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Detection of nonopioid β-endorphin receptor in the rat myocardium

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Two selective agonists of nonopioid β-endorphin receptor, synthetic peptides TPLVTLFK (octarphin) and SLTCLVKGFY (immunorphin), were labeled with tritium to specific activity of 29 and 25 Ci/mmol, respectively. Both labeled peptides were found to bind to high-affinity naloxone-insensitive binding sites on the membranes isolated from the rat myocardium (Kd = 2.0 ± 0.2 and 2.5 ± 0.3 nM, respectively). The [^{3}H]octarphin specific binding to the myocardial membranes was inhibited by unlabeled β-endorphin (Ki = 1.9 ± 0.2 nM) and immunorphin (Ki = 2.2 ± 0.3 nM). The [^{3}H]immunorphin specific binding with the membranes was inhibited by unlabeled β-endorphin (Ki = 2.3 ± 0.3 nM) and octarphin (Ki = 2.4 ± 0.3 nM). The binding specificity study revealed that these binding sites were insensitive not only to naloxone but also to α-endorphin, γ-endorphin, [Met^5]enkephalin and [Leu^5]enkephalin. Thus, β-endorphin, immunorphin and octarphin bind to the common high-affinity naloxone-insensitive receptor of the rat myocardial membranes. Copyright © 2011 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: β-endorphin; peptides; receptors; myocardium

Introduction

It is known that β-endorphin binds (besides the opioid μ-receptor and δ-receptor [1]) to the nonopioid receptor (insensitive to the opioid antagonist naloxone) described for the first time by Hazum et al. [2]. In 1980, Julliard et al. found corticotrophin-like and β-endorphin-like sequences in the human IgG H-chain [3]. Then, Houck et al. synthesized the tetradecapeptide SLTCLVKGFYPSDI corresponding to the β-endorphin-like sequence of IgG (fragment 364–377 of the H-chain CH_3-domain) and showed that it competed with ^{125}I-labeled β-endorphin for binding to rat brain membranes [4]. Later on, we synthesized and investigated the β-endorphin-like decapeptide SLTCLVKGFY (referred to as immunorphin) corresponding to sequence 364–373 of the human IgG(1–4) H-chain [5]. Experiments show that immunorphin labeled with ^{125}I or tritium binds with high affinity and specificity to the nonopioid β-endorphin receptor of human T-lymphocytes [6–8], mouse peritoneal macrophages [9,10], rat brain synaptic membranes [11] and cells of the human T-lymphoblast Jurkat line [12]. The study of biological activity of immunorphin has shown that it enhances the mitogen-induced proliferation of human T-lymphocytes in vitro [6–8], activates mouse peritoneal macrophages in vitro and in vivo [10,11], stimulates the growth of human T-lymphoblast Jurkat and MT-4 cell lines [13], inhibits the activity of adenylate cyclase of the membranes of rat adrenal cortex and inhibits the secretion of glucocorticoids from adrenal glands into blood [14]. The study of distribution of the nonopioid β-endorphin receptor in rats shows its presence in cells of the immune (macrophages and lymphocytes), nervous (synaptic brain membranes), endocrine (adrenal cortex membranes) and cardiovascular (myocardium membranes) systems [15].

Recently, for the purpose of identifying the shortest β-endorphin fragment that can bind to the nonopioid receptor with high affinity, we synthesized a panel of β-endorphin fragments and studied the ability of each of them to inhibit specific binding of [^{3}H]immunorphin to mouse peritoneal macrophages. As a result, we established that the synthetic peptide TPLVTLFK corresponding to β-endorphin sequence 12–19 (octarphin) is the shortest fragment of the hormone having practically the same affinity to the nonopioid receptor as immunorphin and β-endorphin [16]. Our experiments showed that octarphin can be successfully used for the detection and investigation of the nonopioid β-endorphin receptor [17,18].

The goal of the present work was to investigate the binding of [^{3}H]octarphin and [^{3}H]immunorphin to the membranes isolated from the rat myocardium.

Materials and Methods

The chemicals used in this study were naloxone, aluminum oxide (Al_2O_3) (Sigma, USA); sucrose, bovine serum albumin, ethylenediaminetetraacatate (EDTA), ethylene glycole-tetraactate (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 from Sigma. α-Endorphin, β-endorphin, γ-endorphins and [Met^5]enkephalin were obtained from Sigma. Other reagents were of extra purity grade. Distilled water was additionally purified using Mono-Q system (Millipore, USA).

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Adult male Wistar rats (200–250 g) were obtained from the Breeding Facility at the Branch of the Institute of Bioorganic Chemistry of the Russian Academy of Sciences. All experiments with animals were performed in accordance with the legal requirements of the Russian Academy of Sciences.

Immunorphin (SLTCLVKGFY), octarphin (TPLVTLFK) and its analogs (LPLVTLFK, TLLVTLFK, TPLVLLFK, TPLVTLLK, TPLVTLFL) were synthesized on an Applied Biosystems Model 430A automatic synthesizer (Applied Biosystems, Foster City, CA, USA) using the Boc/Bzl tactics of peptide chain elongation as described previously [19]. The peptides were purified to homogeneous state by preparative reverse-phase HPLC (Gilson chromatograph, France) on a Delta Pack C18 column, 100A (39 × 150 mm, mesh size 5 μm; flow rate 10 ml/min, elution with 0.1% TFA, gradient of acetonitrile 10–40% in 30 min). The purity of the peptides was better than 97%. The molecular masses of the peptides were determined by mass spectrum analysis (Finnigan mass spectrometer, San Jose, CA, USA). The data of amino acid analysis (hydrolysis by 6 M HCl, 22 h, 110 °C; LKB 4151 Alpha Plus amino acid analyzer, Sweden) are presented in Table 1.

[^{3}H]octarphin and [^{3}H]immunorphin were obtained by the reaction of high-temperature solid-phase catalytic isotope exchange [20]. Aluminum oxide (50 mg) was added to a solution of immunorphin (2 mg) or octarphin (2 mg) in water (0.5 ml), and the solution was evaporated on a rotor evaporator. Aluminum oxide with the peptide applied was mixed with 10 mg of catalyst (5% Rh/Al_2O_3). The solid mixture obtained was placed in a 10-ml ampoule. The ampoule was evacuated, 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 the solid reaction mixture by two portions of 50% aqueous ethanol (3 ml each), and the combined solution was evaporated. Labile tritium was removed by repeating the procedure twice. [^{3}H]octarphin and [^{3}H]immunorphin were purified using HPLC with a Beckman spectrophotometer at 254 and 280 nM on a column of Kromasil (4 × 150 mm; the granulation was 5 μm, 20 °C). The elution was with 0.1% TFA using a gradient of methanol gradient 42–70% in 20 min; the flow rate was 3 ml/min. The incorporation of tritium into the peptide was calculated by liquid scintillation counting.

Membrane fractions were isolated from the rat myocardium as described in Ref. [21]. Protein content was measured as described in Ref. [22].

The binding of [^{3}H]octarphin and [^{3}H]immunorphin to the rat myocardial membranes was measured as follows: membrane suspension (2 mg protein) was incubated with the labeled peptide (10^{-10} to 10^{-7} M, each concentration point in triplicate) in 1 ml 50 mM Tris-HCl buffer, pH 7.4, containing PMSF (0.6 g/l) at 4 °C for 1 h. The incubation was terminated by rapid filtration through Whatman GF/B fiberglass filters. Filters were rinsed three times with 5 ml volumes of ice-cold 50 mM Tris-HCl buffer, pH 7.4. Radioactivity was counted using LS 5801 beta counter (Beckman, USA). Nonspecific binding of each labeled peptide to the membranes was measured in the presence of a 1000-fold excess of the same unlabeled peptide (100 μM). The binding characteristics of the labeled peptides (the equilibrium dissociation constant, Kd, and the maximal binding capacity, Bmax) were estimated from the plots of the ratio of the bound labeled peptide molar concentration (B) to that of the free labeled peptide (F) versus bound labeled peptide molar concentration (B) [23].

To study the inhibitory effect of unlabeled peptides (β-endorphin, immunorphin, α-endorphin, γ-endorphin, [Leu5]-enkephalin and [Met^5]enkephalin, octarphin and its analogs) on the specific binding of [^{3}H]octarphin or [^{3}H]immunorphin to the membrane suspension (1.5 mg protein) was incubated with labeled peptide (5 nM) and unlabeled peptides (10^{-10} to 10^{-6} M, each concentration point in triplicate) as described previously. The inhibition constant (Ki) was estimated from the equation Ki = [IC]50/(1 + [L]/Kd) [24], where [L] is a molar concentration of labeled peptide, Kd is the dissociation constant of the labeled peptide/receptor complex and [I]50 is the concentration of the unlabeled peptide causing half-maximum inhibition of the labeled peptide specific binding. [IC]50 was estimated graphically from the inhibition curve. The Kd values were determined previously as described above.

The data are presented as the means ± standard error of the mean of at least three independent experiments.

Results

The sequences and main characteristics of synthesized peptides are given in Table 1.

We obtained after purification [^{3}H]immunorphin with a specific activity of 25 Ci/mmol and [^{3}H]octarphin with a specific activity of 29 Ci/mmol. The retention times for [^{3}H]immunorphin and unlabeled immunorphin on a Kromasil C18 column were 18 min, and the retention times for [^{3}H]octarphin and unlabeled octarphin were 15 min. The ratios of the coefficients of molar absorption at 254 and 280 nM for the labeled and unlabeled immunorphin and octarphin also coincided, indicating that the chemical structures of both peptides are retained if hydrogen is exchanged by tritium.

Studies on [^{3}H]octarphin and [^{3}H]immunorphin binding to the rat myocardial membranes have revealed that both the peptides

Table 1. Main characteristics of peptides

Peptide	Purity, %	Amino acid analysis data	Molecular mass, D
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)

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bind specifically to the membranes, and this binding is reversible, saturable and naloxone-insensitive. Figure 1. A shows the total (plot 1), specific (plot 2) and nonspecific (plot 3) binding of [^{3}H] octarphin to the membranes as a function of incubation time. It is evident that dynamic equilibrium in the system [^{3}H] octarphin-receptor was established approximately after 1 h and remained in this state for at least 2 h. Therefore, to assess the equilibrium dissociation constant (Kd), the reaction of [^{3}H] octarphin binding to the membranes was carried out for 1 h. The nonspecific binding of [^{3}H]octarphin under these conditions was 6.8 ± 0.9% of the total binding.

An analysis of the specific binding of [^{3}H]octarphin to the membranes in the Scatchard coordinates (Figure 2, plot 1) showed that there is one class of binding sites (receptors) for this peptide on their surface: the plot represents a straight line. The Kd value equal to 2.0 ± 0.2 nM indicates a high affinity of labeled peptide to the receptor; Bmax was 280 ± 50 fmol/mg. The results on inhibition of the specific binding of [^{3}H]octarphin by naloxone and unlabeled peptides are presented in Table 2. It is evident that only unlabeled β-endorphin, immunorphin and octarphin effectively competed with [^{3}H]octarphin (Ki 1.9 ± 0.2, 2.2 ± 0.3 and 2.1 ± 0.2 nM, respectively). The inhibition curve for unlabeled β-endorphin is presented on Figure 3. Five synthetic analogs of octarphin possessed very low inhibitory capacity; naloxone and the rest of peptides were inactive (Ki > 10 μM). These data indicate that immunorphin, β-endorphin and octarphin bind with a high affinity and specificity to the common naloxone-insensitive receptor of the rat myocardial membranes.

Figure 1B shows the total (plot 1), specific (plot 2) and nonspecific (plot 3) binding of [^{3}H] immunorphin to the membranes as a function of incubation time. It is clear that the dynamic equilibrium in the system [^{3}H]immunorphin-receptor was established approximately after 1 h and persisted for at least 2 h. Therefore, the reaction of the binding of [^{3}H]immunorphin to the membranes was carried out for 1 h. The nonspecific binding of [^{3}H]immunorphin was estimated in the presence of 10^{-4} M unlabeled immunorphin; it was 8.0 ± 0.7% of the total binding of the labeled peptide. Scatchard analysis of the specific binding of [^{3}H]immunorphin to the membranes (Figure 2, plot 2) showed the binding to one type of receptors (Kd = 2.5 ± 0.3 nM, Bmax = 280 ± 62 fmol/mg).

To characterize the specificity of binding of [3H]immunorphin to the membranes, we tested as potential competitors, unlabeled naloxone, octarphin, α-endorphin, β-endorphin, γ-endorphin,

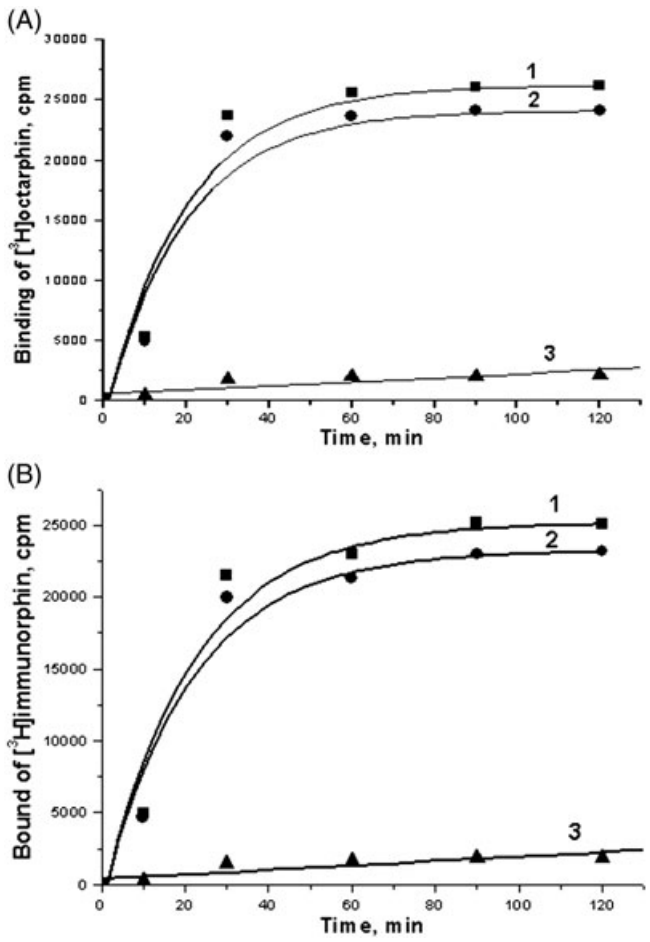

Figure 1. Total (1), specific (2) and nonspecific (3) binding of [^{3}H] octarphin (A) and [^{3}H]immunorphin (b) to the membranes of the rat myocardium. Incubation at 4 °C was terminated after 10, 30, 60, 90 and 120 min, and the specific binding was calculated by subtracting the nonspecific binding (measured in the presence of 10^{-4} M unlabeled peptide from the total binding).

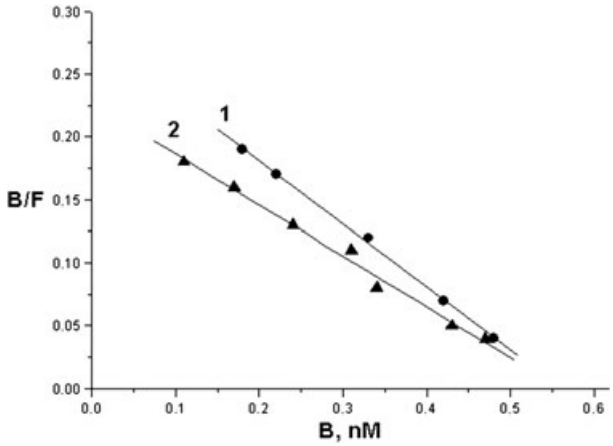

Figure 2. Scatchard analysis of the specific binding of [^{3}H]octarphin (1) and [^{3}H]immunorphin (2) to the membranes from the rat myocardium. B and F are the molar concentrations of the bound and free labeled peptide, respectively.

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

Ligand	[IC]50 (nM)	Ki (nM)
Naloxone	>10 000	>10 000
β-Endorphin	6.8 ± 0.2	1.9 ± 0.2
Immunorphin	7.8 ± 0.3	2.2 ± 0.3
α-Endorphin	>10 000	>10 000
γ-Endorphin	>10 000	>10 000
[Met5]enkephalin	>10 000	>10 000
[Leu5]enkephalin	>10 000	>10 000
Octarphin	7.4 ± 0.3	2.1 ± 0.2
LPLVTLFK	>1 000	>1 000
TLLVTLFK	>1 000	>1 000
TPLVLLFK	>1 000	>1 000
TPLVTLLK	>1 000	>1 000
TPLVTLFL	>1 000	>1 000

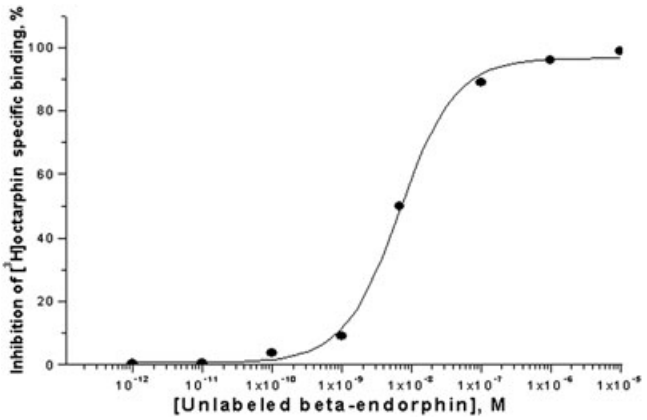

Figure 3. Inhibition of the specific binding of [^{3}H]octarphin to the membranes from the rat myocardium by unlabeled β-endorphin.

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

Ligand	[IC]50 (nM)	Ki (nM)
Naloxone	>10 000	>10 000
β-Endorphin	7.0 ± 0.3	2.3 ± 0.3
α-Endorphin	>10 000	>10 000
γ-Endorphin	>10 000	>10 000
[Met5]enkephalin	>10 000	>10 000
[Leu5]enkephalin	>10 000	>10 000
Octarphin	7.2 ± 0.3	2.4 ± 0.3

[Met^5]-enkephalin and [Leu^5]-enkephalin. The results of the experiments (Table 3) indicated that only β-endorphin and octarphin inhibited effectively the binding of [^{3}H]immunorphin to the membranes (Ki = 2.3 ± 0.3 and 2.4 ± 0.3 nM, respectively). Naloxone and the other peptides were ineffective (Ki > 10 μM). Thus, β-endorphin, immunorphin and octarphin bind to the common high-affinity naloxone-insensitive receptor of the rat myocardial membranes.

Discussion

Proopiomelanocortin (POMC) peptides were first identified in heart by Saito et al. [25], who reported that ACTH immunoreactivity is present in rat heart extracts. Later, Forman et al. [26] provided definitive evidence that β-endorphin and its immediate precursor, β-lipotropin, are detectable in cardiac tissue. They also reported that the ratio of β-endorphin to β-lipotropin is influenced by hemorrhagic shock, cardiac hypertrophy and other physiological stimuli, providing evidence that POMC processing can be regulated in heart [26]. Forman and Bagasra [27] were also the first to provide evidence that POMC may be synthesized by cardiomyocytes. They reported that POMC messenger RNA (mRNA) is detectable in ventricular myocytes by *in situ* hybridization histochemistry using an oligonucleotide probe corresponding to the POMC gene sequence encoding β-endorphin's N-terminus [28]. The results presented in the study by Millington et al. [29,30] support the hypothesis that

Figure 4. Comparison of β-endorphin amino acid sequences of man, mouse and rat. Fragment 12–19 (octarphin sequence) is designated with bold.

cardiomyocytes synthesize POMC peptides β-endorphin and αN-acetyl-β-endorphin immunoreactivities were localized in atrial myocytes, particularly in the atrial appendages, but not to a significant extent in ventricular myocytes. Cardiac nerves were not immunostained. Reverse transcription-PCR amplification showed that full-length POMC mRNA transcripts were present in both atrial and ventricular tissue and provides evidence that 5′ truncated POMC mRNA is expressed in heart. Nevertheless, the exact role of POMC peptides, in particular β-endorphin, in the normal or pathogenic regulation of myocyte function remains to be definitively elucidated.

β-Endorphin is known to act not only via opioid but also via nonopioid (naloxone-insensitive) receptor mechanisms. Our previous studies showed that synthetic peptides TPLVTLFK (octarphin) and SLTCLVKGFY (immunorphin) can be used for the detection and investigation of nonopioid β-endorphin receptor. To detect nonopioid β-endorphin binding sites in myocardium, we prepared [^{3}H]octarphin and [^{3}H]immunorphin that were subjected to study of their interaction with the rat myocardial membranes. Scatchard analysis showed that [^{3}H]octarphin and [^{3}H]immunorphin bind to the membranes with high affinity, Kd = 2.0 ± 0.2 and 2.5 ± 0.3 nM, respectively (Figure 2). The study of the binding specificity demonstrated that only unlabeled β-endorphin could replace labeled peptides in the ligand–receptor complex (Tables 2 and 3). Unlabeled naloxone, α-endorphin, γ-endorphin, [Met^5]enkephalin and [Leu^5]enkephalin that were tested as potential competitors were inactive. These results suggest that the rat myocardial membrane express nonopioid β-endorphin receptor.

Comparison of the amino acid sequences of human, mouse and rat β-endorphins [31] has shown their complete coincidence in the region 12–19 (Figure 4), which is evidence of high conservativeness of the latter. Besides, it allows us to study the binding of octarphin on rat models. For characterization, the specificity of the nonopioid receptor of myocardium five analogs of octarphin (LPLVTLFK, TLLVTLFK, TPLVLLFK, TPLVTLLK, TPLVTLFL) have been synthesized. The ability of unlabeled octarphin analogs to inhibit the specific binding of [3H]octarphin was more than 100 times less than in unlabeled octarphin. This means that even a single amino acid substitution in the octarphin molecule results in an abrupt decrease in affinity to the receptor. Thus, the specificity of the binding of labeled octarphin to nonopioid receptor in myocardium is very high, and it can be successfully used as an instrument for the investigation of nonopioid influence of β-endorphin on myocardial functions.

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