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Binding of synthetic peptide TPLVTLFK to nonopioid beta-endorphin receptor on rat brain membranes

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The synthetic peptide TPLVTLFK corresponding to the sequence 12–19 of β-endorphin (referred to as octarphin) was found to bind to high-affinity naloxone-insensitive binding sites on membranes isolated from the rat brain cortex ($K_d = 2.6 \pm 0.2$ 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, as well. The [^{3}H]octarphin specific binding with brain membranes was inhibited by unlabeled β-endorphin ($K_i = 2.4 \pm 0.2$ nM) and a selective agonist of nonopioid β-endorphin receptor decapeptide immunorphin SLTCLVKGFY ($K_i = 2.9 \pm 0.2$ nM). At the same time, unlabeled octarphin completely (by 100%) inhibited the specific binding of [^{3}H]immunorphin with membranes ($K_i = 2.8 \pm 0.2$ nM). Thus, octarphin binds with a high affinity and specificity to nonopioid receptor of β-endorphin on rat brain cortex membranes. Copyright © 2010 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: β-endorphin; peptides; receptors; brain

Introduction

It is known that β-endorphin binds to opioid (μ, δ, κ) and nonopioid (insensitive to the opioid antagonist naloxone) receptors [1]. Hazum *et al*. [2] were first to describe specific nonopioid receptor of β-endorphin in cultured human lymphocytes.

In 1980, Julliard *et al*. [3] detected a β-endorphin-like sequence in the heavy chain of human immunoglobulin G (IgG). Houck *et al*. [4] synthesized the tetradecapeptide SLTCLVKGFYPSDI corresponding to the β-endorphin-like sequence of human IgG (the fragment 364–377 of C_{H3} domain of heavy chain) and showed that it competes with ^{125}I-labeled β-endorphin for binding to rat brain membranes. We have synthesized the decapeptide SLTCLVKGFY corresponding to the amino acid sequence 364–373 of the heavy chain of human IgG of subclasses 1–4 (referred to as immunorphin) [5] and found that it is a selective agonist of nonopioid (insensitive to naloxone) β-endorphin receptor of human T-lymphocytes [6–9], mouse peritoneal macrophages [10,11], synaptic membranes of rat brain [12], rat adrenal cortex membranes [13,14], and human Jurkat lymphoblastic T-cells [15]. The investigations of biological activity of immunorphin showed that it increases the Con A-induced proliferation of human T-lymphocytes *in vitro* [6–9], activates mouse peritoneal macrophages *in vitro* and *in vivo* [10,11], stimulates the growth of human lymphoblast T-cell lines Jurkat and MT-4 [15,16], inhibits the adenylate cyclase activity in rat adrenocortical membranes and the secretion of corticosterone from the adrenal glands to the bloodstream [13], and stimulates cell division in early mouse blastocysts *in vitro* [17,18]. Study of the distribution of the nonopioid receptor of β-endorphin in the body of the rat showed that it is present on the cells of the immune (macrophages and lymphocytes), nervous (synaptic membranes of the brain), and cardiovascular systems (myocardium membranes) [19].

Recently, we showed that the fragment 12–19 of β-endorphin (TPLVTLFK, the author's name of the peptide octarphin) is the shortest peptide possessing practically the same affinity for nonopioid β-endorphin receptor as β-endorphin itself [20].

The purpose of the present study was to investigate the interaction of [^{3}H]octarphin with the membranes isolated from the rat brain cortex.

Materials and Methods

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

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Table 1. The main characteristics of synthesized peptides

Peptide	Purity (%)	Amino acid analysis data	Molecular mass (calculated value) (Da)
SLTCLVKGFY (immunorphin)	>97	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 (1130.0)
TPLVTLFK (octarphin)	>97	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)

Adult male Wistar rats (200–250 g) were obtained from the Breeding Facility at the Branch of the Institute of Bioorganic Chemistry, 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 (USA) using the Boc/Bzl tactics of peptide chain elongation as described previously [21]. 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]Immunorphin and [^{3}H]octarphin were obtained by the reaction of high-temperature solid-phase catalytic isotope exchange [22]. 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]Immunorphin and [^{3}H]octarphin were purified by 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 brain cortex as described in Ref. 23. Adult male Wistar rats (200–250 g) were euthanized by decapitation; the brains were rapidly removed and placed in a Petri dish. Cerebral cortex was chopped in ice-cold 10 mM Tris–HCl buffer, pH 7.4, supplemented with PMSF (0.6 mg/l) and then homogenized in the same buffer using polytrone (20 s in ice-cold bath). The homogenate was centrifuged at 150 000 *g* for 10 min at 4 °C. The pellet was resuspended in an excess of 10 mM Tris–HCl buffer, pH 7.4, supplemented with 0.15 M NaCl, allowed to stay in ice for 1 h, and again centrifuged as described above. Then the pellet was resuspended in an excess of the buffer free of NaCl and again centrifuged as described above; the new pellet was finally resuspended in 50 mM Tris–HCl buffer, pH 7.4. Protein content was measured as described in Ref. 24.

The binding of [^{3}H]octarphin and [^{3}H]immunorphin to rat brain membranes was measured as follows: membrane suspension (5 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 40 min. 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, K_d, and the maximal binding capacity, B_{max}) 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*) [25].

To study the inhibitory effect of unlabeled peptides (β-endorphin, immunorphin, α-endorphin, γ-endorphin, [Leu^5]- and [Met^5]enkephalins, octarphin, and its analogs) on the specific binding of [^{3}H]octarphin or [^{3}H]immunorphin to the membrane suspension (1 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 above. The inhibition constant (K_i) was estimated from the equation $K_i = [IC]_{50}/(1 + [L]/K_d)$ [26], where [L] is a molar concentration of labeled peptide; K_d is the dissociation constant of the labeled peptide/receptor complex; $[I]_{50}$ 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 K_d values were determined previously as described above.

The data are presented as the means ± SEM (standard error of mean) of 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

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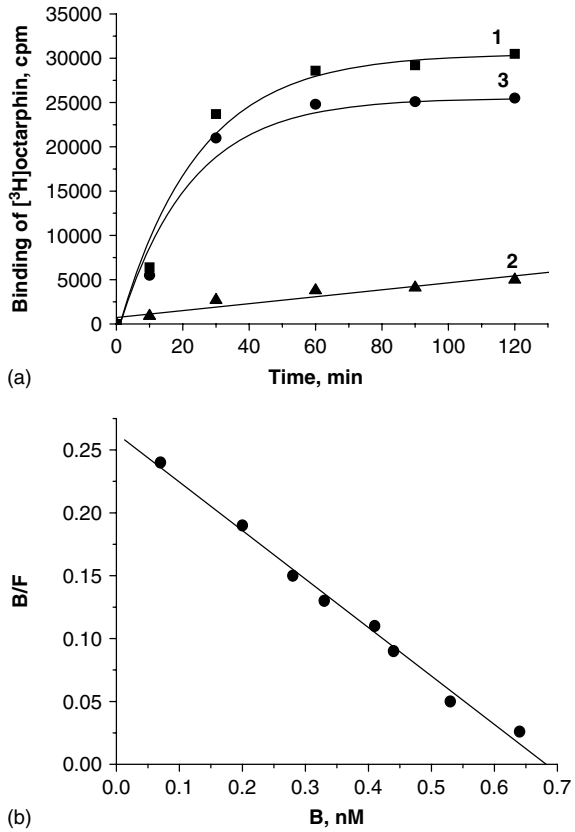

Figure 1. (a) Total (1), nonspecific (2), and specific (3) binding of [^{3}H]octarphin to the membranes of rat brain cortex. Incubation at 4 °C was terminated after 10, 30, 60, 90, and 120 min, and the specific binding was calculated by subtracting the nonspecific binding (measured in the presence of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

of 29 Ci/mmol. The retention times for [^{3}H]immunorphin and unlabeled immunorphin on a Kromasil C18 column were 18 min and 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 rat brain cortex membranes have revealed that both the peptides bind specifically to the membranes, and this binding is reversible, saturable and naloxone-insensitive.

Figure 1(a) shows the total (plot 1), nonspecific (plot 2), and specific (plot 3) binding of [^{3}H]octarphin to rat brain 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 (K_d), 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 13.3 ± 0.4% of total binding.

An analysis of the specific binding of [^{3}H]octarphin to membranes in the Scatchard coordinates (Figure1(b)) showed that there is one class of binding sites (receptors) for this peptide on their surface: the plot represents a straight line. The K_d value equal to 2.6 ± 0.2 nM indicates a high affinity of labeled peptide to the receptor; B_{max} was 136 ± 26 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 (K_i = 2.6 ± 0.2, 2.9 ± 0.2, and 2.7 ± 0.2 nM, respectively). Five synthetic analogs of octarphin possessed very low inhibitory capacity; naloxone and the rest of peptides were inactive (K_i > 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 rat brain membranes.

Table 2. Inhibition of the specific binding of 5 nM [^{3}H]octarphin to membranes of the rat brain cortex by unlabeled peptides and naloxone

Peptide	IC_{50}[a], nM	K_i[a], nM
Immunorphin	8.4 ± 0.6	2.9 ± 0.2
β-Endorphin	7.0 ± 0.5	2.4 ± 0.2
Octarphin	7.8 ± 0.6	2.7 ± 0.2
LPLVTLFK	1234.7 ± 89.7	425.8 ± 37.8
TLLVTLFK	1488.6 ± 121.5	513.1 ± 49.0
TPLVLLFK	1253.4 ± 97.7	432.1 ± 38.6
TPLVTLLK	1708.6 ± 155.7	589.0 ± 54.2
TPLVTLFL	1935.7 ± 182.1	667.5 ± 57.3
α-Endorphin	>10 000	>10 000
γ-Endorphin	>10 000	>10 000
[Met^5]enkephalin	>10 000	>10 000
[Leu^5]enkephalin	>10 000	>10 000
Naloxone	>10 000	>10 000

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

Figure 2(a) shows the total (plot 1), nonspecific (plot 2), and specific (plot 3) binding of [^{3}H] immunorphin to rat brain 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 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 11.6 ± 0.7% of the total binding of the labeled peptide.

Scatchard analysis of the specific binding of [^{3}H]immunorphin to membranes (Figure 2(b)) showed the binding to one type of receptors (K_d = 2.3 ± 0.2 nM, B_{max} = 118 ± 27 fmol/mg).

To characterize the specificity of binding of [^{3}H]immunorphin to membranes, we tested as potential competitors, unlabeled naloxone, octarphin, α-, β-, γ-endorphins, [Leu^5]- and [Met^5]-enkephalins. The results of the experiments (Table 3) indicated that only β-endorphin and octarphin inhibited effectively the binding of [^{3}H]immunorphin to membranes (K_i = 2.2 ± 0.2 and 2.8 ± 0.2 nM, respectively). Naloxone and the other peptides were ineffective (K_i > 10 μM). Thus, β-endorphin, immunorphin, and octarphin bind to the common high affinity naloxone-insensitive receptor of rat brain cortex membranes.

The results presented in this article allow the following conclusions: first, β-endorphin immunorphin, and octarphin share a common receptor on rat brain cortex membranes. This statement is confirmed by complete (100%) inhibition of [^{3}H]immunorphin binding by unlabeled β-endorphin and octarphin and at the same time by complete inhibition of [^{3}H]octarphin binding by

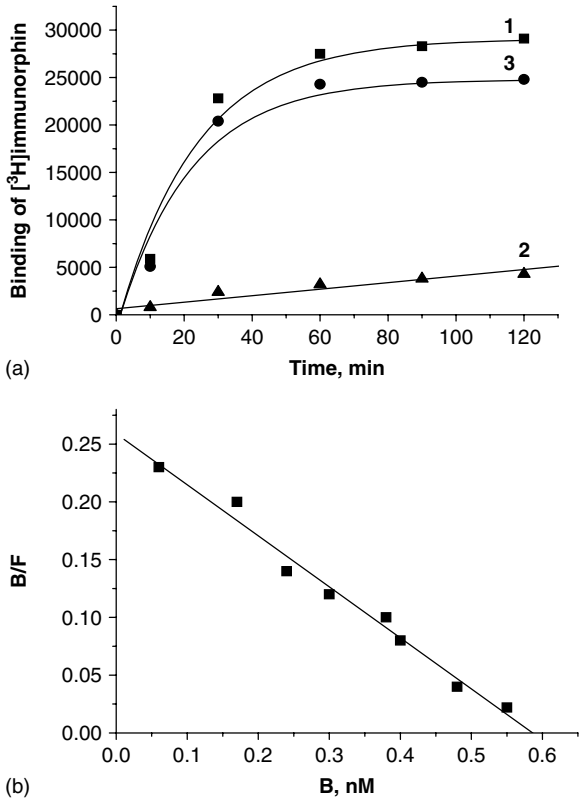

Figure 2. (a) Total (1), nonspecific (2), and specific (3) binding of [^{3}H]immunorphin to the membranes of rat brain cortex. Incubation at 4 °C was terminated after 10, 30, 60, 90, and 120 min, and the specific binding was calculated by subtracting the nonspecific binding (measured in the presence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

Table 3. Inhibition of the specific binding of 5 nM [^{3}H]immunorphin to of the rat brain cortex by unlabeled peptides and naloxone

Peptide	IC_{50}[a], nM	K_i[a], nM
β-Endorphin	7.0 ± 0.4	2.2 ± 0.2
Octarphin	9.0 ± 0.6	2.8 ± 0.2
α-Endorphin	>10 000	>10 000
γ-Endorphin	>10 000	>10 000
[Met^5]enkephalin	>10 000	>10 000
Naloxone	>10 000	>10 000

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

unlabeled β-endorphin and immunorphin, as well as by similar B_{max} values for [^{3}H]immunorphin and [^{3}H]octarphin (118 ± 27 and 136 ± 26 fmol/mg, respectively). Second, the receptors found are insensitive to naloxone and do not bind α- and γ-endorphins and enkephalins.

Discussion

Even early in the 1980s of the last century, the investigators noticed that the effect of β-endorphin on immunocompetent cells does not depend in some cases on the presence of naloxone and hence is not mediated via opioid receptors [1]. Nonopioid receptor for β-endorphin on human cultured lymphocytes was discovered by Hazum *et al*. more than 30 years ago [2]. According to their data, this receptor did not bind naloxone, cyclazocine, morphine, [Met^5]enkephalin, [Leu^5]enkephalin, α-endorphin, β-lipotropin, α-melanocyte-stimulating hormone (α-MSH), corticotropin (ACTH), insulin, and glucagon.

The existence of a β-endorphin-like sequence in the constant part of human IgG heavy (H) chain has been reported by Julliard *et al*. [3]. To isolate ACTH and β-endorphin from human placenta, the authors used immobilized antibodies to these hormones as affinity absorbents. A 50 kDa protein was thereby isolated; it was found to be an H-chain of IgG. The elucidation of the causes of such an effect has led to the discovery of ACTH- and β-endorphin-like sequences in the H-chain. It has been found that the H-chain fragment 364–377 (SLTCLVKGFYPSDI) is 40% homologous to β-endorphin fragment 10–23 (SQTPLVTLFKNAII). An artificial peptide (14 amino acid residues) corresponding to the β-endorphin-like IgG sequence has been synthesized and found to interact with rat brain receptors for β-endorphin [4]. We synthesized the decapeptide SLTCLVKGFY (immunorphin) that competes with ^{125}I-labeled β-endorphin for binding to naloxone-insensitive binding sites on human and mouse immunocompetent cells [5–9] as well as on rat brain membranes [12]. At present β-endorphin is known to interact with immunocompetent cells via not only opioid, but also nonopioid (naloxone-insensitive) receptor mechanisms.

We have shown that naloxone-insensitive receptors common to β-endorphin and immunorphin on mouse peritoneal macrophages and thymocytes are not sensitive to morphine, [Met^5]enkephalin, [Leu^5]enkephalin, and Fc-fragment of HulgG [19]. Although the study of the β-endorphin effect on the immune system is intensively in progress, naloxone-insensitive receptors for β-endorphin still remain 'enigmatic receptors' with an unknown structure and mechanism of action. We have analyzed the possibility that the receptors we have found belong to one of the known type of β-endorphin receptors. Mu, delta, and kappa opioid receptors display high affinity to β-endorphin, but they are all sensitive to naloxone. Therefore, naloxone-insensitive binding sites for β-endorphin that we have found on rat brain membranes cannot be μ, δ, or κ type opioid receptors. It is also unlikely that these receptors are σ or σ-like ones. These receptors are usually classified as nonopioid receptors as they are not sensitive to naloxone. Until recently, it has been believed that σ receptors do not bind endogenous opioid peptides. Ten years ago Wollemann *et al*. [27] studied the endogenous opioid peptide MERF binding to brain membranes of mammals and amphibia. Heptapeptide MERF, [Met^5]enkephalin-Arg^6-Phe^7 (Tyr-Gly-Gly-Phe-Met-Arg-Phe), was initially isolated from the bovine adrenal core and striatum [28]. Then it was found in the brain of various mammals [29–31] and amphibia [32]. Studies on the peptide interaction with the membranes isolated from the brain of rat, guinea pig, and frog have revealed that [^{3}H]MERF binds to opioid (κ_2 and δ) as well as to naloxone-insensitive nonopioid receptors [33]. Naloxone-insensitive binding sites for [^{3}H]MERF have been found on the brain membranes of all the three species. In the frog and guinea pig brains, they comprise approximately 25%, and in the rat brain, about 50% of the total [^{3}H]MERF binding sites. The presence of σ2-like receptors in each of the membrane fractions under study was demonstrated by [^{3}H]MERF (+) and (−)SKF-10.047 binding inhibition experiments. The unlabeled (−)SKF-10.047 possessing high affinity to σ2

receptors and low affinity to $\sigma 1$ ones, actively inhibited [^{3}H]MERF binding to frog, rat, and guinea pig brain membranes (K_i = 0.33, 0.43, and 5 nM, respectively). In contrast, (+)SKF-10.047 having a low affinity to $\sigma 1$ receptors slightly inhibited [^{3}H]MERF binding in all the three cases (K_i = 130, 1721, and 2660 nM, respectively). Apart from the $\sigma 2$-like receptors, nonopioid receptors for MERF included nonsensitive to σ-ligands and naloxone binding sites as well, in which [^{3}H]MERF could be replaced only by endogenous opioid peptides such as β-endorphin, [Met^5]enkephalin, and dynorphin (1–13). On the basis of these data, Wollemann *et al*. [33] conclude that there are naloxone-insensitive receptors common for β-endorphin, [Met^5]enkephalin, dynorphin (1–13), and MERF in the frog, rat, and guinea pig brain. The results presented in this article also indicate that naloxone-insensitive binding sites for β-endorphin are present in the rat brain. However, the binding sites that we have found do not bind [Met^5]enkephalin. Recently, Fontanilla *et al*. [34] discovered that the endogenous compound *N*,*N*-dimethyltryptamine (DMT) is an endogenous agonist for the $\sigma 1$ receptor. In the present study, this ligand has not been tested as a potential competitor of labeled octarphin.

Not long ago, we showed that β-endorphin fragment 12–19 TPLVTLFK (referred to as octarphin) is the shortest peptide that possesses practically the same capacity to inhibit the specific binding of [^{3}H]immunorphin to mouse peritoneal macrophages as β-endorphin (K_i = 3.1 and 2.9 nM, respectively). [^{3}H]Octarphin was found to bind to macrophages with high affinity (K_d = 2.3 nM). The specific binding of [^{3}H]octarphin was inhibited by unlabeled immunorphin and β-endorphin (K_i = 2.4 and 2.7 nM, respectively) [20]. The present study shows that β-endorphin, immunorphin, and octarphin bind to the common high affinity naloxone-insensitive receptor of rat brain cortex membranes. This result suggests with a high degree of probability that fragment 12–19 provides the binding of β-endorphin by the nonopioid receptor of rat brain.

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