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Binding of synthetic fragments of β-endorphin to nonopioid β-endorphin receptor

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Abstract: Selective agonist of nonopioid β-endorphin receptor decapeptide immunorphin (SLTCLVKGFY) was labeled with tritium (the specific activity of 24 Ci/mmol). [^{3}H]Immunorphin was found to bind to nonopioid β-endorphin receptor of mouse peritoneal macrophages ($K_d = 2.0 \pm 0.1$ nM). The [^{3}H]immunorphin specific binding with macrophages was inhibited by unlabeled β-endorphin ($K_i = 2.9 \pm 0.2$ nM) and was not inhibited by unlabeled naloxone, α-endorphin, γ-endorphin and [Met^5]enkephalin ($K_i > 10$ μM). Thirty fragments of β-endorphin have been synthesized and their ability to inhibit the [^{3}H]immunorphin specific binding to macrophages was studied. Unlabeled fragment 12–19 (TPLVTLFK, the author's name of the peptide octarphin) was found to be the shortest peptide possessing practically the same inhibitory activity as β-endorphin ($K_i = 3.1 \pm 0.3$ nM). The peptide octarphin was labeled with tritium (the specific activity of 28 Ci/mmol). [^{3}H]Octarphin was found to bind to macrophages with high affinity ($K_d = 2.3 \pm 0.2$ nM). The specific binding of [^{3}H]octarphin was inhibited by unlabeled immunorphin and β-endorphin ($K_i = 2.4 \pm 0.2$ and 2.7 ± 0.2 nM, respectively). Copyright © 2008 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: β-endorphin; peptides; receptors; macrophages

INTRODUCTION

It is known that β-endorphin binds to both opioid (μ and δ) 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].

The goal of the present study was to examine the ability of unlabeled β-endorphin fragments to inhibit the specific binding of [^{3}H]immunorphin to mouse peritoneal macrophages and determine the shortest fragment capable to binding with a high affinity to the nonopioid receptor of β-endorphin.

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 (USA). Other reagents were of extra purity grade. Distilled water was additionally purified using Mono-Q system (Millipore, USA).

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BALB/c mice 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.

Fragments of β-endorphin were synthesized on an Applied Biosystems Model 430A automatic synthesizer (USA) using the Boc/Bzl tactics of peptide chain elongation as described previously [20]. The peptides were purified to homogeneous state by preparative reverse-phase HPLC (Gilson chromatograph, France) on a Delta Pack C18 column, 100A (39 mm × 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). 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 [21]. 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 be 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 mm × 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.

Macrophages of the peritoneal cavity of mice were isolates and cultured as recommended in [22]. The viability of cells was 93–95%, as determined by the acridine orange test.

The binding of [^{3}H]immunorphin to murine peritoneal macrophages was performed in medium 199 containing Hepes (25 mM), NaN_3 (20 mM), PMSF (0.6 mg/ml), pH 7.4, according to the following scheme: 100 μl of the labeled peptide (10^{-10}–10^{-7} M; three parallel samples for each concentration), 100 μl of medium (total binding), or 100 μl of a 10^{-3} M solution of unlabeled peptide in medium (nonspecific binding), and 800 μl of cell suspension (1.0×10^7 cells in 1 ml of medium) were added to siliconized test tubes. The test tubes were incubated at 4 °C for 1 h. After the incubation, the reaction mixture was filtered through GF/C fiberglass filters (Whatman, UK) to separate the labeled peptide bound to cells from the unbound (free) peptide. The filters were washed three times with 5 ml ice–cold saline. The radioactivity on filters was measured by an LS 5801 liquid scintillation counter (Beckman, USA). Specific binding of [^{3}H]immunorphin to cells was determined as the difference between its total and nonspecific binding, and the nonspecific binding of [^{3}H]immunorphin was determined in the presence of 10^{-4} M unlabeled immunorphin (a 1000-fold excess relative to the highest concentration of labeled immunorphin 10^{-7} M). The parameters of specific binding of labeled immunorphin to macrophages (equilibrium dissociation constant K_d and density of receptors n, the number of sites of specific binding of peptide per one cell) were determined from the plot of the ratio of the molar concentration of bound (B) and free (F) labeled immunorphin versus the molar concentration of the bound labeled peptide (B). The density of receptors (n) was determined by the formula: $n = (R_0 \times A)/N$, where R_0 is the molar concentration of receptors, A is the Avogadro's number, and N is the amount of cells in 1l [23].

To determine the ability of unlabeled peptides, amino acids and naloxone to inhibit the specific binding of [^{3}H]immunirphin to macrophages, cells (1.0×10^7 cells in 1 ml) were incubated with labeled immunorphin (5 nM) and one of potential competitors (concentration range 10^{-12}–10^{-5} M; triplicate for each concentration) as described above. The inhibition constant (K_i) was determined by the formula: $K_i = IC_{50}/(1 + [L]/K_d)$ [24], where [L] is the molar concentration of [^{3}H]immunorphin, K_d is the equilibrium dissociation constant for the complex [^{3}H]immunorphin–receptor, and IC_{50} is the molar concentration of unlabeled peptide that causes a 50% inhibition of specific binding of labeled immunorphin. The value of IC_{50} was determined graphically from the inhibition plots (the percentage of inhibition versus the molar concentration of inhibitor). The K_d value was determined preliminarily as described above.

The binding of [^{3}H]octarphin to macrophages was assayed as described above for the [^{3}H]immunorphin binding. The nonspecific binding of [^{3}H]octarphin was determined in the presence of 10^{-4} M unlabeled octarphin. To characterize a specificity of the [^{3}H]octarphin binding unlabeled immunorphin, α-, β-, γ-endorphins, [Met^5]enkephalin, and naloxone were tested as potential binding inhibitors. Macrophages (1.0×10^7 cells in 1 ml)) were incubated with labeled peptide (5 nM) and one of potential competitors (concentration range 10^{-12}–10^{-5} M; triplicate for each concentration) as described above. The inhibition constant K_i was determined as described above.

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 24 Ci/mmol and [^{3}H]octarphin with a specific activity of 28 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.

Binding of (^{3}H)immunorphin to Murine Peritoneal Macrophages

The experiments showed that [^{3}H]immunorphin under the conditions chosen binds specifically to murine peritoneal macrophages. The degree of specific binding

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Table 1 Main characteristics of synthetic fragments of β-endorphin

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)
SQTPLVTLFKNAII (β-endorphin fragment 10–23)	>97	Thr 1.94; Ser 0.93; Gln 0.96; Val 1.00; Leu 1.98; Pro 0.94; Phe 0.99; Lys 0.92; Ala 1.00; Ile 1.98; Asn 0.96	1543.6 (1544.99)
QTPLVTLFKNAII (11–23)	>97	Thr 1.96; Gln 0.97; Val 1.00; Leu 1.98; Pro 0.98; Phe 0.97; Lys 0.95; Ala 0.99; Ile 1.98; Asn 0.94	1456.9 (1457.9)
SQTPLVTLFKNAI (10–22)	>97	Thr 1.96; Ser 0.96; Gln 0.95; Val 0.99; Leu 1.96; Pro 0.96; Phe 0.98; Lys 0.94; Ala 0.98; Ile 0.97; Asn 0.93	1432.4 (1431.82)
QTPLVTLFKNAI (11–22)	>97	Thr 1.98; Gln 0.94; Val 0.98; Leu 1.98; Pro 0.95; Phe 0.99; Lys 0.96; Ala 0.98; Ile 0.98; Asn 0.94	1345.9 (1347.73)
TPLVTLFKNAII (12–23)	>97	Thr 1.98; Val 1.00; Leu 1.98; Pro 0.94; Phe 0.97; Lys 0.95; Ala 0.99; Ile 0.98; Asn 0.95	1324.8 (1326.73)
SQTPLVTLFKNA (10–21)	>97	Thr 1.98; Ser 0.96; Gln 0.94; Val 0.99; Leu 1.98; Pro 0.97; Phe 0.99; Lys 0.95; Ala 0.98; Asn 0.95	1319.6 (1318.65)
TPLVTLFKNA (12–21)	>97	Thr 1.98; Val 1.00; Leu 1.98; Pro 0.96; Phe 0.98; Lys 0.94; Ala 0.98; Asn 0.96	1102.5 (1103.3)
QTPLVTLFKNA (11–21)	>97	Thr 1.96; Gln 0.96; Val 1.00; Leu 1.98; Pro 0.96; Phe 0.98; Lys 0.94; Ala 0.96; Asn 0.96	1232.0 (1231.56)
TPLVTLFKNAI (12–22)	>97	Thr 1.96; Val 1.00; Leu 1.97; Pro 0.96; Phe 0.96; Lys 0.93; Ala 0.99; Ile 0.96; Asn 0.97	1214.9 (1216.6)
PLVTLFKNAII (13–23)	>97	Val 1.00; Leu 1.98; Pro 0.96; Phe 0.96; Lys 0.96; Ala 0.99; Ile 1.99; Asn 0.98	1226.2 (1228.65)
PLVTLFKNAI (13–22)	>97	Val 1.00; Leu 1.96; Pro 0.98; Phe 0.96; Lys 0.98; Ala 0.99; Ile 0.98; Asn 0.96	1116.9 (1115.48)
PLVTLFKNA (13–21)	>97	Val 1.00; Leu 1.98; Pro 0.95; Phe 0.97; Lys 0.96; Ala 0.99; Asn 0.95	1000.4 (1002.31)
PLVTLFKN (13–20)	>97	Val 1.00; Leu 1.98; Pro 0.95; Phe 0.97; Lys 0.96; Ala 0.99	932.7 (931.22)
SQTPLVTLFKN (10–20)	>97	Thr 1.98; Ser 0.96; Gln 0.96; Val 0.99; Leu 1.99; Pro 0.96; Phe 0.96; Lys 0.94; Ala 0.99; Asn 0.94	1246.0 (1247.56)
QTPLVTLFKN (11–20)	>97	Thr 1.96; Gln 0.96; Val 0.99; Leu 1.99; Pro 0.96; Phe 0.96; Lys 0.94; Ala 0.99; Asn 0.94	1162.1 (1160.47)
TPLVTLFKN (12–20)	>97	Thr 1.98; Val 0.98; Leu 1.98; Pro 0.97; Phe 0.98; Lys 0.95; Ala 0.99; Asn 0.96	1033.8 (1032.34)
SQTPLVTLFK (10–19)	>97	Thr 1.98; Ser 0.97; Gln 0.96; Val 0.99; Leu 1.98; Pro 0.96; Phe 0.95; Lys 0.94	1130.2 (1133.46)
QTPLVTLFK (11–19)	>97	Thr 1.98; Gln 0.95; Val 0.99; Leu 1.99; Pro 0.96; Phe 0.96; Lys 0.96	1043.5 (1046.37)
TPLVTLFK (12–19)	>97	Thr 1.98; Val 0.99; Leu 1.99; Pro 0.96; Phe 0.96; Lys 0.96	918.9 (918.24)
PLVTLFK (13–19)	>97	Thr 0.97; Val 1.00; Leu 1.99; Pro 0.95; Phe 0.96; Lys 0.94	818.2 (817.12)
LVTLFK (14–19)	>99	Thr 0.98; Val 1.00; Leu 1.98; Phe 0.96; Lys 0.96	720.4 (719.99)
VTLFK (15–19)	>99	Thr 0.98; Val 1.00; Leu 0.97; Phe 0.98; Lys 0.96	606.0 (606.82)
TLFK (16–19)	>99	Thr 0.98; Leu 0.98; Phe 0.98; Lys 0.94	508.5 (507.67)
LFK (17–19)	>99	Leu 0.99; Phe 0.98; Lys 0.97	405.7 (406.55)
FK (18–19)	>99	Phe 0.96; Lys 0.96	293.6 (293.38)
SQTPLVTLF (10–18)	>99	Thr 1.96; Ser 0.96; Gln 0.95; Val 0.99; Leu 1.96; Pro 0.95; Phe 0.98	1045.8 (1046.31)
QTPLVTLF (11–18)	>99	Thr 1.96; Gln 0.96; Val 0.99; Leu 1.98; Pro 0.96; Phe 0.98	917.7 (918.18)
TPLVTLF (12–18)	>99	Thr 1.96; Val 0.98; Leu 1.98; Pro 0.97; Phe 0.99	790.2 (790.06)
PLVTLF (13–18)	>99	Thr 0.98; Val 0.99; Leu 1.98; Pro 0.99; Phe 0.96	686.8 (688.93)
SQTPLVTL (10–17)	>97	Thr 1.96; Ser 0.97; Gln 0.96; Val 0.98; Leu 1.99; Pro 0.96	860.0 (858.08)

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of [^{3}H]immunorphin was determined as the difference between its total and nonspecific binding. The nonspecific binding of [^{3}H]immunorphin was estimated in the presence of 10^{-4} M unlabeled immunorphin, it was $9.7 \pm 0.8\%$ of the total binding of the labeled peptide. It is evident from Figure 1(a) 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 macrophages was carried out for 1 h.

An analysis of the specific binding of [^{3}H]immunorphin to macrophages in the Scatchard coordinates (Figure 1(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 2.0 ± 0.1 nM indicates a high affinity of immunorphin to the receptor. The density of receptors (the number of sites of specific binding of the labeled peptide per one macrophage) was $39\,140 \pm 3800$.

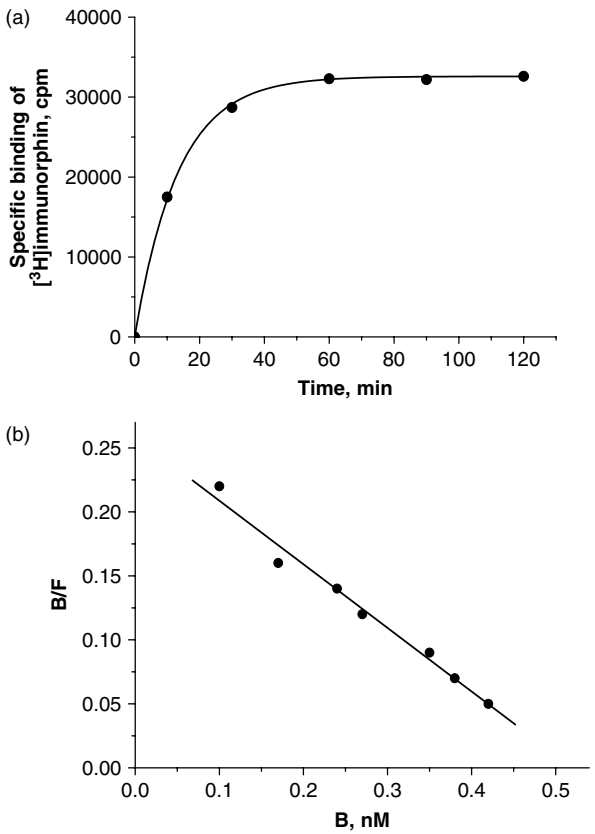

Figure 1 (a) Specific binding of [^{3}H]immunorphin to murine peritoneal macrophages. Incubation at 4 °C was terminated after 10, 30, 60, 90 and 120 min, and the specific binding was calculated by subtracting the nonspecific binding (measured in the presence of 10^{-4} M unlabeled immunorphin) from the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

To characterize the specificity of binding of [^{3}H]immunorphin to macrophages, we tested as potential competitors, unlabeled naloxone, α-, β-, γ-endorphins and [Met^5]enkephalin. The results of the experiments indicated that only β-endorphin inhibited effectively the binding of [^{3}H]immunorphin to macrophages ($K_i = 2.9 \pm 0.2$ nM). Naloxone and the other peptides were ineffective ($K_i > 10$ μM). Thus, immunorphin and β-endorphin bind with a high affinity and specificity to the common, naloxone-insensitive receptor of murine peritoneal macrophages.

Inhibition of the Specific Binding of (^{3}H)immunorphin to Murine Peritoneal Macrophages by Unlabeled Synthetic Fragments of β-Endorphin

To estimate the binding capacity of synthetic fragments of β-endorphin (30 peptides) to the nonopiod β-endorphin receptor, we studied the ability of each fragment to inhibit the specific binding of [^{3}H]immunorphin to murine peritoneal macrophages. It is evident from the Table 2 that β-endorphin fragment 12–19 (TPLVTLFK) is the shortest peptide that possesses practically the same inhibitory activity as β-endorphin ($K_i = 3.1 \pm 0.2$ and 2.9 ± 0.2 nM, respectively; the inhibition curve is shown in Figure 2), and the activity of fragments 13–19 and 12–18 is by more than one order of magnitude lower ($K_i = 51.2 \pm 4.1$ and 41.5 ± 3.4 nM, respectively). Dipeptide FK (fragment 18–19) also inhibited the binding, however, its activity was more than 200 times lower than that of β-endorphin and fragment 12–19 ($K_i = 623.9 \pm 52.0$ nM), Fragment 10–17 and amino acids *L*-Phe and *L*-Lys were inactive. Thus, octapeptide TPLVTLFK (fragment 12–19) inhibits the specific binding of [^{3}H]immunorphin to peritoneal macrophages by 100% and has almost the same affinity to receptor as β-endorphin.

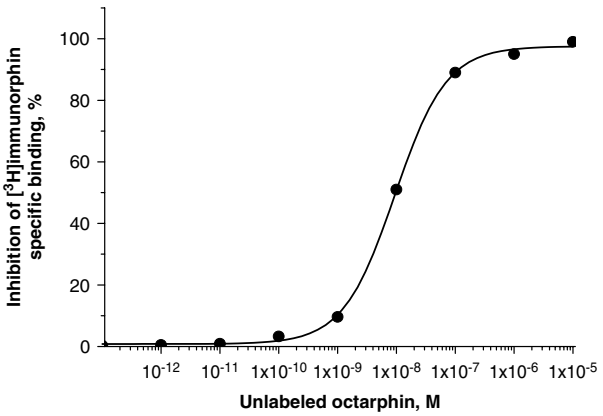

Figure 2 Inhibition by unlabeled octarphin of the specific binding of [^{3}H]immunorphin (5 nM) to murine peritoneal macrophages.

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Table 2 Inhibition of the specific binding of immunorphin to mouse peritoneal macrophages by unlabeled β-endorphin fragments and amino acids

Peptide	IC_{50}[a], nM	K_i[a], nM
β-Endorphin	8.9 ± 0.5	2.9 ± 0.2
β-Endorphin (10–23)	9.3 ± 0.6	3.0 ± 0.2
β-Endorphin (11–23)	9.4 ± 0.7	3.0 ± 0.2
β-Endorphin (10–22)	9.3 ± 0.6	3.0 ± 0.2
β-Endorphin (11–22)	9.2 ± 0.8	3.0 ± 0.2
β-Endorphin (12–23)	9.4 ± 0.5	3.0 ± 0.2
β-Endorphin (10–21)	9.5 ± 0.8	3.1 ± 0.3
β-Endorphin (12–21)	9.2 ± 0.6	3.0 ± 0.2
β-Endorphin (11–21)	9.3 ± 0.8	3.0 ± 0.3
β-Endorphin (12–22)	9.3 ± 0.8	3.0 ± 0.3
β-Endorphin (13–23)	13.2 ± 0.9	4.3 ± 0.3
β-Endorphin (13–22)	13.6 ± 0.9	4.4 ± 0.3
β-Endorphin (13–21)	13.4 ± 0.7	4.3 ± 0.3
β-Endorphin (13–20)	13.7 ± 0.8	4.4 ± 0.3
β-Endorphin (10–20)	9.3 ± 0.5	3.0 ± 0.2
β-Endorphin (11–20)	9.3 ± 0.6	3.0 ± 0.2
β-Endorphin (12–20)	9.2 ± 0.6	3.0 ± 0.2
β-Endorphin (10–19)	9.5 ± 0.7	3.1 ± 0.3
β-Endorphin (11–19)	9.6 ± 0.8	3.1 ± 0.3
β-Endorphin (12–19)	9.5 ± 0.9	3.1 ± 0.3
β-Endorphin (13–19)	158.6 ± 12.7	51.2 ± 4.6
β-Endorphin (14–19)	248.6 ± 20.0	80.2 ± 7.9
β-Endorphin (15–19)	976.6 ± 78.4	315.0 ± 25.2
β-Endorphin (16–19)	1422.4 ± 127.9	458.8 ± 36.7
β-Endorphin (17–19)	1500.9 ± 128.6	484.2 ± 38.7
β-Endorphin (18–19)	1934.1 ± 174.1	623.9 ± 52.0
L-Phe	>10 000	>10 000
L-Lys	>10 000	>10 000
β-Endorphin (10–18)	125.5 ± 11.6	40.5 ± 4.2
β-Endorphin (11–18)	127.4 ± 12.3	41.1 ± 4.3
β-Endorphin (12–18)	128.6 ± 11.8	41.5 ± 4.4
β-Endorphin (13–18)	318.7 ± 14.2	102.8 ± 9.7
β-Endorphin (10–17)	>10 000	>10 000

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

Binding of (^{3}H)octarphin to Murine Peritoneal Macrophages

Figure 3(a) shows the total (Plot 1), specific (Plot 2), and nonspecific (Plot 3) binding of [^{3}H]octarphin to murine peritoneal macrophages 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 under these conditions was 7.8 ± 0.6% of total binding.

Scatchard analysis of the specific binding of [^{3}H]octarphin to macrophages (Figure 3(b)) showed the binding to one type of receptors (K_d = 2.3 ± 0.2 nM and n = 43 600 ± 3900). The results on inhibition of the specific binding of [^{3}H]octarphin by naloxone and unlabeled peptides are presented in Table 3. It is evident that only immunorphin and β-endorphin effectively competed with [^{3}H]octarphin (K_i2.4 ± 0.2 and 2.7 ± 0.2 nM, respectively). Naloxone and the other 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 nonopiod receptor of murine peritoneal macrophages.

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]. The nonopioid (insensitive to the opioid antagonist naloxone) receptor of β-endorphin was identified by Hazum and coworkers on transformed human lymphocytes [2]. A study of the

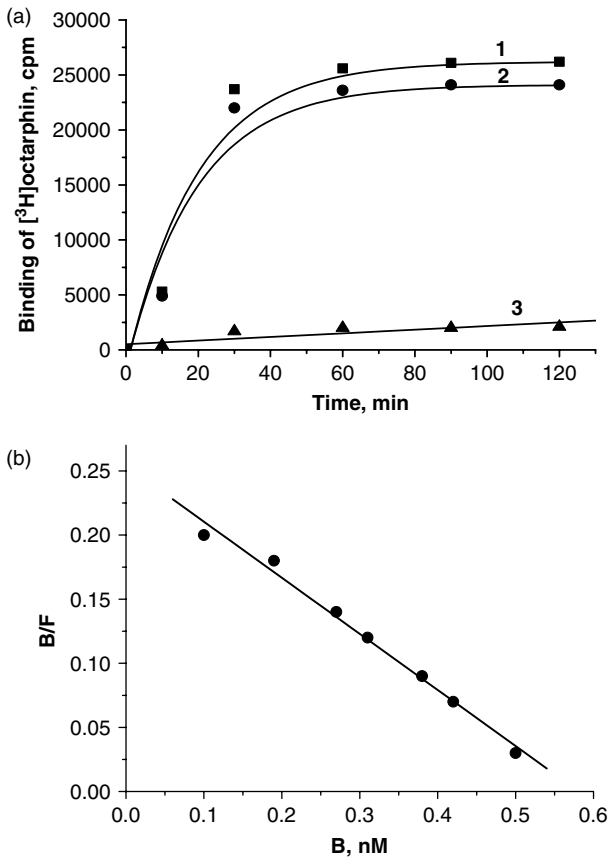

Figure 3 (a) Total (Plot 1), specific (Plot 2) and nonspecific (Plot 3) binding of [^{3}H]octarphin to murine peritoneal macrophages. (b) Scatchard analysis of the specific binding of [^{3}H]octarphin to peritoneal macrophages. B and F are the molar concentrations of the bound and free labeled peptide, respectively.

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Table 3 Inhibition of the specific binding of 5 nM [^{3}H]octarphin to mouse peritoneal membranes by unlabeled peptides

Peptide	IC_{50}[a], nM	K_i[a], nM
Immunorphin	7.7 ± 0.6	2.4 ± 0.2
β-Endorphin	8.6 ± 0.5	2.7 ± 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.

specificity of the receptor showed that it does not bind α- and γ-endorphins, [Met^5]- and [Leu^5]enkephalins, β-lipotropin, corticotrophin, α-melanocyte-stimulating hormone, insulin and glucagon.

It was found almost simultaneously by several research groups that β-endorphin is capable of affecting the proliferation of T lymphocytes *in vivo* (both the stimulating and inhibitory effects were reported [25–27]. In an effort to determine the reason for such a substantial disparity, Van Den Bergh and coworkers [28] studied the effect of five opioid peptides (α-, β-, and γ-endorphins, [Met^5]- and [Leu^5]enkephalins) on the Con A-induced proliferation of rat spleen T cells. It was found that the constant presence of any of these peptides in a medium for culturing splenocytes did not affect their proliferative response. However, a preliminary 30-min incubation of cells with β-endorphin (but not with the other peptides) increased the proliferation by 50–100% in a dose-dependent manner. Naloxone did not inhibit the stimulating effect of β-endorphin; consequently, the effect of β-endorphin on the proliferation of T cells was not mediated via naloxone-sensitive opioid receptors. It was shown simultaneously that the constant presence of β- or α-endorphin in a culture of T cells preliminarily incubated with β-endorphin eliminated completely the stimulating effect of the latter. The authors proposed that in the absence of opioid peptides on the surface of T lymphocytes only nonopioid receptors of β-endorphin are accessible for binding. The addition of β-endorphin to medium leads to an increase in the proliferative response mediated via these receptors. If β-endorphin (or another opioid peptide) is constantly present in the culture medium, opioid receptors appear on the surface of T cells, and, when binding to these receptors, β-endorphin inhibits its own stimulating effect [28].

To prove that β-endorphin molecule contains a binding site for nonopioid receptor, Van Deb Bergh and coworkers studied the effect of synthetic fragments of β-endorphin: 6–31, 18–31, 24–31, 28–31, and 1–27 on the proliferation of T lymphocytes (the peptides were added to the cultivation medium prior to the stimulation of cells by mitogen, and 72 h later the proliferation level was determined) [29]. The study showed that fragments 6–31 and 18–31 increase the proliferation, the first fragment being considerably more active than the second. At the same time, fragments 1–27, 24–31, and 28–31 were inactive. Based on the results obtained, the authors proposed that region 6–23 is important for the realization of the effect of β-endorphin on T lymphocytes and, consequently, for the interaction with the naloxone-insensitive receptor through which the effect is mediated; in their opinion, the key role in binding is played by fragment 18–23. Simultaneously, these authors showed that β-endorphin-(18–23) increases the production interleukin-2 and interleukin-4 by CD4$^+$ T cells.

Shahabi and coworkers identified naloxobe-insensitive β-endorphin receptors on murine splenocytes [30] and a human mononuclear cell line U937 [31] and characterized their specificity. According to their results, *N*-Ac-β-endorphin caused a 100% inhibition of the binding of ^{125}I-labeled β-endorphin to both cell types; β-endorphin fragment 6–31 had a somewhat lower inhibitory activity; and naloxone, α- and γ-endorphins, and [Met^5]- and [Leu^5]enkephalins, as well as β-endorphin fragments 1–16 and 28–31 were inactive. The high inhibitory capacity of *N*-Ac-β-endorphin and fragment 6–31 and the absence of activity in the case of fragments 1–16, 28–31, α- and γ-endorphins, and the enkephalins indicated that the site of the binding of the β-endorphin molecule to the nonopioid receptor is localized in its central part.

We previously showed that β-endorphin in the concentration range of 10^{-11}–10^{-7} M considerably enhances the Con A-induced proliferation of donor blood T lymphocytes; the maximum activity of the peptide was observed at a concentration of 10^{-10} M [8]. Immunorphin produced practically the same effect on proliferating T lymphocytes as β-endorphin did: it increased the proliferation in the concentration range of 10^{-11}–10^{-7} M with the maximum at the concentration of 10^{-10} M. Naloxone and [Met^5]enkephalin did not affect the proliferative response of T lymphocytes. What in the main thing, naloxone did not inhibit the activating effect of β-endorphin and immunorphin.

A comparison of the amino acid sequences of β-endorphin and immunorphin (Figure 4) shows that immunorphin and β-endorphin fragment 10–19 have five coincident amino acid residues. We previously showed that immunorphin causes a 100% inhibition of the specific binding of ^{125}I-labeled β-endorphin to T lymphocytes [8]. Summarizing the data presented above, we proposed that the central moiety of the β-endorphin molecule, tentatively region 10–23 or its shorter fragment is involved in the binding of the peptide to the nonopioid receptor. To verify

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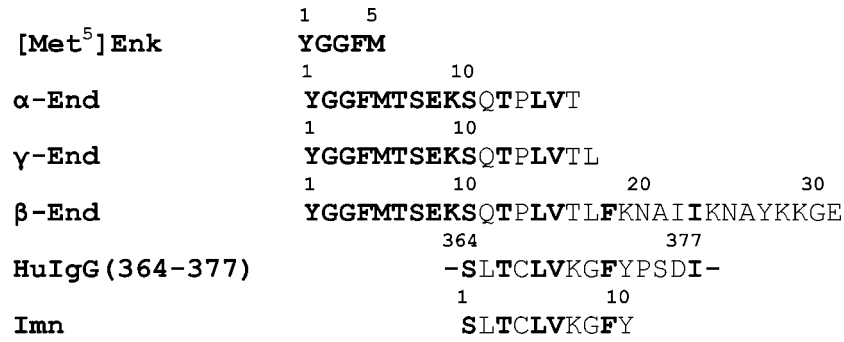

Figure 4 Comparison of amino acid sequences of [Met^5]enkephalin ([Met^5]ENK), α-, γ-, β-endorphins (α-, γ-, β-ENDs), β-endorphin-like fragment from heavy chain of human IgG subclasses 1–4 (HuIgG), and immunorphin (IMN). The numbers of the amino acid residues are marked with numerals. The coinciding residues are marked with bold letters.

this suggestion, we synthesized 30 fragments of β-endorphin (Table 1) and studied the ability of each fragment to inhibit the specific binding of [^{3}H]immunorphin to peritoneal macrophages of the mouse. It is evident from the Table 2 that β-endorphin fragment 12–19 TPLVTLFK (referred to as octarphin) is the shortest peptide that possesses practically the same inhibitory activity as β-endorphin ($K_i = 3.1 \pm 0.2$ nM). The inhibition curve (Figure 2) shows that unlabeled octarphin inhibits the specific binding of [^{3}H]immunorphin to peritoneal macrophages by 100% and has almost the same affinity to receptor as β-endorphin.

To characterize the interaction of octarphin with murine peritoneal macrophages we prepared [^{3}H] octarphin. Scatchard analysis showed that [^{3}H] octarphin binds to macrophages with high affinity, $K_d = 2.3 \pm 0.2$ nM (Figure 3(b)). The study of binding specificity demonstrated that only unlabeled β-endorphin and immunorphin could replace labeled octarphin in the ligand–receptor complex (Table 3). Other unlabeled peptides and naloxone that were tested for potential competition were inactive. This result suggests with a high degree of probability that fragment 12–19 provides the binding of β-endorphin by the nonopioid receptor of murine peritoneal macrophages.

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