ISSN 0006-2979, Biochemistry (Moscow), 2011, Vol. 76, No. 12, pp. 1337-1341. © Pleiades Publishing, Ltd., 2011.
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Interaction of Synthetic Peptide Octarphin with Rat Myocardium Membranes

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Received April 19, 2011
Revision received May 21, 2011

Abstract—A selective agonist of non-opioid β-endorphin receptor synthetic peptide octarphin (TPLVTLFK, specific activity 28 Ci/mmol) was prepared. The [^{3}H]octarphin binding to rat myocardium membranes before and after experimental myocardial infarction (EMI) was studied. It was found that [^{3}H]octarphin with high affinity and specificity binds to non-opioid β-endorphin receptor of rat myocardium membranes before EMI: K_{d1} value of the [^{3}H]octarphin specific binding to membranes was 1.8 ± 0.2 nM. In 3 h after EMI a sharp lowering in affinity of the binding is observed (K_{d2} = 13.3 ± 0.4 nM), and in 48 h its almost complete restoration (K_{d4} = 2.2 ± 0.3 nM). The results indicate participation of non-opioid β-endorphin receptor in the regulation of myocardial activity.

DOI: 10.1134/S0006297911120066

Key words: β-endorphin, naloxone, peptide, receptor, myocardium

It is known that β-endorphin, in addition to opioid μ and δ receptors [1], binds to non-opioid receptor (which is resistant to opioid antagonist naloxone) as first described by Hazum et al. [2]. In 1980 Julliard et al. discovered corticotrophin and β-endorphin-like sequences in heavy chain of human IgG [3]. Then Houck et al. synthesized the tetradecapeptide SLTCLVKGFYPSDI corresponding to β-endorphin-like sequence in IgG (fragment 364-377 of heavy chain domain C_{H3}) and showed that it competes with ^{125}I-labeled β-endorphin for binding to membranes in rat brain [4]. Later we synthesized decapeptide SLTCLVKGFY (dubbed "immunorphin") corresponding to sequence 364-373 in human IgG heavy chain [1-4], and it was shown that it is a selective agonist of non-opioid β-endorphin receptor [5]. Using immunorphin labeled with tritium, distribution of non-opioid β-endorphin receptor in the rat organism was studied. It was found that this type of receptors is present on the cells of immune (peritoneal macrophages, T lymphocytes from donor blood, spleen T and B lymphocytes), nervous (synaptic membranes in brain), and endocrine (adrenal cortex membranes) systems [6]. Now there are data concerning the action of immunorphin on the cells of the immune and endocrine systems: it has been shown that the peptide increases proliferation induced by mitogen of human T lymphocytes *in vitro* [7-9], activates mouse peritoneal macrophages *in vitro* and *in vivo* [10, 11], and suppresses secretion of glucocorticoids from the adrenal body into the blood in rats *in vivo* [12].

Recently we localized the region of the β-endorphin molecule that is responsible for binding to non-opioid receptor: it appeared to be fragment 12-19 (TPLVTLFK) [13]. The peptide (dubbed "octarphin") corresponding to this sequence was synthesized. Conducted experiments showed that octarphin possesses nearly the same affinity to non-opioid receptor as β-endorphin and immunorphin and can be successfully used as a tool for its detection and study [14, 15].

The aim of presented study was to study the binding of [^{3}H]octarphin to rat myocardium membranes in health and after infarction.

MATERIALS AND METHODS

[Met^5]Enkephalin, α-, β-, γ-endorphin, and naloxone (Sigma, USA), BSA, EDTA, EGTA, Tris, and

Abbreviations: ECG, electrocardiogram; EMI, experimental myocardial infarction.

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phenylmethylsulfonyl fluoride (PMSF) (Serva, Germany), scintillator Unisolv 100 (Amersham, GB) were used in the study. *N*-Methylpyrrolidone, diisopropylcarbodiimide, 1-hydroxybenzotriazole, and thioanisole were purchased from Merck (Germany). The remaining reagents and solvents were domestic products and were used after appropriate purification.

Male rats of SD line weighing 180-200 g were obtained from the vivarium of the Branch of the Institute of Bioorganic Chemistry, Russian Academy of Sciences.

Immunorphin (SLTCLVKGFY), octarphin (TPLVTLFK), and its analogs (LPLVTLFK, TLLVTLFK, TPLVLLFK, TPLVTLLK, TPLVTLFL) were synthesized using an automatic synthesizer (Applied Biosystems model 430A and Vega Coupler model C250 (USA)) using the Boc/Bzl technique of peptide chain elongation and were purified by preparative reversed-phase chromatography (Gilson chromatograph, France; column Waters Symmetry Prep C18 (19 × 300 mm) (Malva, Greece)) as described earlier [16]. 2-Chlorobenzyloxycarbonyl and dichlorobenzyl for lysine and tyrosine, respectively, were used as constant protective groups. Upon completing assembly of the protected polypeptide chain, the final product was unblocked with its simultaneous cleavage from polymer using anhydrous hydrogen fluoride in the presence of scavengers.

The synthesized peptides were characterized by analytic reversed-phase HPLC (Gilson chromatograph, France; XTerra RP18 column (Malva, Greece)), amino acid analysis (hydrolysis by 6 M HCl, 24 h, 110°C; amino acid analyzer LKB 4151 (Alpha Plus, Sweden)), and mass spectrometric analysis (Finnigan mass spectrometer, USA).

[^{3}H]Octarphin was obtained by high-temperature solid-phase catalytic isotope exchange [17]. Fifty milligram of alumina was added to octarphin solution (2 mg in 0.5 ml of water) and the sample was evaporated using a rotary evaporator. The alumina with applied peptide was mixed with 10 mg of catalyzer (5% Rh/Al_2O_3). The resulting solid mixture was put into a 10 ml ampule. The ampule was filled with tritium gas at pressure 250 mm Hg, warmed to 170°C, and incubated at this temperature for 20 min. Then the ampule was cooled, scavenged with hydrogen, and the air was evacuated from it. The labeled peptide was extracted from the solid reaction mixture with two 3-ml portions of 50% aqueous ethanol, and the resulting solution was combined and evaporated. To remove labile tritium the procedure was repeated twice. The [^{3}H]octarphin was purified by HPLC on a 4 × 150-mm Kromasil column (particle size 5 μm) at 20°C with elution by 0.1% trifluoroacetic acid with 42-70% methanol gradient (20 min, flow velocity 3 ml/min) while monitoring spectrophotometrically (Beckman spectrophotometer, USA) at 254 and 280 nm. Tritium incorporation into the peptide was measured using a liquid scintillation counter.

Membranes from rat myocardium were isolated according to a published technique [18]; protein concentration was determined by Lowry [19] using BSA as a standard.

The reaction of [^{3}H]octarphin binding to membranes was performed in 50 mM Tris-HCl buffer containing PMSF (0.6 mg/ml), pH 7.5, according to the following scheme: 100 μl of labeled peptide from solution with concentration 10^{-10}-10^{-7} M (three parallel samples for each concentration), 100 μl of buffer (total binding) or 100 μl of 10^{-3} M unlabeled peptide solution in buffer (nonspecific binding), and 800 μl of newly isolated membrane suspension (2 mg of protein) were placed in glass siliconized tubes. The tubes were incubated at 4°C for 1 h, and the reaction mixtures were filtered through glass microfiber filters (GF/B; Whatman, GB). The filters were rinsed three times with 5 ml portions of ice-cold buffer solution. Radioactivity on the filters was measured using an LS 5801 liquid scintillation counter (Beckman). The value of [^{3}H]octarphin specific binding to membranes was determined from the difference between its total and nonspecific binding. To determine parameters of [^{3}H]octarphin specific binding to membranes (equilibrium dissociation constant K_d and density of receptors B_{max} – maximal binding ability per mg protein), a plot of the relation between molar concentrations of bound (B) and free (F) labeled peptide on molar concentration of bound labeled peptide (B) (Scatchard plot) was constructed [20].

To estimate the ability of naloxone and unlabeled peptides to inhibit [^{3}H]octarphin specific binding to rat myocardial membranes, membrane suspension (1.5 mg of protein, 800 μl) was incubated with [^{3}H]octarphin (5 nM, 100 μl) and with one of the potential inhibitors (concentration range 10^{-10}-10^{-4} M, three repeats for each concentration) in 50 mM Tris-HCl buffer containing PMSF (0.6 mg/ml), pH 7.5, at 4°C for 1 h. Then the reaction mixture was filtered through Whatman GF/B filters. Filters were rinsed three times with 5-ml portions of ice-cold buffer solution. Radioactivity on filters was measured using the LS 5801 liquid scintillation counter. Inhibition constant (K_i) was determined according to the formula: $K_i = [IC]_{50}/(1 + [L]/K_d)$ where [L] is molar concentration of [^{3}H]octarphin, K_d is equilibrium dissociation constant for the complex [^{3}H]octarphin–receptor, $[IC]_{50}$ is concentration of unlabeled ligand that causes 50% inhibition of labeled [^{3}H]octarphin specific binding [21]. The value of IC_{50} was determined graphically based on the inhibition curve (plot of relationship between inhibition (%) and inhibitor molar concentration).

Myocardium infarction was modeled in rats by ligature of the left coronary artery. Skin and subcutaneous fat were dissected with etherization, and pectoral and intercostal muscles were separated along the fiber line. After opening the breast, the heart was moved to the operative wound, and the left coronary artery was stitched and ligatured. The beginning of myocardium ischemia was con-

trolled electrocardiographically. The wound was closed by suturing.

The ECG was registered on unanesthetized rats using an RM-6000 polygraph (Japan). The animals were in special plastic cages. Thin steel needles placed subcutaneously were used as electrodes. ECG was registered for lead I. The electrocardiographic characteristic of EMI in the rats was registration of the rise in ST segment 1 h after surgery.

RESULTS AND DISCUSSION

The main characteristics of synthesized peptides (purity, amino acid analysis data, and molecular weights) are summarized in Table 1.

[^{3}H]Octarphin with specific activity 28 Ci/mmol was collected after purification by high-temperature solid-phase catalytic isotope exchange. The retention time of [^{3}H]octarphin and unlabeled octarphin on the column Kromasil C18 coincided and was 15 min; extinction ratio at 254 and 280 nm for labeled and unlabeled peptide also coincided, which indicates that the chemical structure of octarphin is maintained during the hydrogen/tritium substitution.

[^{3}H]Octarphin binding to rat myocardial membranes. The relationship between values of total, nonspecific, and specific [^{3}H]octarphin binding to membranes at 4°C and incubation time is shown in Fig. 1. It appears that dynamic equilibrium in the labeled peptide–receptor system was achieved in ~1 h and lasted at least for 2 h. For this reason, to determine the value of the equilibrium dissociation constant (K_d) the reaction of [^{3}H]octarphin binding to membranes was conducted for 1 h. Nonspecific binding of [^{3}H]octarphin under these conditions was very low – 6.8 ± 1.2% of the value of its total binding.

A Scatchard plot (*1*) is presented in Fig. 2; it characterizes [^{3}H]octarphin specific binding to rat myocardial membranes before EMI. Linearity of the plot indicates the presence in myocardium of one receptor type for the peptide, and K_{d1} value (1.8 ± 0.2 nM) – indicating high affinity of the peptide to the receptor. The receptor density (B_{max}) was 0.28 ± 0.05 pmol/mg protein.

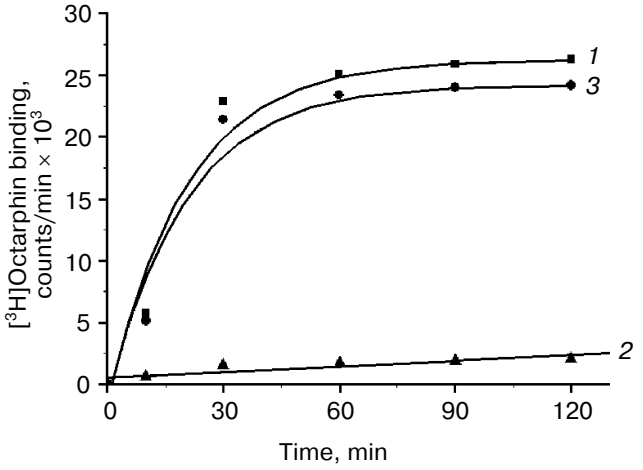

Fig. 1. Time course of total (*1*), nonspecific (*2*), and specific (*3*) [^{3}H]octarphin binding to rat myocardial membranes.

Plots *2*, *3*, and *4* in Fig. 2 characterize [^{3}H]octarphin binding to membranes collected from rat myocardium at 3, 24, and 48 h after EMI, respectively. It is obvious that receptor density is changed only slightly while affinity was decreased from 3 h after the operation (K_{d2} = 13.3 ± 0.4 nM, plot *2*). In 24 h after EMI, [^{3}H]octarphin affinity to the receptor was significantly increased (K_{d3} = 3.6 ± 0.3 nM, plot *3*), and at 48 h it was almost completely restored (K_{d4} = 2.2 ± 0.3 nM, plot *4*).

Inhibition of specific [^{3}H]octarphin binding to rat myocardial membranes by naloxone and unlabeled peptides. Results of experiments presented in Table 2 show

Table 1. Main peptide characteristics

Peptide	Purity, %	Amino acid analysis data	Molecular mass, Da
SLTCLVKGFY (immunorphin)	>99	Thr 0.89; Ser 0.92; Gly 1.00; Val 1.00; Leu 1.94; Tyr 1.03; Phe 1.00; Lys 0.91	1129.3 (calculated value – 1130.0)
TPLVTLFK (octarphin)	>99	Thr 1.96; Pro 0.97; Leu 2.02; Val 1.00; Phe 1.00; Lys 0.95	917.9 (918.24)
LPLVTLFK	>97	Thr 0.95; Pro 0.99; Leu 2.98; Val 0.99; Phe 0.97; Lys 0.95	930.4 (930.29)
TLLVTLFK	>97	Thr 1.98; Leu 2.99; Val 0.98; Phe 0.98; Lys 0.97	934.4 (934.28)
TPLVLLFK	>97	Thr 0.99; Pro 0.98; Leu 3.04; Val 1.00; Phe 1.02; Lys 0.97	930.5 (930.29)
TPLVTLLK	>97	Thr 1.97; Pro 0.96; Leu 3.00; Val 1.01; Lys 0.98	884.6 (884.22)
TPLVTLFL	>97	Thr 1.98; Pro 0.98; Leu 2.96; Val 0.99; Phe 1.03	902.9 (903.22)

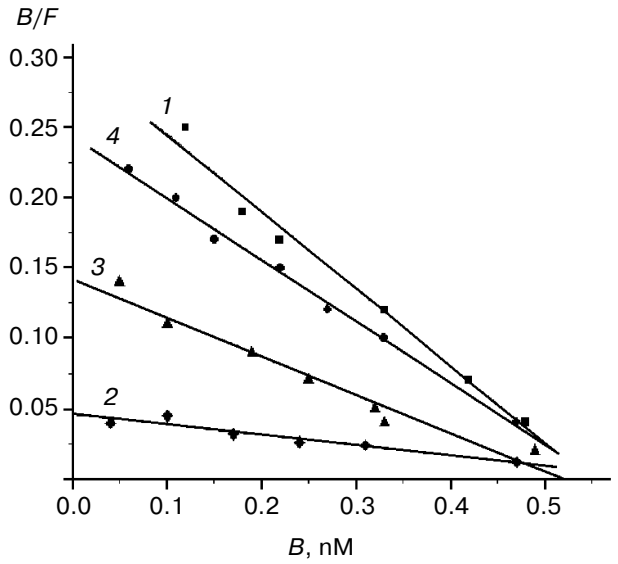

Fig. 2. Analysis of [^{3}H]octarphin specific binding to rat myocardial membranes under normal conditions (*1*) and in 3 (*2*), 24 (*3*), and 48 h (*4*) after EMI in Scatchard coordinates. *B* and *F* are molar concentrations of bound and free labeled peptide, respectively.

that specific binding of [^{3}H]octarphin to membranes was inhibited by β-endorphin and immunorphin (K_i = 1.8 ± 0.2 and 2.1 ± 0.3 nM, respectively). Naloxone, α- and γ-endorphins, and [Met^5]enkephalin did not inhibit binding (K_i > 10 μM). Inhibiting activity of unlabeled octarphin analogs LPLVTLFK, TLLVTLFK, TPLVLLFK, TPLVTLLK, and TPLVTLFL was more than 500 times lower (K_i > 1 μM) than the activity of unlabeled octarphin (K_i = 2.0 ± 0.3 nM).

Previously we synthesized a panel of β-endorphin fragments and tested the ability of each to inhibit specific binding of [^{3}H]immunorphin to mouse peritoneal macrophage. We found that the fragment of β-endorphin 12-19 TPLVTLFK was the shortest peptide possessing nearly the same inhibiting activity as β-endorphin (K_i 3.1 ± 0.3 nM, synthetic peptide "octarphin") [15]. The results of studies concerning the interaction of octarphin with rat myocardial membranes are presented in an article where we previously reported the discovery of a non-opioid β-endorphin receptor [6].

According to current ideas, the heart is a complicated endocrine organ that is mainly regulated by the adrenergic and opioid systems [22-24]. The results of recent studies show that β-endorphin together with other opioid peptides is involved in cardiac cycle regulation. It was found that cardiomyocytes synthesize and secrete proopiomelanocortin (POMC) peptides, particularly β-endorphin [25]. Evidence of the participation of β-endorphin in development of a variety of heart pathologies has been obtained. Thus, in patients with ischemia increased concentration of β-endorphin was noted in myocardium and peripheral blood [26, 27]. Using a model of chronic hypertension, change in κ- and δ-opioid receptor expression and activity in myocardium has been shown, as well as decrease in β-endorphin concentration in plasma [28]. Change in response of κ- and δ-opioid myocardial receptors to the action of selective agonists leading to the depression of heart function was also noted during cardiomyopathy and evolving heart failure [29].

Octarphin labeled with tritium ([^{3}H]octarphin) was used to reveal and characterize non-opioid β-endorphin receptor of myocardium. Results of the experiments showed that [^{3}H]octarphin binding to rat myocardial membranes is characterized by high affinity (K_{d1} = 1.8 ± 0.2 nM) (Fig. 2, plot *1*) and specificity and is not sensitive to naloxone (Table 2). The ability of unlabeled octarphin analogs (LPLVTLFK, TLLVTLFK, TPLVLLFK, TPLVTLLK, TPLVTLFL) to inhibit [^{3}H]octarphin specific binding was more than two orders lower than the ability of unlabeled octarphin. Therefore, even single amino acid substitutions in the octarphin molecule resulted in significant decrease in its affinity to the receptor, which indicates high specificity of binding.

Experimental myocardium infarction (EMI) is characterized by development of a region of necrosis, insufficiency of coronary blood flow, depression in contractive myocardium activity, and failure in atrioventricular and intraventricular conduction, cell respiration and metabolism, and activation of lipid peroxidation and decrease in antioxidant defense.

Comparative analysis of [^{3}H]octarphin binding to the non-opioid β-endorphin receptor of rat myocardium before and after EMI showed that the affinity of the

Table 2. Inhibition of [^{3}H]octarphin specific binding to rat myocardial membranes by naloxone and unlabeled peptides

Ligand	$[IC]_{50}$, nM	K_i, nM
Naloxone	>10 000	>10 000
β-Endorphin	7.0 ± 0.3	1.8 ± 0.2
Immunorphin	7.8 ± 0.4	2.1 ± 0.3
α-Endorphin	>10 000	>10 000
γ-Endorphin	>10 000	>10 000
[Met^5]Enkephalin	>10 000	>10 000
Tuftsin	>10 000	>10 000
Octarphin	7.4 ± 0.4	2.0 ± 0.3
LPLVTLFK	>1000	>1000
TLLVTLFK	>1000	>1000
TPLVLLFK	>1000	>1000
TPLVTLLK	>1000	>1000
TPLVTLFL	>1000	>1000

labeled peptide to the receptor decreased 7.4-fold 3 h after operation (K_{d2} = 13.3 ± 0.4 nM) (Fig. 2, plot *2*). Then gradual recovery of binding affinity was seen (Fig. 2, plots *3* and *4*), and after 48 h [^{3}H]octarphin affinity to the receptor was near that before EMI (K_{d4} = 2.2 ± 0.3 nM, plot *4*). These results are the first experimental evidence for the presence of non-opioid β-endorphin receptor in myocardium and change in its properties during experimental myocardial infarction.

This study was supported by the Russian Foundation for Basic Research (project No. 11-04-00208-a) and by International Science and Technology Center (project No. 2615).

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