Journal of Peptide Science
J. Pept. Sci. 2007; **13**: 513–518
Published online 6 July 2007 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/psc.873

JOURNAL OF PEPTIDE SCIENCE

Interaction of ACTH synthetic fragments with rat adrenal cortex membranes

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Received 12 February 2007; Revised 2 April 2007; Accepted 10 April 2007

Abstract: Synthetic peptide, corresponding to the amino acid sequence 11–24 of human adrenocorticotropic hormone (ACTH), was labeled with tritium (specific activity of 22 Ci/mmol). [^{3}H]ACTH (11–24) was found to bind to rat adrenal cortex membranes with high affinity and specificity ($K_d = 1.8 \pm 0.1$ nM). Twenty nine fragments of ACTH (11–24) have been synthesized and their ability to inhibit the specific binding of [^{3}H]ACTH (11–24) to adrenocortical membranes has been investigated. Unlabeled fragment ACTH 15–18 (KKRR) was found to replace in a concentration-dependent manner [^{3}H]ACTH (11–24) in the receptor–ligand complex ($K_i = 2.3 \pm 0.2$ nM). ACTH (15–18) was labeled with tritium (specific activity of 20 Ci/mmol). [^{3}H]ACTH (15–18) was found to bind to rat adrenal cortex membranes with high affinity ($K_d = 2.1 \pm 0.1$ nM). The specific binding of [^{3}H]ACTH (15–18) was inhibited by unlabeled ACTH (11–24) ($K_i = 2.2 \pm 0.1$ nM). ACTH (15–18) at the concentration range of 1–1000 nM did not affect the adenylate cyclase activity in adrenocortical membranes. Copyright © 2007 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: adrenocorticotropic hormone (ACTH); peptide; receptor; adenylate cyclase; adrenal cortex

INTRODUCTION

The main function of adrenocorticotropic hormone (ACTH) is the stimulation of synthesis and secretion of glucocorticoids by adrenal zona fasciculata/reticularis cells. The sensitivity of adrenal cortex cells to ACTH depends on the expression and function of the G-protein-coupled receptor (MC2-R, melanocortin receptor of type 2), which belongs to the melanocortin receptor subfamily [1–4]. The hormone binding to MC2-R leads to the increase of adenylate cyclase activity followed by the activation of protein kinase A [5–7]. Kapas *et al.* studied the binding of ACTH fragments with the cloned mouse ACTH receptor expressed in human HeLa cells [8]. They found the ACTH fragment 11–24 to strongly compete with ^{125}I-labeled ACTH for the receptor binding (with I_{50} about 1 nM), but in contrast to the full-size ACTH, it was unable to activate adenylate cyclase thus acting as an antagonist.

The goal of this study was to analyze the interaction of [^{3}H]ACTH (11–24) and its unlabeled synthetic fragments with rat adrenal cortex membranes and find the shortest fragment capable of binding to ACTH receptor with high affinity.

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MATERIALS AND METHODS

The chemicals used in this study were 1,3,4,6-tetrachloro-3α,6α-diphenylglycouril (Iodogen), sucrose, bovine serum albumin, EDTA, EGTA, Tris, phenylmethylsulfonyl fluoride (PMSF), sodium azide (Serva, Germany), *N*-methylpyrrolidone, *N*,*N*′-diisopropylcarbodiimide, 1-hydroxybenzotriazole, thioanisole (Merck, Germany), scintillation fluid Unisolv 100 (Amersham, UK) and other chemicals (Sigma St Louis, MO).

Adult male Sprague-Dawley rats, 180–210 g by weight, were obtained from the Breeding Facility at the Branch of the Institute of Bioorganic Chemistry, Russian Academy of Sciences. All experiments with animals were performed in accordance with the legal requirements of the Russian Academy of Sciences.

ACTH (1–24), ACTH (4–10), somatostatin, β-endorphin, and [Met^5]enkephalin were obtained from Sigma (St Louis, MO). ACTH (11–24) and its fragments (29 peptides) have been synthesized. The synthesis was carried out on a Vega Coupler C250 automatic peptide synthesizer (USA), using the Boc/Bzl peptide elongation technique [9,10]. Peptides were purified to homogeneity by reverse-phase HPLC (chromatograph Gilson, France) on Delta Pack C18 column (100 Å, 39 × 150 mm, mesh size 5 µm), elution with 0.1% trifluoroacetic acid and acetonitrile gradient 10–40% within 30 min, flow rate 10 ml/min. The purity of peptides was >95%. Molecular mass of peptides was determined by mass spectrometric analysis using Finnigan spectrometer (San Jose, CA). Amino acid analysis was performed on LKB 4151 Alpha Plus analyzer (Sweden) following 22-h hydrolysis with 6 N HCl at 110 °C. The main characteristics of synthesized peptides are presented in Table 1.

Table 1 Main characteristics of synthetic fragments of ACTH (11–24)

Peptide	Purity, %	Amino acid analysis data	Molecular mass, D
ACTH (11–24) KPVGKKRRPVKVYP	>99	Gly 1.04; Val 3.04; Tyr 0.96; Pro 3.02; Lys 3.96; Arg 1.98	1652.3 (calculated value – 1652.26)
ACTH (12–24) PVGKKRRPVKVYP	>95	Gly 1.06; Val 3.00; Tyr 0.99; Pro 3.08; Lys 2.96; Arg 2.00	1523.9 (1523.81)
ACTH (11–23) KPVGKKRRPVKVY	>95	Gly 1.06; Val 3.03; Tyr 0.99; Pro 2.01; Lys 3.98; Arg 2.00	1555.3 (1555.13)
ACTH (13–24) VGKKRRPVKVYP	>95	Gly 1.04; Val 3.00; Tyr 0.99; Pro 2.07; Lys 2.99; Arg 2.04	1454.2 (1454.26)
ACTH (11–22) KPVGKKRRPVKV	>95	Gly 1.05; Val 3.02; Pro 2.07; Lys 3.95; Arg 2.00	1392.1 (1391.94)
ACTH (14–24) GKKRRPVKVYP	>95	Gly 1.04; Val 2.00; Tyr 0.96; Pro 2.07; Lys 2.96; Arg 1.98	1327.9 (1327.79)
ACTH (11–21) KPVGKKRRPVK	>95	Gly 1.04; Val 2.01; Pro 2.00; Lys 3.96; Arg 1.98	1292.7 (1292.79)
ACTH (15–24) KKRRPVKVYP	>95	Val 2.00; Tyr 0.94; Pro 2.03; Lys 2.98; Arg 1.97	1270.6 (1270.72)
ACTH (11–20) KPVGKKRRPV	>95	Gly 1.04; Val 2.01; Pro 2.00; Lys 3.06; Arg 1.98	1164.8 (1164.6)
ACTH (16–24) KRRPVKVYP	>95	Val 2.02; Tyr 0.98; Pro 2.01; Lys 2.00; Arg 1.97	1142.6 (1142.53)
ACTH (11–19) KPVGKKRRP	>95	Gly 1.06; Val 1.00; Pro 2.08; Lys 3.04; Arg 1.92	1065.6 (1065.45)
ACTH (17–24) RRPVKVYP	>95	Val 2.00; Tyr 0.96; Pro 2.05; Lys 1.02; Arg 1.98	1014.5 (1014.34)
ACTH (11–18) KPVGKKRR	>95	Gly 1.02; Val 1.01; Pro 1.07; Lys 3.06; Arg 1.90	968.4 (968.32)
ACTH (18–24) RPVKVYP	>95	Val 2.02; Tyr 0.97; Pro 2.03; Lys 1.00; Arg 0.96	858.3 (858.12)
ACTH (11–17) KPVGKKR	>95	Gly 1.02; Val 1.01; Pro 1.07; Lys 3.06; Arg 0.98	806.2 (806.12)
ACTH (19–24) PVKVYP	>95	Val 2.00; Tyr 0.97; Pro 2.02; Lys 0.98	702.2 (701.92)
ACTH (11–16) KPVGKK	>95	Gly 1.00; Val 1.00; Pro 1.04; Lys 2.96	650.3 (649.92)
ACTH (20–24) VKVYP	>95	Val 2.04; Tyr 0.96; Pro 1.04; Lys 0.98	605.2 (604.81)
ACTH (11–15) KPVGK	>95	Gly 1.02; Val 1.00; Pro 1.08; Lys 1.97	528.0 (527.73)
ACTH (21–24) KVYP	>95	Val 1.00; Tyr 0.96; Pro 1.01; Lys 0.96	505.3 (505.66)
ACTH (11–14) KPVG	>95	Gly 1.00; Val 1.03; Pro 1.00; Lys 0.99	399.2 (399.54)
ACTH (12–23) PVGKKRRPVKVY	>95	Gly 1.00; Val 3.09; Tyr 0.96; Pro 2.02; Lys 2.97; Arg 1.98	1427.2 (1426.94)
ACTH (13–22) VGKKRRPVKV	>95	Gly 1.00; Val 3.02; Pro 1.00; Lys 2.96; Arg 1.96	1166.8 (1166.62)
ACTH (14–21) GKKRRPVK	>95	Gly 1.00; Val 1.04; Pro 1.04; Lys 2.98; Arg 1.96	968.1 (968.32)
ACTH (15–20) KKRRPV	>95	Val 1.02; Pro 1.04; Lys 2.04; Arg 1.96	782.8 (783.06)
ACTH (16–19) KRRP	>95	Pro 1.04; Lys 1.00; Arg 1.98	556.0 (555.72)
ACTH (14–19) GKKRRP	>95	Gly 1.00; Pro 1.00; Lys 2.02; Arg 1.98	741.2 (740.98)
ACTH (15–19) KKRRP	>95	Pro 1.00; Lys 2.02; Arg 1.98	684.1 (683.91)
ACTH (15–18) KKRR	>95	Lys 2.00; Arg 1.98	586.9 (586.78)
ACTH (16–18) KRR	>95	Lys 1.03; Arg 1.96	458.7 (458.59)

[^{3}H]ACTH (11–24) and [^{3}H]ACTH (15–18) were obtained by the high-temperature solid-state catalytic isotope exchange (HSCIE) reaction [11]. Solution of 2.0 mg ACTH (11–24) or ACTH (15–18) in 0.5 ml H_2O was mixed with 50 mg aluminum oxide. Water was removed by evaporation. Aluminum oxide with applied peptide was mixed with 10 mg catalyst (5% Rh/Al_2O_3) and transferred into a 10-ml ampoule. The ampoule was vacuumized, filled with gaseous tritium to a pressure of 250 Torr, heated to 170 °C, and kept at this temperature for 20 min. The ampoule was then cooled, vacuumized, blown with hydrogen, and vacuumized again. The labeled peptide was extracted from solid reaction mixture with two portions of 3 ml 50% ethanol in water, and the solutions were combined and evaporated. The procedure was repeated twice to remove the unbound tritium. The [^{3}H]ACTH (11–24) and [^{3}H]ACTH (15–18) were purified by HPLC on a Kromasil column (4 × 150 mm, mesh size 5 μm at 20 °C),with a Beckman spectrophotometer monitoring at 254 and 280 nm, elution with 0.1% trifluoroacetic acid and methanol gradient 42–70% within 20 min, and a flow rate 3 ml/min. Labeling efficiency was determined by liquid scintillation counting.

Membrane fraction was isolated from the rat adrenal glands as described [12]. Protein concentration was determined by the Lowry method [13] with bovine serum albumin as standard.

The binding of [^{3}H]ACTH (11–24) to the adrenocortical crude membranes was assayed in 50 mM Tris-HCl, pH 7.5,

DOI: 10.1002/psc

as follows: 100 μl of labeled peptide (concentration range of $10^{-10} - 10^{-7}$ M, each concentration point in triplicate) plus 100 μl of buffer (for total binding) or 100 μl of 10^{-3} M unlabeled ACTH (11–24) (for nonspecific binding) was added to 800 μl of membrane suspension (0.2 mg protein) in siliconized tubes and incubated at 4 °C for 1h. The samples were then filtered through GF/C glass fiber filters (Whatman, UK). The filters were washed three times with 5 ml ice-cold saline. Radioactivity was measured using a LS 5801 liquid scintillation counter (Beckman, USA). Specific binding of [^{3}H]ACTH (11–24) to the membranes was determined as the difference between total and nonspecific binding and expressed in moles per mg protein. The characteristics of labeled ACTH (11–24) specific binding to the membranes (the equilibrium dissociation constant K_d and the maximal binding capacity B_{max} per mg protein) were determined graphically from the plotted molar concentration ratio of bound (B) to free (F) labeled peptide as a function of bound labeled peptide molar concentration (B) [14].

To test the inhibitory effects of unlabeled ACTH (1–24), ACTH (4–10), somatostatin, β-endorphin, [Met^5]enkephalin and unlabeled fragments of ACTH (11–24) on the specific binding of [^{3}H]ACTH (11–24), the membrane suspension (0.2 mg protein) was incubated with 10 nM labeled peptide and in the presence or absence of various concentrations ($10^{-12} - 10^{-5}$ M) of one of unlabeled peptides, listed in Table 2. All competition binding experiments were performed in triplicates. The inhibition constant K_i was determined by the equation: $K_i = IC_{50}/(1 + [L]/K_d)$ [15], where [L] is the molar concentration of [^{3}H]ACTH (11–24), K_d is the dissociation constant of the [^{3}H]ACTH (11–24)–receptor complex, and IC_{50} is the molar concentration of unlabeled peptide causing 50% inhibition of specific binding at the given [^{3}H]ACTH (11–24) concentration. The IC_{50} values were determined graphically from the inhibition plots (the percentage of inhibition plotted against the molar concentration of inhibitor).

The binding of [^{3}H]ACTH (15–18) to the adrenocortical membranes was assayed as described above for the [^{3}H]ACTH (11–24) binding. The nonspecific binding of [^{3}H]ACTH (15–18) was determined in the presence of 10^{-4} M unlabeled ACTH (15–18). To characterize a specificity of the [^{3}H]ACTH (15–18) binding unlabeled ACTH (11–24), ACTH (4–10), somatostatin, β-endorphin, and [Met^5]enkephalin were tested as potential binding inhibitors. The membrane suspension (0.2 mg protein) was incubated with 10 nM labeled peptide and in the presence or absence of various concentrations ($10^{-12} - 10^{-5}$ M) of one of unlabeled peptides. All competition binding experiments were performed in triplicates. The inhibition constant K_i was determined as described above.

Adenylate cyclase activity was determined using α[^{32}P]ATP as described [16], and expressed as the amount of cAMP produced in 10 min (in nmol per 1 mg protein of adrenal cortex membranes).

The data are presented as the means ±SEM of at least three independent experiments.

RESULTS

The HSCIE reaction yielded [^{3}H]ACTH (11–24) with specific activity of 22 Ci/mmol and [^{3}H]ACTH (15–18) with specific activity of 26 Ci/mmol. The retention time for [^{3}H]ACTH (11–24) and unlabeled ACTH (11–24) was 18 min and for [^{3}H]ACTH (15–18) and unlabeled ACTH (15–18) was 14 min on Kromasil C18 column; the 254/280 nm absorbance ratio was also the same, which indicated the conservation of chemical structure of both peptides upon the exchange of hydrogen for tritium.

Table 2 Inhibition of the specific binding of 10 nM [^{3}H]ACTH (11–24) to rat adrenal cortex membranes by unlabeled peptides

Peptide	IC_{50}[a], nM	K_i[a], nM
ACTH (1–24)	11.3 ± 0.1	1.7 ± 0.1
ACTH (4–10)	>10 000	>10 000
Somatostatin	>10 000	>10 000
β-Endorphin	>10 000	>10 000
[Met^5]enkephalin	>10 000	>10 000
ACTH (12–24)	12.0 ± 0.2	1.9 ± 0.1
ACTH (11–23)	11.8 ± 0.2	1.8 ± 0.1
ACTH (13–24)	12.2 ± 0.2	1.9 ± 0.1
ACTH (11–22)	12.6 ± 0.2	1.9 ± 0.2
ACTH (14–24)	11.9 ± 0.2	1.8 ± 0.2
ACTH (11–21)	12.9 ± 0.2	2.0 ± 0.2
ACTH (15–24)	12.9 ± 0.2	2.0 ± 0.2
ACTH (11–20)	13.2 ± 0.2	2.0 ± 0.2
ACTH (16–24)	23.8 ± 0.4	3.6 ± 0.3
ACTH (11–19)	12.9 ± 0.3	2.0 ± 0.2
ACTH (17–24)	1890 ± 170	286.4 ± 25.8
ACTH (11–18)	13.6 ± 0.3	2.1 ± 0.3
ACTH (18–24)	>10 000	>10 000
ACTH (11–17)	>10 000	>10 000
ACTH (19–24)	>10 000	>10 000
ACTH (11–16)	>10 000	>10 000
ACTH (20–24)	>10 000	>10 000
ACTH (11–15)	>10 000	>10 000
ACTH (21–24)	>10 000	>10 000
ACTH (11–14)	>10 000	>10 000
ACTH (12–23)	12.7 ± 0.1	1.9 ± 0.1
ACTH (13–22)	12.7 ± 0.2	1.9 ± 0.2
ACTH (14–21)	12.8 ± 0.2	1.9 ± 0.2
ACTH (15–20)	12.8 ± 0.2	1.9 ± 0.2
ACTH (16–19)	187.9 ± 0.6	28.5 ± 2.4
ACTH (14–19)	12.8 ± 0.2	1.9 ± 0.2
ACTH (15–19)	12.9 ± 0.1	2.0 ± 0.1
ACTH (15–18)	15.1 ± 0.2	2.3 ± 0.2
ACTH (16–18)	7362 ± 589	1116 ± 90

[a] Values are means ± SEM of two independent experiments, each performed in triplicates.

Our experiments demonstrated that [^{3}H]ACTH (11–24) specifically got bound to rat adrenocortical membranes under the conditions chosen. Figure 1(a) shows the total (plot 1), specific (plot 2), and nonspecific (plot 3) binding of [^{3}H]ACTH (11–24) to the membranes at 4 °C as a function of incubation time. The [^{3}H]ACTH (11–24)–receptor system reached dynamic equilibrium in 1 h and remained in this state for at least 2 h. Therefore, to assess the equilibrium dissociation constant

DOI: 10.1002/psc

(K_d), the reaction of [^{3}H]ACTH (11–24) binding to the membranes was carried out for 1 h. The nonspecific binding under these conditions was $6.4 \pm 0.8\%$ of total binding.

Scatchard analysis of [^{3}H]ACTH (11–24) specific binding resulted in a straight line (Figure 1(c), plot 1), suggesting that this peptide binds to one type of receptors on rat adrenal cortex membranes. The K_d value was 1.8 ± 0.1 nM, indicating high affinity of [^{3}H]ACTH (11–24) binding to the receptor. The maximal binding capacity of the membranes B_{max} was 2.8 ± 0.2 pmol per 1 mg protein.

To characterize the specificity of [^{3}H]ACTH (11–24) binding, we tested unlabeled ACTH (1–24), ACTH (4–10), somatostatin, β-endorphin, and [Met^5]enkephalin for potential competition with [^{3}H]ACTH (11–24). The results presented in Table 2 showed that only ACTH (1–24) was able to inhibit the specific binding of [^{3}H]ACTH (11–24) to the membranes, with $K_i = 1.7 \pm 0.1$. Thus, [^{3}H]ACTH (11–24) binds to ACTH receptor on rat adrenal cortex membranes with high affinity and can be used as a marker of this receptor.

To estimate the binding capacity of synthetic fragments of ACTH (11–24) (29 peptides) to ACTH receptor, the inhibition potencies of these peptides in competing for [^{3}H]ACTH (11–24) binding sites in adrenocortical membranes were investigated. The results of experiments are presented in Table 2. From these data it can be concluded that ACTH (15–18) is the shortest peptide capable of inhibition of the [^{3}H]ACTH (11–24) specific binding ($K_i = 2.3 \pm 0.2$).

Figure 1(b) shows the total (plot 1), specific (plot 2), and nonspecific (plot 3) binding of [^{3}H]ACTH (15–18) to rat adrenal cortex membranes at 4 °C as a function of incubation time. The [^{3}H]ACTH (11–24)-receptor system reached dynamic equilibrium in 1 h and remained in this state for at least 2 h. Therefore, to assess the equilibrium dissociation constant (K_d), the reaction of [^{3}H]ACTH (15–18) binding to the membranes was carried out for 1 h. The nonspecific binding under these conditions was $6.4 \pm 0.6\%$ of total binding.

Scatchard analysis of [^{3}H]ACTH (15–18) specific binding to the adrenocortical membranes (Figure 1(c), plot 2) showed the binding to one type of receptors, with $K_d = 2.1 \pm 0.1$ nM and $B_{max} = 2.6 \pm 0.2$. The results on inhibition of the specific binding of [^{3}H]ACTH (15–18) by unlabeled ACTH (1–24), ACTH (11–24), ACTH (4–10), somatostatin, β-endorphin, and [Met^5]enkephalin are presented in Table 3. Only ACTH (1–24) and ACTH (11–24) effectively competed with [^{3}H]ACTH (15–18), with $K_i = 1.9 \pm 0.1$ and 2.0 ± 0.1 nM, respectively. Other peptides were inactive, $K_i > 10$ μM. These data indicate that ACTH (15–18) binds to the ACTH receptor on rat adrenal cortex membranes with high affinity.

The effects of ACTH (1–24), ACTH (11–24), and ACTH (15–18) on adenylate cyclase activity in rat adrenocortical membranes are presented in Table 4.

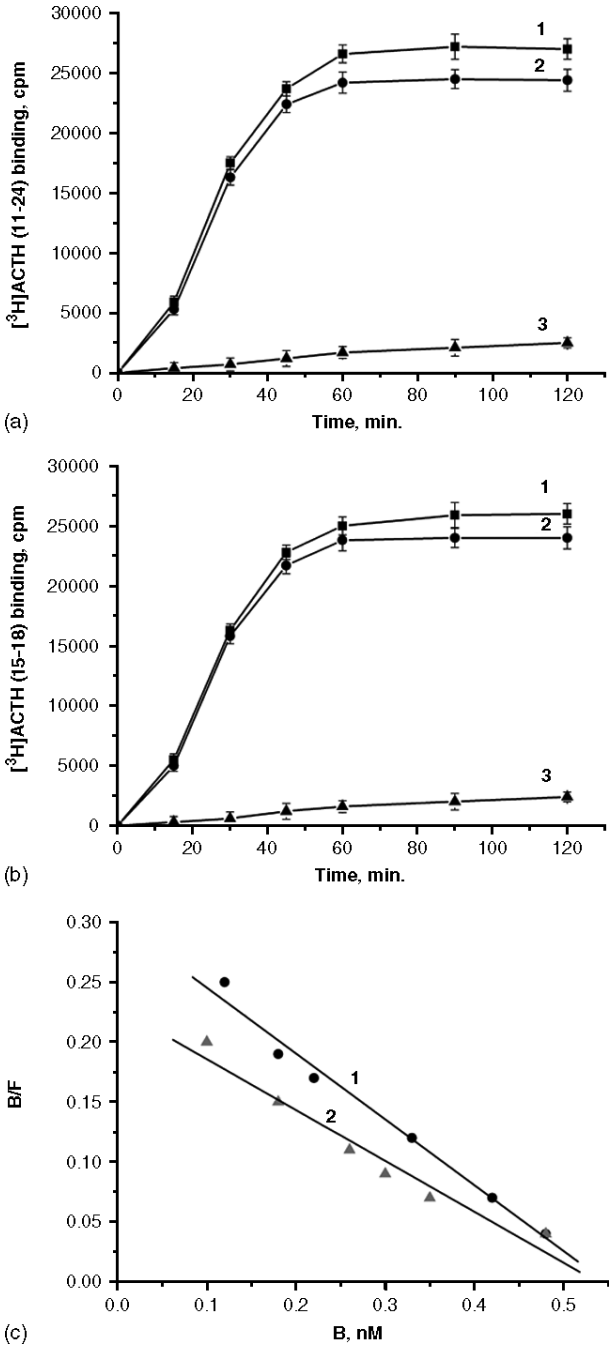

Figure 1 (a) Total (1), specific (2), and nonspecific (3) binding of [^{3}H]ACTH (11–24) to rat adrenal cortex membranes. Incubation at 4 °C was terminated after 10, 30, 60, 90 and 120 min, and the specific binding was calculated by subtracting nonspecific binding [measured in the presence of 10^{-4} M unlabeled ACTH (11–24)] from total binding. (b) Total (1), specific (2), and nonspecific (3) binding of [^{3}H]ACTH (15–18) to rat adrenal cortex membranes. Incubation at 4 °C was terminated after 10, 30, 60, 90 and 120 min, and the specific binding was calculated by subtracting nonspecific binding [measured in the presence of 10^{-4} M unlabeled ACTH (15–18)] from total binding. (c) Scatchard analysis of the specific binding of [^{3}H]ACTH (11–24) (plot 1) and [^{3}H]ACTH (15–18) (plot 2) to adrenocortical membranes. B and F – molar concentrations of bound and free labeled peptide, respectively.

DOI: 10.1002/psc

Table 3 Inhibition of the specific binding of 10 nM [^{3}H]ACTH (15–18) to rat adrenal cortex membranes by unlabeled peptides

Peptide	IC_{50}[a], nM	K_i[a], nM
ACTH (1–24)	11.0 ± 0.1	1.9 ± 0.1
ACTH (11–24)	11.3 ± 0.1	2.0 ± 0.1
ACTH (4–10)	>10 000	>10 000
Somatostatin	>10 000	>10 000
β-Endorphin	>10 000	>10 000
[Met^5]enkephalin	>10 000	>10 000

[a] Values are means ± SEM of two independent experiments, each performed in triplicates.

These data show that ACTH (11–24) and ACTH (15–18) at concentrations of 1–1000 nM did not affect the enzymatic activity, while ACTH (1–24) increased it.

DISCUSSION

The sequence 1–24 of the human and rat ACTH is the same. ACTH (1–24), the minimal ACTH sequence required for full activity, and α-melanocyte stimulating hormone (MSH) differ only by the ten *C*-terminal amino acids of ACTH (1–24). Interestingly, these ten *C*-terminal residues have been highly conserved throughout vertebrate evolution. To understand the functional constraints of these ten amino acids, Costa *et al.* [17] analyzed the effects of mutating these residues on steroidogenic activity *in vivo* and *in vitro*. Alanine substitutions of some of the first four amino acid residues (the basic core residues KKRR, 15–18) greatly reduces ACTH activity *in vitro* and *in vivo*. Thus, for ACTH receptor binding and activation, the amino acid residues 15–18 are important for their side chains. Surprisingly, conversion of the five *C*-terminal residues (20–24) to alanines increases ACTH activity *in vivo* over that of native ACTH. With respect to receptor binding and activity, the last five amino acid residues are important only for the peptide length they contribute; however, with respect to serum stability, their side chains are significant.

The data of Kapas *et al.* [8] suggest that the ACTH fragment 11–24 acts as ACTH receptor antagonist, strongly competing with ^{125}I-labeled ACTH for binding with the cloned receptor (IC_{50} about 1 nM), but not activating adenylate cyclase in contrast to ACTH. We obtained [^{3}H]ACTH (11–24) and found it to bind the ACTH receptor on rat adrenal cortex with high affinity, $K_d = 1.8 \pm 0.1$ nM (Figure 1(c), plot 1). Our experiments also showed that ACTH (11–24) binding to the adrenocortical membranes did not change the adenylate cyclase activity (Table 4). To estimate the binding ability of unlabeled fragments of ACTH (11–24) (29 peptides) to ACTH receptor, the inhibition potencies of these peptides in competing for [^{3}H]ACTH (11–24) binding sites in adrenocortical membranes were investigated. Additionally, ACTH (4–10), somatostatin, β-endorphin, and [Met^5]enkephalin were tested as potential inhibitors of specific binding of [^{3}H]ACTH (11–24). The results of the experiments are given in Table 2. It is clear that the peptides containing the sequence KKRR kept the capacity to inhibit [^{3}H]ACTH (11–24) specific binding. The activity of the rest fragments was very low, $K_i \geq 1$ μM. The peptide KKRR was the shortest fragment of ACTH (11–24) capable of inhibition of [^{3}H]ACTH (11–24) binding to ACTH receptor ($K_i = 2.2 \pm 0.1$ nM).

To characterize the interaction of ACTH (15–18) to rat adrenal cortex membranes, we prepared [^{3}H]ACTH (15–18). Scatchard analysis showed that [^{3}H]ACTH (15–18) bound to adrenocortical membranes with high affinity, $K_d = 2.1 \pm 0.1$ nM (Figure 1(c), plot 2). The study of binding specificity demonstrated that only unlabeled ACTH (1–24) and ACTH (11–24) could replace labeled ACTH (15–18) in the ligand–receptor complex (Table 3). Other unlabeled peptides that were tested for potential competition were inactive. The ACTH (15–18) binding to the adrenocortical membranes did not change the adenylate cyclase activity (Table 4).

Table 4 Effects of ACTH (1–24), ACTH (11–24), and ACTH (15–18) on adenylate cyclase activity in rat adrenal cortex membranes

Peptide concentration (nM)	Adenylate cyclase activity (nmoles of cAMP per 1 mg protein in 10 min)		
	ACTH (1–24)	ACTH (11–24)	ACTH (15–18)
0		1.43 ± 0.12	
0.1	1.47 ± 0.15	1.42 ± 0.12	1.46 ± 0.13
1	1.82 ± 0.17	1.50 ± 0.18	1.48 ± 0.15
10	2.23 ± 0.19	1.52 ± 0.14	1.44 ± 0.18
100	2.68 ± 0.24	1.43 ± 0.12	1.49 ± 0.16
1000	2.56 ± 0.16	1.40 ± 0.12	1.42 ± 0.18

The data are presented as mean ± SEM. of three independent experiments.

DOI: 10.1002/psc

Therefore, similar to ACTH (11–24), ACTH (15–18) is an antagonist of the ACTH receptor.

Acknowledgements

This work was supported by the Russian Foundation for Basic Research (grant No. 05-04-48060), by the programs Molecular and Cellular Biology (chairman V.M. Lipkin), and Naukograd (grant No. 04-04-97200), and by the International Science and Technology Center (project No. 2615). V.V. Yurovsky is supported by the American Heart Association (grant No. 0555415U).

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