N. 2007, Neurosci. Behav. Physiol., 37, 705.
9. Shpakov, A. O., Shpakova, E. A., Tarasenko, I. I., Derkach, K. V. and Vlasov, G. P. 2010, Int. J. Pept. Res. Ther., 16, 95.

10. Shpakov, A. O., Shpakova, E. A.,Tarasenko, I. I., Derkach, K. V., Chistyakova, O. V., Avdeeva, E. A. and Vlasov, G. P. 2011, Global J. Biochem., 2, 59.
11. Shpakov, A. O., Shpakova, E. A. and Derkach, K. V. 2012, Current Topics Pept. Protein Res., 13, 61.
12. Covic, L., Tchernychev, B., Jacques, S. and Kuliopulos, A. 2007, Handbook of Cell-Penetrating Peptides, Taylor & Francis, New York, 245.
13. Shpakova, E. A., Skvortsova, E. A., Tarasenko, I. I. and Shpakov, A. O. 2012, Tsitologiia, 54, 119.
14. O'Callaghan, K., Kuliopulos, A. and Covic, L. 2012, J. Biol. Chem., 287, 12787.
15. Guidelines for the treatment of animals in behavior research and teaching. 2006, Animal Behavior, 71, 245.
16. Shpakov, A. O., Chistyakova, O. V., Derkach, K. V., Moiseyuk, I. V. and Bondareva, V. M. 2012, Central Eur. J. Biol., 7, 33.
17. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. 1951, J. Biol. Chem., 193, 265.
18. Salomon, Y., Londos, C. and Rodbell, M. A. 1974, Anal. Biochem., 58, 541.
19. Freissmuth, M. and Gilman, A. G. 1989, J. Biol. Chem., 264, 21907.
20. Quist, E. E., Coyle, D. L., Vasan, R., Satumtira, N., Jacobson, E. L. and Jacobson, M. K. 1994, J. Mol. Cell. Cardiol., 26, 251.
21. Katada, T. 2012, Biol. Pharm. Bull., 35, 2103.
22. Qanbar, R., Cheng, S., Possmayer, F. and Schürch, S. 1996, Am. J. Physiol., 271, L572.
23. Gustafsson, M., Griffiths, W. J., Furusjö, E. and Johansson, J. 2001, J. Mol. Biol., 310, 937.
24. Shpakov, A. O. 2002, Tsitologiia, 44, 242.
25. Shpakova, E. A., Shpakov, A. O., Chistyakova, O. V., Moyseyuk, I. V. and Derkach, K. V. 2012, Dokl. Biochem. Biophys., 433, 64.
26. Sevigny, L. M., Zhang, P., Bohm, A., Lazarides, K., Perides, G., Covic, L. and Kuliopulos, A. 2011, Proc. Natl. Acad. Sci. USA, 108, 8491.
27. Kaufmann, R., Hascher, A., Mussbach, F., Henklein, P., Katenkamp, K., Westermann, M. and Settmacher, U. 2012, Histochem. Cell. Biol., 138, 913.
28. Michael, E. S., Kuliopulos, A., Covic, L., Steer, M. L. and Perides, G. 2013, Am. J. Physiol., 304, G516.
29. Leger, A. J., Jacques, S. L., Badar, J., Kaneider, N. C., Derian, C. K., Andrade-Gordon, P., Covic, L. and Kuliopulos, A. 2006, Circulation, 113, 1244.
30. Agarwal, A., Tressel, S. L., Kaimal, R., Balla, M., Lam, F. H., Covic, L. and Kuliopulos, A. 2010, Cancer Res., 70, 5880.
31. Swift, S., Leger, A. J., Talavera, J., Zhang, L., Bohm, A. and Kuliopulos, A. 2006, J. Biol. Chem., 281, 4109.
32. Shpakov, A. O. and Shpakova, E. A. 2011, Biochemistry (Moscow). Suppl. Ser. B: Biomed. Chem., 5, 246.
33. Licht, T., Tsirulnikov, L., Reuveni, H., Yarnitzky, T. and Ben-Sasson, S. A. 2003, Blood, 102, 2099.
34. Leger, A. J., Jacques, S. L., Badar, J., Kaneider, N. C., Derian, C. K., Andrade-Gordon, P., Covic, L. and Kuliopulos, A. 2006, Circulation, 113, 1244.
35. Shpakov, A. O. 2010, Current Topics Pept. Protein Res., 11, 1.
36. Shpakov, A. O., Tarasenko, I. I., Shpakova, E. A., Guryanov, I. A., Derkach, K. V. and Vlasov, G. P. 2010, Current Topics Pept. Protein Res., 11, 49.
37. Lambright, D. G., Sondek, J., Bohm, A., Skiba, N. P., Hamm, H. E. and Sigler, P. B. 1996, Nature, 379, 311.