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The sensitivity of the adenylyl cyclase system in rat thyroidal and extrathyroidal tissues to peptides corresponding to the third intracellular loop of thyroid-stimulating hormone receptor

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ABSTRACT

The importance of selective regulators of thyroid functions is dictated by a key role the hypothalamic-pituitary-thyroid axis plays in the control of biochemical and physiological processes and by the fact that only a limited number of drugs with thyrotropic potency is available today. We synthesized peptide corresponding to 612-627 region of third intracellular loop of rat thyroid-stimulating hormone (TSH) receptor and its palmitoylated analogue 612–627-K(Pal)A and studied their influence on adenylyl cyclase (AC) signaling system in the thyroid and the extrathyroidal tissues. Peptide 612–627-K(Pal)A at micromolar concentrations stimulated basal AC activity and GppNHp binding of G_s and G_q proteins and inhibited TSH-stimulating AC activity in the thyroidal membranes but had little influence on AC system in synaptosomal, myocardial and testicular membranes, which indicates the receptor and tissue specificity of 612–627-K(Pal)A. In the thyroid 612–627-K(Pal)A also decreased PACAP-38- and human chorionic gonadotropin-stimulated AC activity, which is most likely to be due to inhibiting of TSH-mimicking effect of these hormones on AC. Unmodified peptide 612–627-KA was effective to a much lesser extent, indicating the importance of hydrophobic moiety for 612–627-K(Pal)A activity. The obtained data suggest the possibility to develop thyroid regulators and modulators on the basis of TSH receptor-derived peptides.

KEYWORDS: adenylyl cyclase, GTP-binding protein, peptide, thyroid, thyroid-stimulating hormone, thyroid-stimulating hormone receptor, third intracellular loop

ABBREVIATIONS

AC, adenylyl cyclase; CTX, cholera toxin; FSH, follicle-stimulating hormone; GPCR, G protein-coupled receptor; 5-HTR, 5-hydroxytryptamine receptor; hCG, human chorionic gonadotropin; ICL3, third intracellular loop; LH, luteinizing hormone; PACAP-38, pituitary adenylyl cyclase-activating peptide-38; Pal, palmitoyl; PAR, protease-activated receptor; PLC, phospholipase C; PTX, pertussis toxin; TSH, thyroid-stimulating hormone; TSHR, TSH receptor

INTRODUCTION

The search of new regulators of thyroid-stimulating hormone (TSH)-sensitive signaling pathways in the thyroid gland is today one of the most important directions in molecular endocrinology

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as they are used for effective treatment and prevention of TSH-dependent thyroid and non-thyroid tumors and the diseases associated with hypo- and hyperthyroidism. Recombinant human TSH is difficult to produce, therefore its cost is high, and it is not used for regulation of the thyroid function in clinical practice. TSH has limited application in the monitoring of patients who are taking thyroid hormones after thyroid ectomy [1]. Recently small-molecule ligands for the TSH receptor (TSHR) with agonistic and antagonistic activity were developed and shown to regulate TSH-dependent effector systems in the primary cultures of human thyrocytes *in vitro* and to control the thyroid function in mice *in vivo*, inducing changes in serum thyroxine level and in thyroidal radioiodide uptake [2]. Another, also a very promising approach for developing the selective regulators of thyroid function is the peptide strategy based on the synthesis and study of peptides corresponding to functionally important regions of signal proteins, the components of TSH-regulated signaling pathways, TSHR in particular [3-6].

The efficiency of G protein recognition by heptahelical G protein-coupled receptors (GPCR) and the interaction between hormone-activated GPCR and G protein are determined by intracellular domains of the receptors, the third intracellular loop (ICL3) primarily [7]. This applies to the TSHR, which, like the human chorionic gonadotropin (hCG)/luteinizing hormone (LH) and the follicle-stimulating hormone (FSH) receptors, belongs to the subfamily of glycoprotein hormone receptors [8-10]. The N-terminal and central portions of the second intracellular loop (ICL2) as well as the N- and C-terminal regions of the ICL3 of TSHR participate in the activation of both G_s and G_q proteins and, thus, are involved in adenylyl cyclase (AC)- and phospholipase C (PLC)-mediated intracellular signaling [11].

We and the other authors showed that the synthetic peptides derived from the intracellular loops of GPCR have the ability to activate the signaling cascades including the homologous receptors and to influence the transduction of hormonal signal through them [3, 12-21]. The peptides corresponding to the ICL3 of hCG/LH receptor stimulated the activity of adenylyl cyclase (AC) signaling system and decreased the stimulating effects of hCG on the AC activity and the GTP binding capacity of G proteins, preferably G_s proteins, in the rat testicular membranes [19]. The peptide 534-556 corresponding to the entire ICL3 of FSH receptor stimulated G_s proteins, inhibited the coupling between hormone-activated receptor and G_s protein and decreased FSH-induced AC stimulation and steroid hormones production in the testicular tissue of rats and cultured Sertoli cells [12]. In the last few years, the evidences were obtained that GPCR-peptides are active not only *in vitro* but also influence the physiological and biochemical processes *in vivo*, this opens the way to develop a new generation of peptide-based drugs regulating and modulating the GPCR-coupled signaling systems [5, 6].

The aim of this work was, first, to study the influence of peptide Gln-Tyr-Asn-Pro-Arg-Asp-Lys-Asp-Thr-Lys-Ile-Ala-Lys-Arg-Nle-Ala$^{612–627}$-Lys-Ala-amide (612–627-KA) corresponding to the C-terminal portion of the ICL3 of rat TSH receptor and its lysine-palmitoylated analogue (612–627-K(Pal)A) on the functional activity of AC system in the thyroid, the main target of TSH, and in the extrathyroidal tissues, and second, to study the specificity and selectivity of their action. We showed that in the rat thyroid palmitoylated peptide 612–627-K(Pal)A activated TSH-dependent AC signaling system, decreased the transduction of TSH-induced signal via this system, and its regulatory action on the AC system was receptor- and tissue-specific. At the same time, non-palmitoylated peptide had a rather low activity, indicating an important role of the hydrophobic palmitoyl residue for efficient interaction of TSHR-peptides with proteins, the components of TSH-activated signaling cascades.

MATERIALS AND METHODS

Animals

In experiments adult 6 month-old male Wistar rats were used. The animals were housed in plastic sawdust-covered cages with a normal light-dark cycle and free access to food and water. The experiments were carried out under the guidelines of the National Institutes of Health regulations for the Care and Use of Animals for Scientific Purposes

according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" and in the "Guidelines for the treatment of animals in behavior research and teaching" [22]. All efforts were made to minimize animal suffering and reduce the number of animals used.

Chemicals and radiochemicals

The chemicals, such as TSH from bovine pituitary, hCG, pituitary adenylyl cyclase-activating peptide-38 (PACAP-38), isoproterenol, serotonin, forskolin, cAMP, ATP, GTP, and 5'-β,γ-imidotriphosphate (GppNHp), were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Calbiochem (San Diego, CA, USA). The peptide Lys-Asp-Thr-Ile-Leu-Gln-Leu-Asn-Leu-Lys-Glu-Tyr-Asn-Leu-Val$^{345–359}$ corresponding to the last C-terminal region of $G\alpha_q$ subunit was kindly provided by Dr. Valery I. Korolkov (Institute of Macromolecular Compounds, St. Petersburg, Russia). [α-^{32}P]-ATP (4 Ci/mmol) was purchased from Isotope Company (St. Petersburg, Russia), β,γ-imido[8-^{3}H]-guanosine-5'-triphosphate ([8-^{3}H]-GppNHp) (5 Ci/mmol) was from Amersham (UK); the type HA 0.45 μm nitrocellulose filters were from Sigma-Aldrich Chemie GmbH (Germany).

The synthesis of peptides

The peptide Gln-Tyr-Asn-Pro-Arg-Asp-Lys-Asp-Thr-Lys-Ile-Ala-Lys-Arg-Nle-Ala$^{612–627}$-Lys-Ala-amide (612–627-KA) and its palmitoylated analogue Gln-Tyr-Asn-Pro-Arg-Asp-Lys-Asp-Thr-Lys-Ile-Ala-Lys-Arg-Nle-Ala$^{612–627}$-Lys(Pal)-Ala-amide (612–627-K(Pal)A) with the residue Met^{626} replaced with norleucine were synthesized by the standard solid phase reaction procedures using *p*-methylbenzhydrylamine resin (capacity 1.16 mmols per g) using a semiautomatic synthesizer NPS-400 (Neosystem Laboratories, France). The side chain groups of amino acids were protected with *tert*-butyloxycarbonyl (Boc), benzyl, mesitylenesulfonyl and 2-chlorobenzyloxycarbonyl groups. The coupling of amino acids was developed by water-soluble 1,3-diisopropylcarbodiimide in the presence of 1-hydroxybenzotriazole (1-2 h per residue). Boc-groups were removed for 1 h using 50% TFA in methylene chloride. Palmitoylated lysine for the synthesis of peptide 612–627-K(Pal)A was obtained as a result of coupling of pentafluorophenyl palmitate and *N*α-*t*-BOC-lysine using dicyclohexylcarbodiimide in the presence of triethylamine. The cleavage from resin and the removal of protecting groups were performed using a mixture of 1 ml trifluoromethanesulfonic acid, 10 ml TFA, 1 ml thioanisole and 0.5 ml ethandithiol for 1.5 h at 4°C and for 2 h at room temperature (the quantities of reagents were calculated per 1 g of peptidyl-polymer). The crude peptides were isolated by gel-filtration on Sephadex G-10 in 6% acetic acid and purified by RP-HPLC on a Vydac C18 218TP column using water–acetonitrile linear gradient containing 0.1% TFA. All peptides were purified up to and over 95% homogeneities (detection at 230 nm). The identity and purification degree of peptides were assessed using RP-HPLC and electrospray ionization mass spectrometry. The resulting mass spectrometry data were found to be as follows: the M_{exp} of peptide 612–627-KA was 1915.10 (M_{calc}, 1915.08) and the M_{exp} of peptide 612–627-K(Pal)A was 2336.50 (M_{calc}, 2336.45).

Preparation of plasma membranes from the thyroid and extrathyroidal tissues

The preparation of plasma membranes from the rat thyroid was performed according to Heyma and Harrison [23], with some modifications. The thyroid tissue was obtained from decapitated rats and washed in ice-cold 40 mM Tris-HCl buffer (pH 7.4) containing 5 mM $MgCl_2$ (Buffer A). The tissue was cut into small pieces, homogenized with a Polytron in 10 volumes of Buffer A containing a cocktail of protease inhibitors 500 μM *O*-fenantrolin, 2 μM pepstatin and 100 μM phenylmethylsulphonyl fluoride and centrifuged at 500 g for 15 min at 4°C. The pellet was discarded, and the supernatant was centrifuged at 10000 g for 30 min at 4°C.

The preparation of cardiac membranes from the myocardium, of synaptosomal membranes from the brain (cerebral cortices, hippocampi and striata) and testicular membranes from the testes was carried out as described previously [18, 19]. The final pellet was resuspended in 50 mM Tris-HCl buffer (pH 7.4) to produce the membrane fraction with a protein concentration range of 1-3 mg/ml and stored at -70°C. The protein concentration of each membrane preparation was measured by the method of Lowry and colleagues using BSA as a standard.

Adenylyl cyclase assay

Adenylyl cyclase (EC 4.6.1.1) activity was measured using the method of Salomon and colleagues [24], with some modifications [18]. The reaction mixture (final volume 50 µl) contained 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM ATP, 1 µCi [α-^{32}P]-ATP, 0.1 mM cAMP, 20 mM creatine phosphate, 0.2 mg/ml creatine phosphokinase, and 15-45 µg of membrane protein. Incubation was carried out at 37°C for 20 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 activity was measured in the absence of hormones, peptides, forskolin and guanine nucleotides. To study the influence of TSHR-peptides on AC activity, the membrane fractions were pre-incubated at 4°C for 10 min with and without peptides.

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

[8-^{3}H]-GppNHp binding to G proteins was estimated as described earlier [25]. The reaction 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. The reaction was started by the addition of 50-100 µg of membrane protein and carried out at 30°C for 45 min. Being incubated, the reaction mixture was rapidly diluted with 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 three times with 2 ml of washing buffer and dried. The filter-bound radioactivity was estimated in a toluene scintillator using a LKB 1209/1215 RackBeta scintillation counter. 10 mM GppNHp was added to the reaction mixture to estimate non-specific binding. The specific GppNHp binding was taken to be the difference between a total and non-specific binding. 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 hormones and peptides. To study the influence of TSHR-peptides and $G\alpha_q$-derived peptide on GppNHp binding, the membrane fractions were pre-incubated at 4°C for 10 min with and without peptides.

Cholera and pertussis toxin-catalyzed ADP-ribosylation

ADP-ribosylation of the thyroidal plasma membranes by bacterial toxins was performed as described earlier [18]. The membrane samples (protein concentration was 0.9-1.0 mg/ml) were incubated at 37°C for 45 min with 100 µg/ml of cholera toxin (CTX) or 10 µg/ml 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. 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. After ADP-ribosylation reaction, the suspension was diluted to a final volume of 5 ml using ice-cold 50 mM Tris-HCl buffer (pH 7.5) with 5 mM $MgCl_2$ and centrifuged at 37000 × g for 15 min. The pellet was resuspended in the same buffer and immediately used to estimate AC activity and GppNHp binding. Control membranes were treated similarly but without toxins.

Statistical analysis

The data is presented as the mean ± SEM. The difference in the enzyme activity, and in GppNHp binding in control and 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 basal activity of AC in the rat thyroidal membranes was 17.7 ± 1.1 pmol cAMP/min per mg of membrane protein, and the basal level of GppNHp binding of heterotrimeric G proteins was 1.24 ± 0.06 pmol [8-^{3}H]GppNHp/mg of membrane protein. Diterpene forskolin (10^{-5} M), directly interacting with the catalytic site of AC, and non-hydrolysable GTP analogue GppNHp (10^{-5} M), interacting with the guanine nucleotide-binding site of G protein α-subunit, increased the basal AC activity by 328 and 184%, respectively. The bovine TSH (10^{-8} M) stimulated AC activity in the thyroidal membranes by 244% and increased

GppNHp binding by 87% (Table 1). The corresponding effects of PACAP-38 (10^{-6} M) and hCG (10^{-8} M) were much lower. The AC effects of PACAP-38 and hCG were 68 and 45% and their stimulating effects on GppNHp binding were 39 and 17%, respectively.

In the thyroidal membranes peptide 612–627-K(Pal)A increased in a dose-dependent manner the basal AC activity, and its half-maximal stimulation of AC was observed at 11 μM, also increased was the basal level of GppNHp binding (Fig. 1). The maximal AC effect of the peptide was observed at 10^{-4} M, but as far as GppNHp binding is concerned, the stimulating effect was increased at the peptide concentration in the range 10^{-4} to 10^{-3} M. The corresponding effects of unmodified analogue 612–627-KA were much less pronounced and did not reach maximum at the peptide concentrations in the above range.

To estimate the tissue specificity of TSHR-peptides, their regulatory effects on the activity of AC system in the extrathyroidal tissues were studied. In the membrane preparations isolated from the rat brain and myocardium no stimulating effects of TSHR-peptides on the basal AC activity and GppNHp binding level were revealed, even in the case of more active peptide 612–627-K(Pal)A (Table 2). But they were detected in the testicular membranes, being however much weaker compared with those in the thyroidal membranes. Thus, the action of TSHR-peptides, like that of TSH, was tissue specific, being most pronounced in the thyroidal gland enriched with TSHR, but was weakly pronounced in the extrathyroidal tissues, where the expression of TSHR is either low or altogether absent.

It is well known that TSHR are capable of coupling all four G-protein families, namely G_s, $G_{i/o}$, $G_{q/11}$ and $G_{12/13}$ proteins [26]. To identify the types of heterotrimeric G proteins, which are the targets for TSHR-peptides, we used the technique of ADP-ribosylation by bacterial toxins, and the 15-mer peptide corresponding to the C-terminal

Table 1. The stimulating influence of TSHR-peptides on AC activity and GppNHp binding in the thyroidal membranes treated by bacterial toxins.

	Without toxins	PTX	CTX
Adenylyl cyclase activity, pmol cAMP/min per mg of membrane protein			
Without	17.7 ± 1.1	16.6 ± 0.8	53.7 ± 3.7
612–627-KA, 10^{-4} M	25.0 ± 1.5* (+41)	25.7 ± 2.9* (+51)	51.2 ± 3.0 (-)
612–627-K(Pal)A, 10^{-4} M	38.1 ± 3.2* (+115)	39.5 ± 1.0** (+129)	56.8 ± 3.6 (+18)
TSH, 10^{-8} M	60.9 ± 2.9** (+244)	58.0 ± 2.9** (+234)	62.1 ± 5.6 (+47)
GppNHp binding, pmol [8-^{3}H]GppNHp/mg of membrane protein			
Without	1.24 ± 0.06	1.27 ± 0.07	0.98 ± 0.11
612–627-KA, 10^{-4} M	1.44 ± 0.07 (+16)	1.50 ± 0.12 (+19)	1.10 ± 0.09 (+10)
612–627-K(Pal)A, 10^{-4} M	2.01 ± 0.05** (+62)	1.96 ± 0.04** (+56)	1.31 ± 0.07 (+27)
TSH, 10^{-8} M	2.32 ± 0.11** (+87)	2.19 ± 0.12** (+74)	1.54 ± 0.05* (+45)

Figures in parentheses designate the stimulating effects of TSHR-peptides and TSH on AC activity and GppNHp binding in percentage over the basal AC activity and the basal level of GppNHp binding, respectively, taken as 100%. Values are expressed as the mean ± SEM for three individual experiments. *, ** - $P < 0.05$ and $P < 0.001$, respectively.

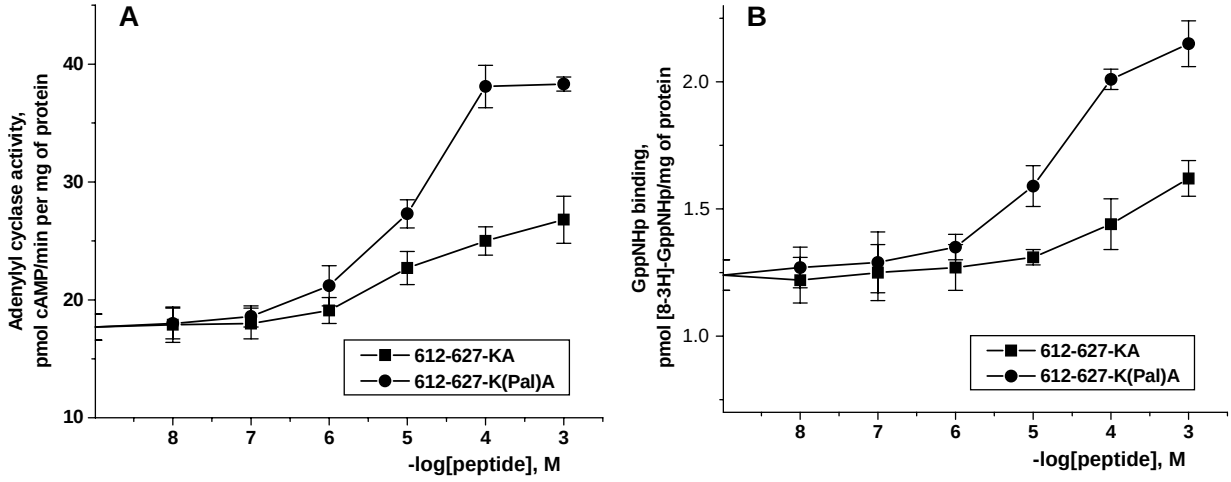

Fig. 1. The stimulating effects of TSHR-peptides on the basal AC activity (A) and the basal level of GppNHp-binding (B) in the rat thyroidal membranes.
1 - 612–627-KA; *2* - 612–627-K(Pal)A. The basal AC activity (17.7 ± 1.1 pmol cAMP/min per mg of membrane protein) and the basal level of GppNHp-binding (1.24 ± 0.06 pmol [8-^{3}H]GppNHp/mg membrane protein) are taken as 100%. Values are the mean ± SEM of three individual experiments, each performed in triplicate. The membranes were pre-incubated in the presence of peptides as described in Materials and Methods.

Table 2. The stimulating influence of TSHR-peptides on AC activity and GppNHp binding in the non-thyroid tissues of rats.

	Brain	Myocardium	Testes
Adenylyl cyclase activity, pmol cAMP/min per mg of membrane protein			
Without	20.2 ± 0.5	26.8 ± 1.6	13.6 ± 1.0
612–627-KA, 10^{-4} M	22.1 ± 1.4 (+9)	28.2 ± 1.0 (+5)	13.7 ± 1.1 (-)
612–627-K(Pal)A, 10^{-4} M	22.0 ± 1.1 (+9)	25.9 ± 1.8 (-)	15.8 ± 1.2 (+16)
TSH, 10^{-8} M	24.3 ± 1.3* (+20)	27.0 ± 0.9 (-)	19.5 ± 1.8* (+43)
GppNHp binding, pmol [8-^{3}H]GppNHp/mg of membrane protein			
Without	4.1 ± 0.1	3.9 ± 0.1	2.3 ± 0.3
612–627-KA, 10^{-4} M	4.3 ± 0.1 (+5)	4.0 ± 0.1 (-)	2.3 ± 0.2 (-)
612–627-K(Pal)A, 10^{-4} M	4.4 ± 0.4 (+7)	4.2 ± 0.4 (+8)	2.6 ± 0.1 (+13)
TSH, 10^{-8} M	4.7 ± 0.3 (+15)	4.2 ± 0.1 (+8)	3.0 ± 0.3 (+30)

Figures in parentheses represent the stimulating effects of TSHR-peptides and TSH on AC activity and GppNHp binding in percentage over the basal AC activity and the basal level of GppNHp binding, respectively, taken as 100%. Values are expressed as the mean ± SEM for three individual experiments. * – $P < 0.05$.

region 345-359 of G_q protein α-subunit responsible for interaction between activated receptor and G_q protein. The treatment by CTX leads to the modification of the guanidine group of arginine residue in α_s subunit with ADP-ribosyl moiety that blocks GTPase activity of G_s protein and converts it into a permanently active GTP-bound state insensitive to hormone regulation, so that the treatment by PTX leads to the ADP-ribosylation of the sulfhydryl group of cysteine residue in the C-terminal region of $\alpha_{i/o}$ subunit and blocks the interaction between hormone-activated receptor and $G_{i/o}$ protein [27, 28]. The peptides corresponding to the C-terminal region of G protein α subunit, $G\alpha_q$ in particular, in a competitive manner occupy the G protein-binding site of GPCR and impair the interaction between the activated receptor and G protein α subunit [29-31].

In the thyroidal membranes treated by CTX the basal AC activity was enhanced by 203%, while GppNHp binding was decreased by 21%. AC stimulating effects of the peptide 612–627-K(Pal)A (10^{-4} M) and TSH (10^{-8} M) in CTX-treated membranes underwent five-six fold reduction, and the corresponding effect of unmodified peptide was not detected at all (Table 1). The stimulating effects of the peptide 612–627-K(Pal)A (10^{-4} M) and TSH (10^{-8} M) on GppNHp binding in CTX-treated membranes showed two-three fold reduction. In PTX-treated thyroidal membranes the corresponding effects of the TSHR-peptides and TSH changed but not significantly. A less pronounced decrease of the stimulating effect of TSHR-peptides on GppNHp-binding in comparison with those on AC activity in CTX-treated membranes, and insensitivity of these effects to PTX treatment may be due to the ability of these peptides, similar to TSH, to activate G_q proteins, insensitive to CTX and PTX, which are involved in the functional coupling between activated TSHR and PLC. This suggestion finds support in the data that the stimulating effects of the peptide 612–627-K(Pal)A on GppNHp binding in the presence of C-terminal peptide 345-359 of $G\alpha_q$ subunit (10^{-3} M) decreased significantly in CTX-treated as well as -untreated thyroidal membranes (Fig. 2). A similar picture was observed in the case of TSH (data not shown). It should be noted that in the control membranes the $G\alpha_q$-peptide-

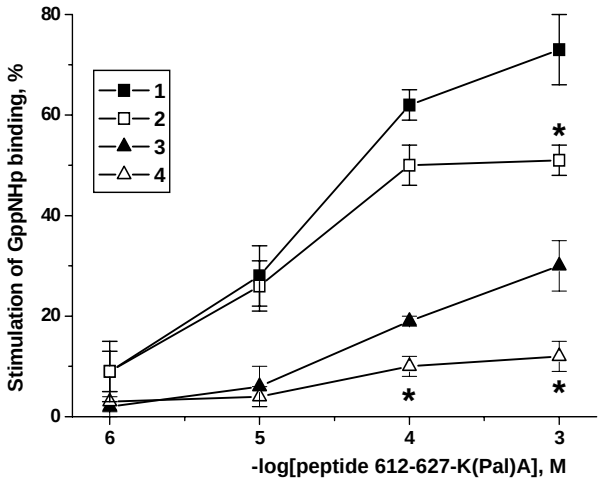

Fig. 2. The inhibitory influence of C-terminal peptide 345–359, derivative of **$G\alpha_q$ subunit, on t**he stimulating effects of the peptide 612–627-K(Pal)A on GppNHp binding in the control and the cholera toxin-treated membranes.
1 - control membranes; *2* - control membranes pre-incubated with **$G\alpha_q$**-peptide 345-359 (10^{-3} M); *3* - CTX-treated membranes; *4*- CTX-treated membranes pre-incubated with **$G\alpha_q$**-peptide 345-359 (10^{-3} M). * - $P < 0.05$.

induced decrease of GppNHp binding stimulating effect of the peptide 612–627-K(Pal)A taken at 10^{-4} and 10^{-3} M was 19 and 30%, respectively, but was not detected at lower concentrations of the peptide. In addition, in CTX-treated membranes $G\alpha_q$-peptide significantly inhibited the residual stimulating effect of 10^{-4}-10^{-3} M 612–627-K(Pal)A on the GppNHp binding, due to activation of CTX-insensitive G_q proteins. This is the evidence that the concentration of peptide 612–627-K(Pal)A which is effective for activation of G_q protein is higher than that for activation of G_s protein.

Peptide 612–627-K(Pal)A in a dose-dependent manner decreased the stimulating effects of TSH on AC activity and GppNHp binding (Fig. 3). The peptide-induced decrease of hormone-stimulated GppNHp-binding was better expressed compared to a decrease of AC effect due to TSH. For example, the stimulating effects of TSH on AC activity and GppNHp-binding in the presence of peptide 612–627-K(Pal)A taken at 10^{-4} M was reduced by 35 and 48%, respectively. Peptide 612–627-K(Pal)A decreased the stimulating effects

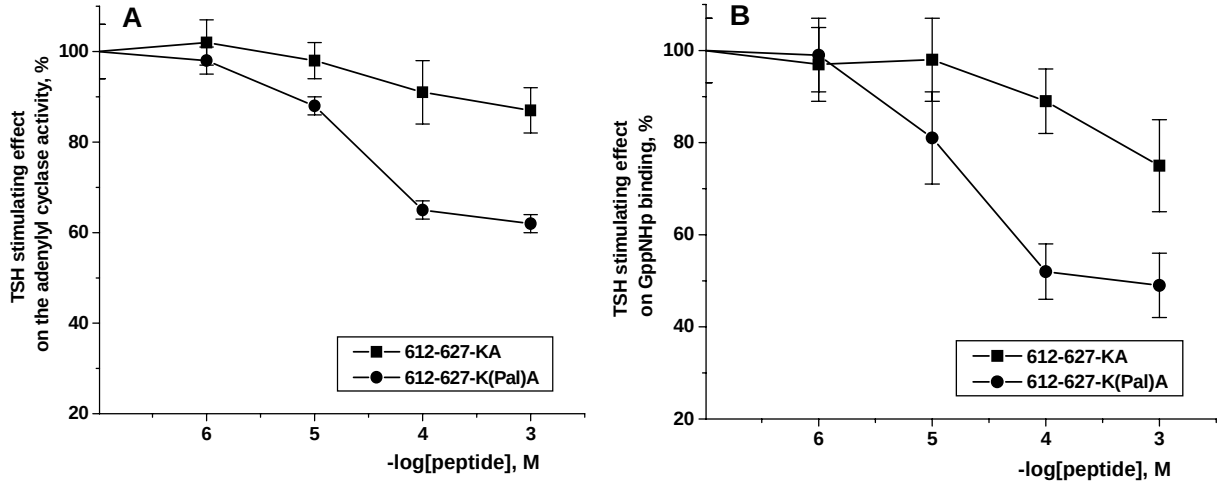

Fig. 3. The influence of TSHR-peptides on TSH-induced stimulation of AC activity (A) and GppNHp-binding (B) in the rat thyroid membranes.
1— 612–627-KA; *2* - 612–627-K(Pal)A. Stimulating effects of TSH (10^{-8} M) on AC activity and GppNHp-binding in the absence of TSHR-peptides are taken as 100%. Values are the mean ± SEM of three individual experiments, each performed in triplicate.

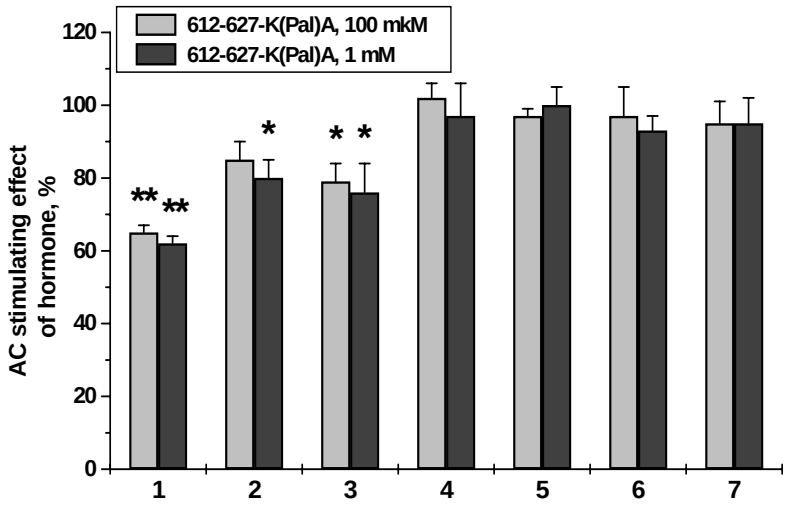

Fig. 4. The effects of the peptide 612–627-K(Pal)A on hormone-stimulated AC activity in the rat tissues.
1 - TSH (10^{-8} M); 2 - PACAP-38 (10^{-6} M); 3 - hCG (10^{-8} M); 4 - isoproterenol (10^{-5} M); 5 - serotonin (10^{-5} M); 6 - PACAP-38 (10^{-6} M); 7 - hCG (10^{-8} M). 1-3 -thyroid; 4 - myocardium; 5 - brain; 6, 7 - testes.
Stimulating effects of the hormones on AC activity in the absence of peptide were taken as 100%. AC stimulating effects of TSH, PACAP-38 and hCG in the thyroidal membranes were 244, 68 and 45% over the basal activity, the same effects of isoproterenol in the myocardial membranes and serotonin in the synaptosomal membranes were 265 and 280%, and AC effects of PACAP-38 and hCG in the testicular membranes were 213 and 610%, respectively. The membranes were pre-incubated in the presence of peptide 612–627-K(Pal)A (10^{-4} and 10^{-3} M) as described in Materials and Methods. Values are the mean ± SEM of three independent experiments, each performed in triplicate. *, ** - $P < 0.05$ and $P < 0.001$, respectively.

of hormone with half-maximal inhibition at 24-28 μM. Peptide 612–627-KA was less effective and when taken at 10^{-4} M reduced TSH-induced stimulation of AC activity and GppNHp binding only by 9 and 11%, respectively.

To test the specificity of TSHR-peptides, the influence of peptide 612–627-K(Pal)A on AC stimulating effects of the hormones, activators of the enzyme, such as PACAP-38 and hCG in the testicular and thyroid membranes, β-agonist isoproterenol in the myocardial membranes and serotonin in the synaptosomal membranes was studied. Both palmitoylated and unmodified TSHR-peptides had very little or no influence on AC effects of these hormones in the extrathyroidal tissues (Fig. 4). Pre-incubation with peptide 612–627-K(Pal)A, however, led to a decrease of AC effects of PACAP-38 and hCG in the thyroidal, but not in the testicular membranes. The inhibitory influence of the peptide on AC effects of PACAP-38 and hCG was less pronounced compared with that of TSH. The peptide 612–627-K(Pal)A at 10^{-4} M decreased AC effects of TSH, PACAP-38 and hCG by 35, 15 and 21%, respectively. It should be noted that in the thyroidal membranes hCG and PACAP-38 are able to bind to TSHR in a non-specific manner and stimulate AC activity via both the cognate receptors and TSHR [32, 33]. These data suggest that the inhibitory influence of peptide 612–627-K(Pal)A on PACAP-38- and hCG-induced AC stimulation in the thyroid can be caused by the peptide-induced impairment of functional coupling between PACAP-38 and hCG-activated TSHR and G_s protein.

DISCUSSION

The first data on the ability of synthetic peptides, the derivatives of the intracellular regions of GPCR, to influence the activity of hormonal signaling systems appeared at the turn of 1980s-90s and referred to rhodopsin and adrenergic receptors [34, 35]. Later it was shown that many of the peptides, derivatives of the ICL2 and ICL3 and the cytoplasmic C-tail of the receptors of biogenic amines, peptide and protein hormones, prostaglandins, fatty acids and cannabinoids possess biological activity and have the ability to regulate and modulate GPCR-dependent signaling cascades *in vitro* and *in vivo* [3-6, 36]. In the present study we showed that peptide 612–627-K(Pal)A corresponding to the C-terminal region of the ICL3 of rat TSH receptor at micromolar concentrations stimulated AC activity in the thyroidal membranes and increased the level of GppNHp binding of G_s proteins responsible for transduction of stimulating hormonal signal to AC. Using the technique of ADP-ribosylation of G protein α subunits with bacterial toxins CTX and PTX selectively blocking G_s- and G_i-dependent signaling pathways, respectively, and the $G\alpha_q$-derived 15-mer peptide disturbing in a competitive manner the interaction between hormonal receptor and $G\alpha_q$ subunit we found that peptide 612–627-K(Pal)A also activated GTP binding capacity of G_q proteins mediating the regulatory effects of TSH on PLC and intracellular calcium signaling. As is well known, in mammals TSHR is functionally coupled with all four G protein families, namely G_s, $G_{i/o}$, $G_{q/11}$, and $G_{12/13}$, but so far biological relevance has only been attributed to the activation of G_s proteins, which is thought to regulate growth and differentiation of the thyroid and, to a lesser degree, to the activation of $G_{q/11}$ proteins, which is thought to stimulate thyroid hormone synthesis and iodination [37, 38]. The key role in the specificity and efficiency of interaction between TSH-activated receptor and G protein α subunits belongs to membrane-proximal regions of the ICL2 and ICL3 of TSHR, especially to ICL3 N- and C-terminal regions involved in binding and activation of G_s and G_q proteins. Thus, the G protein specificity of peptide 612–627-K(Pal)A is similar to that of both the full-length TSHR and its ICL3. Peptide 612–627-K(Pal)A includes the positively charged lysine residues (Lys^{618} and Lys^{621}) that in the case of full-length receptor are most likely directly interacting with C-terminal segment of $G\alpha_q$ subunit, since their replacement by other residues would induce selective interruption of the functional coupling between TSHR and $G\alpha_q$. The sequence 612–627 also includes a non-polar Ile^{622} participating, according to the site-directed mutagenesis data, in the interaction with G_s and G_q proteins. The replacement of Ile^{622} by other amino acids, aspartic acid in particular, leads to the impairment of this interaction [11].

We found that the regulatory action of TSHR-peptides on the AC system is tissue and receptor specific. The stimulating effects of peptide 612–627-K(Pal)A on the basal AC activity and the basal level of GppNHp binding were most pronounced in the thyroid, but were absent or very low in the extrathyroidal tissues under study, such as brain, myocardium and testes, which is in good agreement with the expression and distribution of TSHR in the mammalian tissues. It is well known that TSHR is primarily expressed in the thyroid follicular cells, but in the brain, cardiac muscle and testes the level of its expression is low or not detectable and varies significantly in different species of animals [39-42]. Although in the rat brain cryoslices TSHR mRNA expression was detected both in neuronal cells and in astrocytes, the level of the receptor transcript was significantly lower than in the thyroid [43]. In our previous studies the peptides, derivatives of the ICL3 of hCG/LH-receptor and 5-hydroxytryptamine receptor of the type 6 (5-HT_6R), were most active in the reproductive tissues and the brain, respectively, enriched with the receptors homologous to them and did not influence significantly the AC signaling in the tissues with no or weakly expressed cognate receptors [18, 19]. The regulatory effects of peptide corresponding to N-terminal region of the ICL3 of protease-activated receptor of the type 1 (PAR1) on the PLCβ activity were not detected in the tissues and cells lacking the cognate receptor or having a mutant truncated receptor lacking the C-terminal cytoplasmic domain [14]. It was also shown that palmitoylated peptide 374-386 corresponding to membrane-proximal region of the C-terminal domain of PAR1 regulated phosphoinositide signaling pathway only in the cell cultures with expressed PAR1 [44].

The data obtained in this study showing that TSHR-peptides influence the AC system predominantly in the thyroid enriched with homologous receptors gives evidence for a direct involvement of TSHR in the peptide effects. This is in agreement with the molecular mechanism of action of peptides corresponding to the intracellular regions of GPCR and their hydrophobic radical-modified derivatives, developed by Athan Kuliopulos' group and supported by us [3-6, 13]. This mechanism involves specific interactions of GPCR-peptide with complementary regions localized in the intracellular domains of cognate receptor which have contacts with the region homologous to the peptide. These interactions induce a conformational rearrangement in the intracellular domains and/or in the transmembrane channel resulting in alteration of the ability of the receptor regions to interact both with G proteins and a majority of signal and adaptor intracellular proteins regulating GPCR-mediated signaling.

We and the other authors showed the efficiency of the action of GPCR-peptides to be highly dependent on the peptide structure, first of all, on the presence of the motifs enriched with the positively charged amino acid residues, the hydrophobic radicals mimicking the receptor transmembrane domains and the membrane-permeable sequences [3-6, 13-15, 45]. GPCR-peptides with hydrophobic radicals such as fatty acid residue or transmembrane region fragment, designated as pepducins, possess higher efficiency and selectivity compared with unmodified analogues, which earlier has been demonstrated by us in respect to peptides, derivatives of the ICL3 of G_s-coupled CG/LH receptor, relaxin receptor RXFP1 and 5-HT_6R [17-19], and by the other authors in respect to peptides corresponding to the intracellular regions of different types of $G_{q/11}$- and G_i-coupled PAR and G_s-coupled melanocortin receptor [13, 14]. The present study shows that a more efficient modification of peptide 612–627-K(Pal)A by C_{16}-palmitoyl radical enabled it to stimulate AC activity and GppNHp binding more efficiently and have a more pronounced inhibitory effect on TSH-induced stimulation of AC system in the thyroid membranes, compared with its unmodified analogue. The latter's maximal stimulating effect on the AC system in the basal state was decreased and the inhibitory effect on TSH-activated AC system less pronounced. The half-maximal stimulation and inhibition of these effects were expressed by higher concentration compared with peptide 612–627-K(Pal)A.

The increase of biological activity of GPCR-peptides modified by hydrophobic radical may have the following causes. The peptides anchor in the plasma membrane and form a stable complex

with hydrophobic regions of signal proteins, in particular with transmembrane channel of GPCR and its interface at the junction of the intracellular and transmembrane domains, which leads to high molarity in the perimembranous area where signal proteins, the targets of GPCR-peptides, are localized. It seems likely that the hydrophobic tail of GPCR-peptide penetrates into the transmembrane channel of receptor or into the hydrophobic core formed by its cytoplasmic domains while the polar, usually positively charged, moiety of peptide is in contact with the complementary regions of GPCR or some other signal proteins located on the cytoplasmic side of plasma membrane.

We showed that TSHR-peptides have receptor specificity, which agrees very well with the model of GPCR-peptides action based on their interaction with the complementary regions of cognate receptor. It was also shown in our experiments that in the rat thyroid peptide 612–627-K(Pal)A at micromolar concentrations inhibited TSH-induced stimulation of AC activity and GppNHp binding mediated via TSHR but had no significant influence on the AC effects of the other hormones in the extrathyroidal tissues, such as β-adrenergic agonist isoproterenol in the myocardium, serotonin in the brain and PACAP-38 and hCG in the testes. It should be noted that hCG binds specifically to hCG/LH receptor which, like TSHR, belongs to the subfamily of glycoprotein hormone receptors and by its structural-functional organization and G protein specificity is similar to TSHR [9]. The fact that peptide 612–627-K(Pal)A exerted no influence on hCG-induced AC stimulation in the testes prompted a conclusion that it is not able to interact with the intracellular regions of hCG/LH receptor. The data we obtained extends the number of GPCR-peptides that interact with high specificity with the cognate receptors. Recently we showed that 5-HT_6R-derived peptide reduced the stimulating effects of serotonin and even more of EMD-386088, a selective 5-HT_6R agonist, on AC activity and G_s protein GTP binding, but did not influence the effects of agonists of the other 5-HTR, 5-carboxamidotryptamine and 8-OH-DPAT in particular, which are also able to stimulate the AC system [18]. Peptides 306-316 and 300-316, the derivatives of the ICL3 of 5-HT_{1B}R, decreased the regulatory effects of serotonin and 5-HT_1R agonists, especially of selective 5-HT_{1B}R agonist 5-nonyloxytryptamine, but did not change significantly the regulatory effects of agonists of the other 5-HTR [46]. The palmitoylated 12-mer peptide Pal-RCLSSSAVANRS corresponding to the ICL3 of PAR1 and functioning as PAR1 antagonist inhibited the increase of inositol triphosphate production and decreased the intracellular Ca^{2+} level induced by PAR1 agonists, it also blocked the effects of these agonists on the platelet aggregation but had no influence on vasorelaxation caused by SLIGRL-amide, PAR2 agonist; neither did it inhibit PAR4-dependent platelet aggregation [14, 16].

At the same time, in the thyroidal membranes peptide 612–627-K(Pal)A taken at 10^{-4} and 10^{-3} M decreased AC stimulating effects of PACAP-38 and hCG, although to a lesser extent than in the case of TSH. In our view this is due to the fact that in the thyroid hCG and PACAP-38 are able to bind with TSHR and possess the thyrotropic potency. It was found earlier that hCG, like TSH, stimulates AC activity via TSHR and inhibits the binding of TSH in the thyroidal membranes [33, 47]. PACAP-38 increased cAMP production in porcine thyroid cells *in vitro* and increased T4 production from mouse thyroid gland *in vivo* [32]. It was also shown that PACAP-38 inhibited in a competitive manner [^{125}I]-TSH binding to the thyroidal membranes, which indicates the ability of this hormone to occupy the ligand-binding site of TSHR and induce TSH-related effects. AC stimulating action of PACAP-38 is realized either through TSHR, or the specific PAC_1 and $VPAC_1$ receptors detected in the thyroid follicular cells, parafollicular C-cells and the blood vessels of rats [48]. Therefore, the inhibitory effect of peptide 612–627-K(Pal)A may be due to blocking the signal transduction from PACAP-38- and hCG-activated TSHR to G_s protein. Summing up, we can say that, like in the case of peptides corresponding to the ICL3 of 5-HTR and hCG/LH receptor [18, 19], peptide 612–627-K(Pal)A has receptor specificity, and its influence on AC stimulation induced by PACAP-38 and hCG can be accounted for by their ability to interact with TSHR in a non-specific manner in the thyroid.

In conclusion, we demonstrated that peptides corresponding to the ICL3 of rat TSHR mimic TSH action on the cognate receptor and, like TSH, increase AC activity and GTP binding capacity of G_s and G_q proteins in the thyroidal membranes of rats. Palmitoylated peptide 612–627-K(Pal)A which is much more active than its unmodified analogue decreased TSH-induced AC signaling and its action was receptor specific. The influence of peptide 612–627-K(Pal)A on the AC system and G proteins was revealed predominantly in the thyroid gland and in the extrathyroidal tissues it was rather weak. The selectivity and specificity of the action of peptide 612–627-K(Pal)A make it possible to develop in the near future a new generation of TSHR-peptide-based regulators and modulators of the hypothalamic-pituitary-thyroid axis with the activity of intracellular agonist and antagonist effectively controlling the thyrotropic function.

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