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Elucidation and Characteristics of Non-opioid β-Endorphin Receptors in Rat Adrenal Cortex

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Abstract—β-Endorphin-like decapeptide immunorphin (SLTCLVKGFY), a selective agonist of non-opioid β-endorphin receptor, was labeled with tritium to specific activity of 24 Ci/mmol. It was used for the detection and characterization of non-opioid β-endorphin receptors on rat adrenal cortex membranes (K_{d1} = 39.6 ± 2.0 nM, B_{max1} = 40.7 ± 2.3 pmol/mg protein; K_{d2} = 0.25 ± 0.01 μM, B_{max2} = 187.8 ± 9.4 pmol/mg protein). β-Endorphin was found to inhibit the [^{3}H]immunorphin specific binding to membranes (K_i = 70.0 ± 9.2 nM); naloxone, [Met^5]enkephalin, and α- and γ-endorphins tested in parallel were inactive. Immunorphin at concentrations of 10^{-9}-10^{-6} M was found to inhibit the adenylate cyclase activity in adrenocortical membranes, while intramuscular injection of immunorphin at doses of 10-100 μg/kg was found to reduce the secretion of 11-oxycorticosteroids from the adrenals to the bloodstream.

Key words: β-endorphin, naloxone, peptides, receptors, adrenal cortex

β-Endorphin is known to bind to opioid (μ and δ) receptors [1] and to non-opioid (insensitive to opioid antagonist naloxone) receptors that were first described by Hazum et al. [2]; however, they have not been characterized well in terms of their structure and functions.

In 1980 Julliard et al. found adrenocorticotropic hormone (ACTH)-like and β-endorphin-like sequences in the heavy chain of human immunoglobulin G (IgG) [3]. Houck et al. synthesized the tetradecapeptide SLTCLVKGFYPSDI corresponding to the β-endorphin-like sequence of IgG (fragment 364-377 of the heavy chain C_{H3} domain) and found it to compete with ^{125}I-labeled β-endorphin for the binding to rat brain membranes [4]. We have synthesized the β-endorphin-like decapeptide SLTCLVKGFY (referred to as immunorphin) corresponding to the sequence 364-373 of the heavy chain of human IgG(1-4) [5] and found it to be a selective agonist of non-opioid (naloxone-insensitive) β-endorphin receptor on human T lymphocytes [6-9], mouse peritoneal macrophages [10, 11], rat brain synaptic membranes [12], and human T-lymphoblastoid cell line Jurkat [13]. The study of biologic activity of immunorphin has revealed that it enhances the concanavalin A-induced proliferation of human T lymphocytes *in vitro* [6-9], activates mouse peritoneal macrophages *in vitro* and *in vivo* [10, 11], and stimulates the growth of human T-lymphoblastoid cell lines Jurkat and MT-4 [13, 14]. We have recently identified non-opioid β-endorphin receptors on the membranes isolated from rat adrenal cortex [15]. The aim of the present study was to obtain [^{3}H]immunorphin and to use it for characterization of

Abbreviations: ACTH) adrenocorticotropic hormone; CS) 11-oxycorticosteroids; HSCIE) high-temperature solid-state catalytic isotope exchange; IgG) immunoglobulin G; PMSF) phenylmethylsulfonyl fluoride; SEM) standard error of the mean.

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non-opioid β-endorphin receptors on rat adrenal cortex membranes, and also to examine its effects on the level of 11-oxycorticosteroids (CS) in rat adrenal glands and blood plasma *in vivo*.

MATERIALS AND METHODS

Chemicals used in this study were: α-, β-, γ-endorphins, [Met^5]enkephalin, naloxone, and corticosterone from ICN Biomedicals (USA); phenylmethylsulfonyl fluoride (PMSF) and Tris from Fluka (USA); scintillation fluid Ready Gel from Beckman (USA). All other chemicals were purchased from domestic companies and additionally purified before use.

Animals. Adult male Wistar rats weighing 180-210 g were obtained from the Breeding Facility at the Branch of the Institute of Bioorganic Chemistry, Russian Academy of Sciences.

Immunorphin was synthesized on a Vega Coupler C250 automatic peptide synthesizer (USA) and purified by preparative reverse-phase chromatography (Gilson chromatograph, France) on a Waters SymmetryPrep C18 column (19 × 300 mm; Malva, Greece), as described previously [9]. The peptide was characterized by analytical reverse-phase HPLC (Gilson chromatograph) on an XTerra PR18 column (Malva), amino acid analysis (hydrolysis with 6 N HCl, 24 h, 110°C; LKB 4151 Alpha Plus amino acid analyzer, Sweden), and mass spectrometry (Finnigan mass spectrometer, USA).

[^{3}H]Immunorphin was obtained by the high-temperature solid-state catalytic isotope exchange (HSCIE) reaction [16, 17]. A solution of 2 mg immunorphin 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 ampule. The ampule was evacuated, filled with gaseous tritium to a pressure of 250 mm Hg, heated to 170°C, and kept at this temperature for 20 min. The ampule was then cooled, evacuated, flushed with hydrogen, and evacuated again. The labeled peptide was extracted from the solid reaction mixture with two portions of 3 ml 50% ethanol in water, and then the solutions were combined and evaporated. The procedure was repeated twice to remove the unbound tritium. [^{3}H]Immunorphin was purified by HPLC on a Kromasil C18 column (4 × 150 mm, mesh size 5 μm) at 20°C, with monitoring at 254 and 280 nm using Beckman spectrophotometer, elution with 0.1% trifluoroacetic acid and methanol gradient 42-70% within 20 min, flow rate 3 ml/min. Labeling efficiency was determined by liquid scintillation counting.

Membrane fraction was isolated from rat adrenal glands as described [18]. Protein concentration was determined by the Lowry method [19] with bovine serum albumin as a standard.

The binding of [^{3}H]immunorphin to the adrenocortical membranes was assayed in 50 mM Tris-HCl, pH 7.5, as follows: 100 μl of labeled peptide (concentration range 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 peptide (for nonspecific binding) was added to 800 μl of membrane suspension (0.2 mg protein) in siliconized tubes and incubated at 4°C for 1 h. 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). Specific binding of [^{3}H]immunorphin to the membranes was determined as the difference between total and nonspecific binding and expressed in moles per 1 mg protein. The characteristics of labeled immunorphin specific binding to the membranes (the equilibrium dissociation constant K_d and the maximal binding capacity B_{max} per 1 mg protein) were determined graphically from the plotted molar concentration ratio of bound (B) to free (F) labeled immunorphin as a function of bound labeled peptide molar concentration (B) [20].

To test the inhibitory effects of naloxone and unlabeled peptides on the specific binding of [^{3}H]immunorphin, membrane suspension (0.2 mg protein) was incubated with 20 nM labeled immunorphin and one of the potential inhibitors (concentration range 10^{-12}-10^{-5} M, each concentration point in triplicate) as described above. The inhibition constant K_i was determined by the formula: $K_i = [I]_{50}/(1 + [L]/K_d)$ [21], where [L] is the molar concentration of [^{3}H]immunorphin, K_d is the dissociation constant of the [^{3}H]immunorphin–receptor complex, and $[I]_{50}$ is the concentration of unlabeled peptide giving 50% inhibition of [^{3}H]immunorphin specific binding. The $[I]_{50}$ values were determined graphically from the inhibition plots (the percentage of inhibition plotted against the molar concentration of inhibitor). The K_d values were determined as described above.

Adenylate cyclase activity was determined using [α-^{32}P]ATP as described in [22]. The reaction was performed in 40 mM Tris-HCl, pH 7.4, containing 50 μM ATP, 4 mM cAMP, 12 mM phosphoenolpyruvate, 2 μg/ml pyruvate kinase, and [α-^{32}P]ATP (200,000 to 500,000 cpm). Fifty microliters of this mixture were mixed with 50 μl of rat adrenal cortex membrane suspension or with equal volume of buffer (in control) in glass siliconized conical tubes on ice. The samples were incubated at 34°C for 45 min. The reaction was stopped by adding 0.5 M HCl. The samples were boiled for 15 min, placed on ice, and then 100 μl of 1.5 M imidazole was added to each sample. Each sample was applied to a column with 1 cm^3 Al_2O_3 of Brockman II and washed with 5 ml distilled water. The enzymatic activity was determined by the substrate consumption and expressed as the amount of cAMP produced in 10 min (in nmol/mg protein of adrenocortical membranes).

The level of 11-oxycorticosteroids (CS) was measured in the peripheral blood and adrenal glands of adult male Wistar rats of 180-210 g weight. Control and experimental groups consisted of 10 animals. Immunorphin was administered into the gastrocnemius muscle of rats at doses 10 or 100 μg/kg in 0.5 ml saline. The animals were sacrificed by decapitation 1, 6, or 24 h after the injection, and the CS level was determined in the peripheral blood and adrenal glands of each individual animal by the protocol described in [23]. This method is based on the ability of sulfuric acid/alcohol mixture to induce fluorescence of CS.

Three milliliters of blood plasma were mixed with 15 ml of chloroform in a 50-ml glass-stoppered conical centrifuge tube, shaken, and centrifuged at 4000 rpm for 15 min. The upper (aqueous) layer was removed by aspiration, and the lower (chloroform) layer aliquoted into clean tubes and washed with 4.5 ml 0.1 N NaOH to remove phenolic estrogens. The tubes were shaken for 15 sec and centrifuged. The alkaline layer was removed by aspiration, and two aliquots of CS in chloroform (15 ml each) were transferred into 50-ml glass-stoppered centrifuge tubes containing 3 ml of the fluorescence reagent (2.4 volumes of sulfuric acid mixed with 1 volume of 50% ethyl alcohol). The tubes were shaken for 15 sec and centrifuged. The upper layer was transferred into a fluorimeter cuvette and allowed to stand at room temperature for 2 h. The fluorescence was measured in the Hitachi fluorimeter (Japan) at 530 nm (excitation wavelength 470 nm). A blank, consisting of 3 ml of water instead of plasma, and standards, containing corticosterone of known concentration, were carried through the procedure. It has been shown that the substance obtained from rat plasma by this method has the same fluorescence characteristics as corticosterone, and its fluorescence intensity is increased after the sulfuric acid treatment as it does in standards [24].

Rat adrenal gland was homogenized in 5 ml of chloroform, transferred into a centrifuge tube, and then 10 ml of chloroform was added and the sample was allowed to stand for 15 min. The tube was centrifuged at 4000 rpm for 15 min, and the supernatant was treated as described above. The concentration of CS was obtained using the formula: $X = F_{exp} \times C_{st}/F_{st} \times C_{st}$, where X is the amount of CS in μg/g tissue, F_{exp} is the fluorescence of the experimental sample, C_{st} is the amount of CS in the standard sample in micrograms, F_{st} is the fluorescence of the standard sample.

The average levels of CS in the plasma and adrenal glands of control animals were 0.3 μg/ml and 35 μg/g tissue, respectively. Statistical differences between the control and experimental groups were determined using Student's *t*-test.

RESULTS

The HSCIE reaction yielded 2 mCi [^{3}H]immunorphin with specific activity of 24 Ci/mmol. The retention time for both [^{3}H]immunorphin and unlabeled immunorphin on Kromasil C18 column was 18 min (see the "Materials and Methods" section for chromatography conditions); the 254/280 nm absorbance ratio was also the same, indicating the conservation of chemical structure upon the exchange of hydrogen for tritium.

Binding of [^{3}H]immunorphin to rat adrenocortical membranes. Our experiments demonstrated that [^{3}H]immunorphin specifically bound to rat adrenocorti-

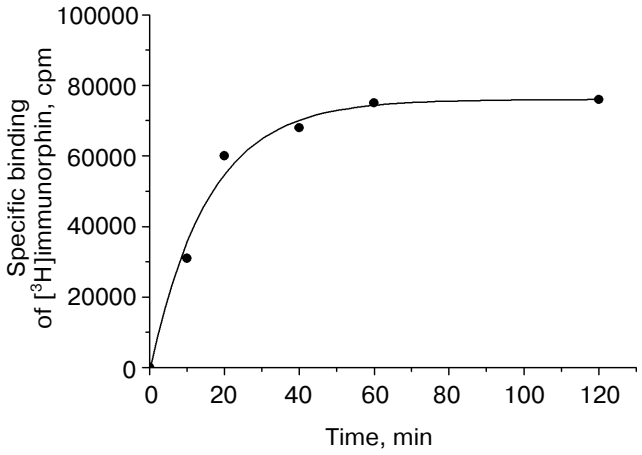

Fig. 1. Dependence of the specific binding of [^{3}H]immunorphin (500,000 cpm) to rat adrenocortical membranes on the incubation time. Specific binding was calculated by subtracting nonspecific binding from total binding. Nonspecific binding was determined in the presence of 10^{-4} M unlabeled immunorphin.

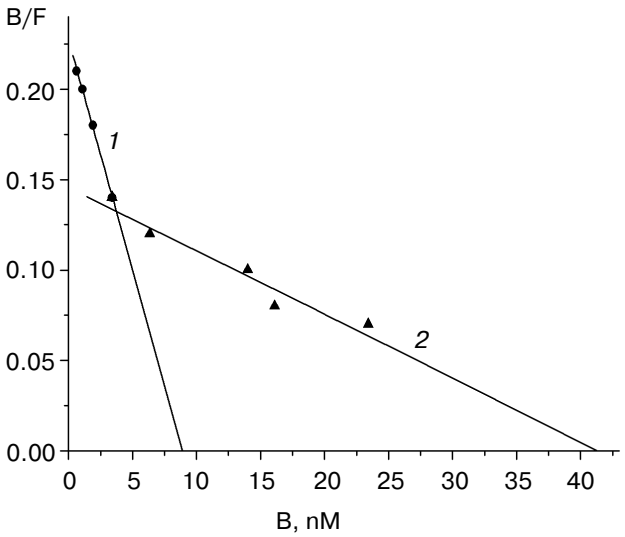

Fig. 2. Scatchard analysis of the specific binding of [^{3}H]immunorphin to rat adrenocortical membranes. B and F are molar concentrations of the bound and free labeled peptide, respectively.

Table 1. Inhibition of [^{3}H]immunorphin specific binding to rat adrenocortical membranes by unlabeled peptides and naloxone

Ligand	$[I]_{50}$ ± SEM, nM	K_i ± SEM, nM
β-Endorphin	105.0 ± 12.3	70.0 ± 9.2*
Naloxone	> 10^6	> 10^6
[Met^5]Enkephalin	> 10^6	> 10^6
α-Endorphin	> 10^6	> 10^6
γ-Endorphin	> 10^6	> 10^6
ACTH (1-24)	> 10^6	> 10^6
Somatostatin	> 10^6	> 10^6

* K_i value was calculated for K_{d1} of [^{3}H]immunorphin–receptor complex.

Table 2. Effects of immunorphin on adenylate cyclase activity in rat adrenocortical membranes

Immunorphin concentration, nM	Adenylate cyclase activity, nmol cAMP/mg protein in 10 min
0	1.57 ± 0.16
0.1	1.55 ± 0.17
1	1.12 ± 0.14
10	0.83 ± 0.09
100	0.86 ± 0.08
1000	0.89 ± 0.11

Note: Data are presented as mean ± SEM of three independent experiments.

cal membranes under the conditions chosen (see "Materials and Methods"). Figure 1 shows the specific binding of [^{3}H]immunorphin to adrenocortical membranes at 4°C as a function of incubation time. The [^{3}H]immunorphin–receptor system reached dynamic equilibrium in 1 h and remained in this state for at least 2 h; therefore, to characterize the binding kinetics, the reaction of [^{3}H]immunorphin binding to the membranes was carried out for 1 h. Nonspecific binding of [^{3}H]immunorphin was 12.3 ± 3.2% of total binding.

Scatchard analysis of the specific binding of [^{3}H]immunorphin to adrenocortical membranes (Fig. 2) suggested the existence of two types of binding sites (receptors) for this peptide, with K_{d1} = 39.6 ± 2.0 nM and K_{d2} = 0.25 ± 0.01 μM. The values of maximal binding capacity (B_{max1} and B_{max2}) were 40.7 ± 2.3 and 187.8 ± 9.4 pmol/mg protein, respectively.

To characterize the specificity of [^{3}H]immunorphin binding to adrenocortical membranes, unlabeled naloxone, [Met^5]enkephalin, α-, β-, and γ-endorphins, ACTH(1-24), and somatostatin were tested for potential competition. The results presented in Table 1 showed that only β-endorphin was able to inhibit the specific binding of [^{3}H]immunorphin, with K_i = 70.0 ± 9.2 nM. These data suggested that immunorphin-binding sites bound also β-endorphin, but not naloxone, [Met^5]enkephalin, or α- or γ-endorphins.

Effects of immunorphin on adenylate cyclase activity in rat adrenocortical membranes. The results presented in Table 2 showed that immunorphin at concentrations of 1-1000 nM down-regulated the adenylate cyclase activity in adrenocortical membranes. The most effective dose of immunorphin was 10 nM, giving 47% inhibition of enzymatic activity. Increasing the peptide concentration (100 and 1000 nM) did not enhance the adenylate cyclase activity.

Effects of immunorphin on 11-oxycorticosteroid (CS) level in the peripheral blood and adrenal glands of rats *in vivo*. The results presented in Table 3 showed that 1 h after the intramuscular injection of immunorphin at dose of 10 μg/kg, the CS level in adrenal glands increased, while in plasma it decreased by approximately 30%. Higher dose of immunorphin (100 μg/kg) induced analogous, but more prominent effect: the increase in adrenal CS level and the decrease in plasma CS level were about 50%. The

Table 3. Effects of immunorphin on the 11-oxycorticosteroid (CS) level in rat adrenals and plasma 1, 6, and 24 h after the intramuscular injection

Immunorphin dose, μg/kg	CS level (experiment/control)					
	1 h		6 h		24 h	
	adrenal glands	plasma	adrenal glands	plasma	adrenal glands	plasma
10	1.32 ± 0.08*	0.67 ± 0.14*	1.29 ± 0.09*	0.65 ± 0.10*	1.07 ± 0.08*	0.59 ± 0.12*
100	1.49 ± 0.06**	0.56 ± 0.11*	1.52 ± 0.09*	0.49 ± 0.08*	1.09 ± 0.09*	0.51 ± 0.09*

*p < 0.02, **p < 0.001 between experimental and control groups as determined by Student's t-test. CS content in plasma and adrenal glands of control animals was on average 0.3 μg/ml and 35 μg/g tissue, respectively.

CS levels in adrenals 6 h after the administration of 10 and 100 μg/kg immunorphin were virtually the same as at 1 h. The changes in CS levels in adrenals and plasma 1- and 6 h post-injection suggest that immunorphin suppresses CS secretion from the adrenals to the bloodstream.

Table 3 also shows that 24 h after the administration of 10 and 100 μg/kg immunorphin, almost 50% decrease in plasma CS level was accompanied by less than 10% increase in adrenal CS level. These results indicate the inhibiting effects of immunorphin on both the secretion and biosynthesis of CS.

DISCUSSION

A non-opioid (insensitive to opioid antagonist naloxone) binding site (receptor) for β-endorphin was first identified on transformed cultured human lymphocytes [2]. Then high affinity non-opioid binding sites for β-endorphin were observed on T lymphocytes from normal human blood [6-9], normal murine splenocytes and peritoneal macrophages [5, 25, 26], mouse thymoma cell line EL-4 [27, 28], human monocyte-like cell line U937 [29] and T-lymphoblastoid cell line Jurkat [14], and rat brain synaptic membranes [12].

Our previous study of the distribution of non-opioid β-endorphin receptor in rat tissues has shown that, in addition to cells of the immune and nervous systems, this receptor type is expressed on adrenal, myocardium, and spleen membranes [15]. In this study, we characterized the affinity and specificity of non-opioid β-endorphin receptors in rat adrenocortical membranes and examined their putative role.

The main function of the adrenal cortex is the production of steroid hormones. More than 50 steroids have been isolated from the adrenal cortex and crystallized. Most of them are the intermediates of biosynthesis. Only glucocorticoids hydrocortisone (11β,17α,21-trihydroxy-4-pregnene-3,20-dione or cortisol) and corticosterone (11β,21-dihydroxy-4-pregnene-3,20-dione), and a mineralocorticoid aldosterone (11β,21-dihydroxy-3,20-dioxo-4-pregnene-18-al) are considered to be the biologically active hormones secreted by the adrenal cortex into the bloodstream. Cortisone (17α,21-dihydroxy-4-pregnene-3,11,20-trione), 11-deoxycortisol (17α,21-dihydroxy-4-pregnene-3,20-dione), prednisone (17α,21-dihydroxy-1,4-pregnadiene-3,11,20-trione), and 11-deoxycorticosterone (21-hydroxy-4-pregnene-3,20-dione) are also adrenal cortex hormones, but the level of their secretion is very low. Hydrocortisone predominates in the peripheral blood of humans, apes, and guinea pigs, while corticosterone predominates in rodents (rats, mice, rabbits). None of the above compounds has significant autofluorescence. However, the treatment with concentrated sulfuric acid or phosphoric acid produces fluorescent derivatives from corticosteroids containing a 11-hydroxyl group, except aldosterone [24]. The excitation wavelength spectrum for these derivatives has a maximum at 470-475 nm, and the fluorescence spectrum has a maximum at 520-530 nm. Corticosterone and hydrocortisone derivatives have the maximum light absorption at 470 nm, while the cortisone derivative obtained under the same conditions does not absorb light at wavelengths above 410 nm. In this study, we used the method of Zenker and Bernstein [23] allowing the reliable measurements of CS content in various tissues (90% of the amount of corticosterone added to the blood plasma can be detected by this method).

The data of Szalay [30], who studied the effects of pro-opiomelanocortin peptides on the functional activity of adrenal cortex, indicate that β-endorphin can enhance, inhibit, or have no effect on corticosteroidogenesis, depending on the dose and functional state of adrenocortical cells. The data of Kapas et al. [31] suggest that stimulatory effect of β-endorphin on aldosterone secretion by *zona glomerulosa* cells is mediated exclusively by μ opioid receptors, and on corticosterone secretion by inner zone cells by both μ and κ receptors. This study also shows that β-endorphin binding to μ and κ opioid receptors on the adrenocortical cells leads to the activation of phospholipase C.

The results of the present study demonstrate that immunorphin binds to non-opioid β-endorphin receptors on rat adrenocortical membranes, inhibits the adenylate cyclase activity, and suppresses the secretion of 11-oxycorticosteroids (corticosterone) from the adrenals to the bloodstream. Thus, the effects of immunorphin are opposite to those of ACTH, which stimulates glucocorticoid synthesis and secretion by inner zone cells through the activation of adenylate cyclase and cAMP production [32]. The analysis of [^{3}H]immunorphin binding to adrenocortical membranes (Fig. 2) revealed the existence of two types of immunorphin binding sites (receptors), with different affinities ($K_{d1} = 39.6 \pm 2.0$ nM, $K_{d2} = 0.25 \pm 0.01$ μM) and binding capacities ($B_{max1} = 40.7 \pm 2.3$ pmol/mg protein, $B_{max2} = 187.8 \pm 9.4$ pmol/mg protein). Therefore, it is possible that signaling through the immunorphin-receptor involves another mechanism in addition to the cAMP-mediated pathway.

Hazum et al. found the non-opioid β-endorphin receptor on transformed cultured human lymphocytes to be insensitive to β-lipotropin, insulin, glucagon, ACTH, and α-melanocyte-stimulating hormone [2]. We have shown previously that non-opioid β-endorphin receptors on human T lymphocytes and mouse peritoneal macrophages bind neither [Met^5]- and [Leu^5]-enkephalins, nor α- and γ-endorphins [6-12]. The results of the present study demonstrate that, similar to the observations on immunocompetent cells, non-opioid β-endorphin receptors on rat adrenocortical membranes do not bind [Met^5]enkephalin, α- or γ-endorphins,

ACTH(1-24), and somatostatin. Only β-endorphin was able to inhibit the specific binding of [^{3}H]immunorphin to the membranes (Table 1).

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