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Effect of Synthetic β-Endorphin-Like Peptide Immunorphin on Human T Lymphocytes

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Abstract—β-Endorphin and the synthetic β-endorphin-like decapeptide Ser-Leu-Thr-Cys-Leu-Val-Lys-Gly-Phe-Tyr (referred to as immunorphin), corresponding to the sequence 364-373 of the CH3 domain of human immunoglobulin G heavy chain, were shown to stimulate concanavalin A-induced proliferation of T lymphocytes from the blood of healthy donors. [Met^5]Enkephalin and the antagonist of opioid receptors naloxone examined in parallel were inactive. The stimulating effect of β-endorphin and immunorphin on T lymphocyte proliferation is not inhibited by naloxone. Studies on receptor binding of ^{125}I-labeled immunorphin to T lymphocytes revealed that it binds with high affinity to naloxone-insensitive receptors (K_d = 7.0 ± 0.3 nM). Unlabeled immunorphin completely inhibits ^{125}I-labeled β-endorphin specific binding to naloxone-insensitive receptors on T lymphocytes (K_i = 0.6 ± 0.1 nM). Thus, β-endorphin and immunorphin interact with common naloxone-insensitive receptors on T lymphocytes.

Key words: β-endorphin, naloxone, immunoglobulin G (IgG), peptides, receptors, T lymphocytes

In the early 1980s, Julliard et al. [1] used immobilized antibodies to β-endorphin as affinity adsorbents for isolation of this hormone from human placenta extract. In so doing, a heavy chain (H-chain) of IgG was isolated in addition to β-endorphin. Elucidation of the causes of such an effect led to the discovery of a β-endorphin-like sequence in the H-chain. It was found that the fragment 364-377 (SLTCLVKGFYPSDI) of the H-chain of IgG subclasses 1-4 was homologous (40%) to β-endorphin's fragment 10-23 (SQTPLVTLFKNAII). The tetradecapeptide SLTCLVKGFYPSDI corresponding to the β-endorphin-like sequence of IgG was synthesized by Houck et al. [2], and shown to inhibit ^{125}I-labeled β-endorphin binding to the receptors on rat brain cells. We have synthesized the decapeptide SLTCLVKGFY corresponding to the amino acid sequence 364-373 of the IgG heavy chain (referred to as immunorphin) and found its ability to inhibit ^{125}I-labeled β-endorphin binding to high-affinity receptors on mouse peritoneal macrophages (K_i = 5.9 nM). Studies on the specificity of the receptors revealed that they are unable to bind natural opioid peptides [Met^5]enkephalin and [Leu^5]enkephalin as well as the antagonist of opioid receptors naloxone [3]. Recently we have found and characterized naloxone-insensitive receptors for β-endorphin on T lymphocytes isolated from the blood of healthy donors (K_d = 0.25 nM) [4].

The purpose of the present study was to investigate *in vitro* the effect of β-endorphin and immunorphin on concanavalin A-induced proliferation of T lymphocytes from healthy donors' blood and to determine kinetic characteristics of ^{125}I-labeled immunorphin binding to these cells.

MATERIALS AND METHODS

The following chemicals were used: [^{125}I-Tyr^{27}]β-endorphin (~2000 Ci/mmol specific activity), [methyl-^{3}H]thymidine (76 Ci/mmol), scintillator Unisolv 100 (Amersham, UK); 1,3,4,6-tetrachloro-3α,6α-diphenylglycouril (Iodogen), [Met^5]enkephalin, β-endorphin, naloxone, cell cultivation media, fetal calf serum (Sigma, USA); concanavalin A (Pharmacia, Sweden); L-glutamine, Hepes (Flow, USA); penicillin and streptomycin (Gibco, USA); agarose, sucrose, BSA, EDTA, EGTA, Tris, phenylmethylsulfonyl fluoride (PMSF), and sodium azide (NaN_3) (Serva, Germany). Other chemicals were chemically pure or especially pure grade.

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Immunorphin was synthesized as described previously [5] using activated esters. The peptide was purified by HPLC on a Zorbax ODS column (4 × 150 mm, 5 μm) using a linear gradient of water acetonitrile (95%) in 0.2% TCA (10-25%, 20 min) at a flow rate of 1 ml/min. The content of the main substance was assessed by measuring the absorbance at 220 nm. The molecular mass of immunorphin was determined by mass spectrometry using a Vision 2000 spectrometer (Thermo Bioanalysis, UK).

Mononuclear cells were separated from the blood of healthy donors as described in [6]. The suspension of lymphocytes was divided into adherent and non-adherent subsets on a nylon wool column [7]. The non-adherent fraction was eluted with RPMI-1640 medium supplemented with 5% heat-inactivated fetal calf serum. This fraction contained T lymphocytes with small amounts of monocytes (less than 1%) and B lymphocytes.

The effect of the peptides on T lymphocyte blast transformation *in vitro* was assayed as in [8]: each well of a 96-well microtiter plate contained 200 μl cell suspension (10^6 cells/ml), pre-incubated for 1 h with one of the peptides tested, and 50 μl concanavalin A (Con A) (0.5 μg/ml). The following samples served as controls: without Con A and peptide; with peptide, without Con A; with Con A, without peptide. The cultures in three replicates were used for each experimental point. The reaction mixture was incubated for 72 h at 37°C in 5% CO_2. After 60 h each well was pulsed with 0.5 μCi [methyl-^{3}H]thymidine. When incubation was terminated the reaction mixture from each well was filtered separately and rinsed with 100-fold excess of physiological solution. Radioactivity on filters was measured in a 1211 Minibeta liquid scintillation counter (LKB, Sweden). The reaction medium was RPMI 1640 supplemented with glutamine (2 mM), streptomycin and penicillin (100 μg/ml each), Hepes (20 mM), and heat-inactivated (56°C, 30 min) fetal calf serum (5% v/v).

Immunorphin (10 μg) was labeled using Na^{125}I (1 mCi) and Iodogen as described in [9]. The iodinated peptide was purified by gel filtration on Sephadex G-10 (0.9 × 10 cm column, 50 mM phosphate buffer, pH 7.4, 5 ml/h). The volume corresponding to the labeled peptide was determined in control experiments using the unlabeled one. Radioactivity was counted using Mini-Gamma Counter (LKB). Fractions with the maximum radioactivity at the positions corresponding to those of the unlabeled peptide peak in control experiments were pooled, and the total as well as the specific radioactivity of the preparation were determined. The purity of the iodinated peptide was tested by thin-layer chromatography on aluminum oxide glass with *n*-butanol–acetic acid–water (4 : 1 : 1 v/v) solvent system, followed by autoradiography. The specific activity of ^{125}I-labeled immunorphin was 232 Ci/mmol.

The binding of ^{125}I-labeled immunorphin to T lymphocytes was measured as follows: 10^6 cells per tube in 1 ml RPMI-1640 medium containing 10 mM Hepes, 20 mM NaN_3 and PMSF (0.6 g/liter), pH 7.4, were mixed with the labeled peptide (10^{-10}-10^{-7} M, each concentration point in triplicate) and incubated at 4°C for 40 min. The reaction mixture was then filtered through Whatman GF/A fiberglass filters. Filters were rinsed three times with 5 ml volumes of ice-cold 0.15 M NaCl supplemented with 10 mM Hepes, pH 7.4. Radioactivity was counted using the Mini-Gamma counter. Nonspecific binding of the labeled peptide was measured in the presence of 10^{-4} M of the unlabeled peptide (1000-fold excess). The results were analyzed according to [10].

To test the inhibitory effect of immunorphin, naloxone, and [Met^5]enkephalin on the specific binding of ^{125}I-labeled β-endorphin, T lymphocytes (10^6 cells/ml) were incubated with the labeled β-endorphin (1 nM) and unlabeled ligands at various concentrations (10^{-10}-10^{-6} M, three replicates for each concentration point) as described earlier. The inhibition constant (K_i) was calculated according to the equation [11]:

$$K_i = [I]_{50}/(1 + [L]/K_d),$$

where [L] is a molar concentration of ^{125}I-labeled β-endorphin, K_d is the equilibrium dissociation constant of ^{125}I-labeled β-endorphin–receptor complex, and $[I]_{50}$ is the concentration of unlabeled ligand causing 50% inhibition of the labeled β-endorphin specific binding. $[I]_{50}$ was estimated graphically from the inhibition curve. The K_d value was determined as described earlier.

Statistical significance was analyzed using Microsoft Excel MS 2000 electronic tables system. Data are presented as the means ± SEM of three independent experiments.

RESULTS

Effect of immunorphin, β-endorphin, and [Met^5]enkephalin on Con A-induced blast transformation of human T lymphocytes *in vitro*. Figure 1 shows that T lymphocyte pre-incubation with immunorphin (*1*) and β-endorphin (*2*) for 1 h results in significant enhancement of cell proliferative response. Plots *1* and *2* indicate that in the concentration range 10^{-11}-10^{-7} M both peptides equally activate T cell proliferation, being the most active at 10^{-10} M. [Met^5]enkephalin at doses ranging from 10^{-12} to 10^{-6} M did not influence the level of [methyl-^{3}H]thymidine incorporation in T lymphocytes (plot *3*). Naloxone at concentration of 10^{-6} M had no effect on T lymphocyte proliferation: in its presence [methyl-^{3}H]thymidine incorporation in the cells was no different from the control (Fig. 2). Naloxone had no influence on the ability of β-endorphin (Fig. 2a) and

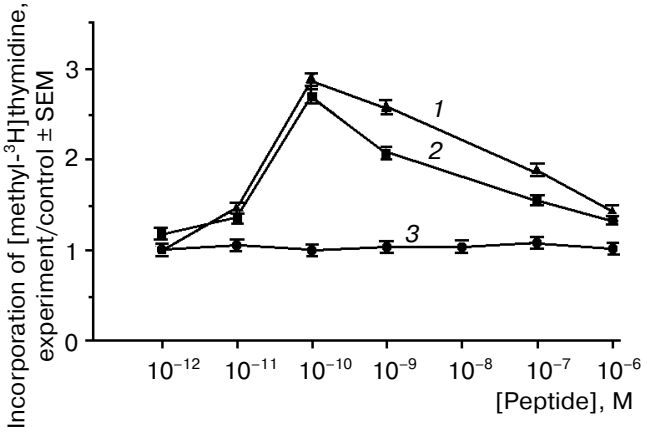

Fig. 1. Influence of immunorphin (*1*), β-endorphin (*2*), and [Met^5]enkephalin (*3*) on Con A-induced blast transformation of T lymphocytes from donor's blood *in vitro*.

immunorphin (Fig. 2b) to enhance T lymphocyte proliferative response as well.

From the results obtained it can be deduced that: first, β-endorphin and immunorphin stimulate, whereas [Met^5]enkephalin and naloxone have no effect on Con A-induced proliferation of T lymphocytes; second, the effect of β-endorphin and immunorphin on T lymphocyte proliferation is independent of the presence of naloxone.

Inhibition of ^{125}I-labeled β-endorphin specific binding to human T lymphocytes by immunorphin, naloxone, and [Met^5]enkephalin. In a previous study we have shown that there is one type of naloxone-insensitive receptors for β-endorphin on human T lymphocytes; $K_d = 0.25 \pm 0.03$ nM [3]. To characterize the specificity of these receptors, unlabeled immunorphin and [Met^5]enkephalin, in addition to naloxone, were tested as potential competitors. Figure 3 shows inhibition curves obtained during these experiments. It is evident that naloxone and [Met^5]enkephalin in the concentration range 10^{-10}-10^{-7} M did not inhibit ^{125}I-labeled β-endorphin binding to T lymphocyte receptors, that is, the receptors found were insensitive to these ligands. At the same time immunorphin actively displaced ^{125}I-labeled β-endorphin from the complex with the receptor: $K_i = 0.6 \pm 0.1$ nM.

Binding of ^{125}I-labeled immunorphin to human T lymphocytes. Studies on receptor binding of ^{125}I-labeled immunorphin to human T lymphocytes revealed that these cells are able to bind the peptide specifically. Figure 4a shows binding isotherm indicating that the peptide binding is saturable. Scatchard plots characterizing ^{125}I-labeled immunorphin specific binding to T lymphocytes in the absence (curve *1*) ($K_d = 7.0 \pm 0.3$ nM), and in the presence of 1 μM naloxone (curve *2*) ($K_d = 7.4 \pm 0.2$ nM) are presented in Fig. 4b. It is apparent that naloxone at this particular concentration does not exert substantial influence on the kinetics of labeled immunorphin binding to the receptor: the Scatchard plot shift in the presence of naloxone was within the limits of experimental error.

Thus, under these experimental conditions, one type of receptors on T lymphocytes was detected, that can bind with high affinity β-endorphin and immunorphin, but not [Met^5]enkephalin and the antagonist of opioid receptors naloxone.

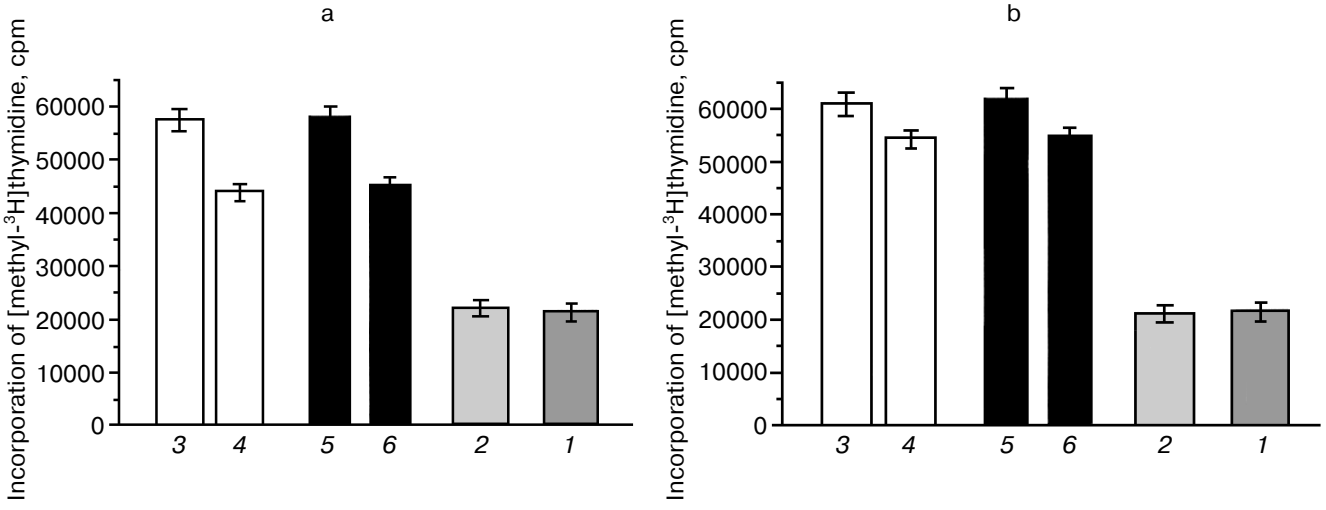

Fig. 2. Effect of naloxone on the ability of β-endorphin (a) and immunorphin (b) to stimulate T lymphocyte blast transformation *in vitro*: *1*, *2*) levels of [methyl-^{3}H]thymidine incorporation in cells in the absence (*1*) and in the presence (*2*) of 10^{-6} M naloxone; *3*, *4*) levels of cell proliferation in the presence of β-endorphin (a) and immunorphin (b) at concentrations of 10^{-10} (*3*) and 10^{-9} M (*4*); *5*, *6*) levels of cell proliferation in the presence of β-endorphin (a) and immunorphin (b) at concentrations of 10^{-10} (*5*) and 10^{-9} M (*6*), and 10^{-6} M naloxone.

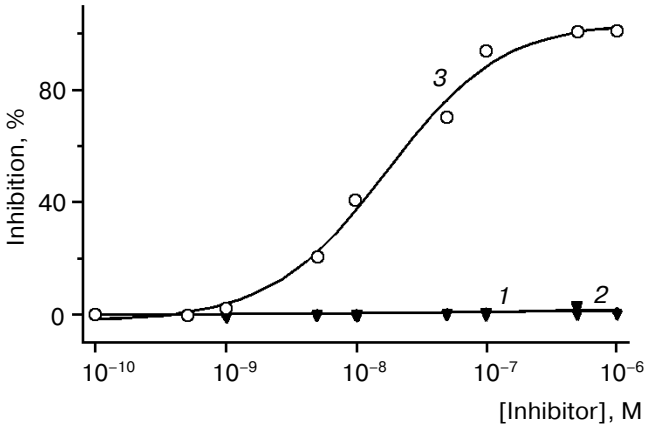

Fig. 3. Inhibition of ^{125}I-labeled β-endorphin (1 nM) specific binding to T lymphocytes from human blood by naloxone (*1*), [Met^5]enkephalin (*2*), and immunorphin (*3*).

DISCUSSION

Several groups of investigators have virtually simultaneously discovered that β-endorphin can influence T lymphocyte proliferation *in vitro* (both stimulating and inhibiting effects were reported) [12-14]. To elucidate the cause of such contradictory data, the effect of five opioid peptides (α-, β-, γ-endorphins, [Met^5]- and [Leu^5]enkephalins) on Con A-induced proliferation of rat spleen T cells was studied [15]. It turned out that none of these peptides constantly present in the cultivation medium of splenocytes has an effect on cell proliferation. At the same time, pre-incubation of T cells with β-endorphin (but not with other peptides) for 30 min resulted in dose-dependent proliferation increase of 50-100%. The presence of naloxone did not inhibit the stimulatory effect of β-endorphin. This suggested that the effect of β-endorphin on T cell proliferation was not mediated by naloxone-sensitive opioid receptors. It was also shown that β-endorphin (or α-endorphin) constantly present in the cultivation medium of T cells pre-incubated with β-endorphin, completely cancelled stimulatory effect of the latter. The authors speculated that, in the absence of opioid peptides, only non-opioid receptors for β-endorphin on rat spleen T lymphocytes are accessible for binding. An addition of β-endorphin to the cultivation medium enhanced the receptor-mediated proliferation response. In circumstances where β-endorphin (or other opioid peptide) is present constantly in the cultivation medium, opioid receptors appear on T cells, and β-endorphin binding to these receptors inhibits its own stimulatory effect.

To prove the hypothesis that the β-endorphin molecule has two different sites, one for binding to opioid and the other to non-opioid receptors, the effect of synthetic β-endorphin fragments 6-31, 18-31, 24-31, 28-31, and 1-27 on T lymphocyte proliferation was studied [16]. The peptides were added to cultivation medium prior to mitogen stimulation, and the level of proliferation was determined after 72 h. It was found that the fragments 6-31 and 18-31 stimulated proliferation (the former being much more active than the latter), while the fragments 1-27, 24-31 and 28-31 were not active. Relying on these results, the authors suggested that the fragment 6-23 is important for β-endorphin action on T lymphocytes (and therefore for binding to naloxone-insensitive receptors mediating this action), with fragment 18-23 playing the key role in binding. At the same time, it has been shown that β-endorphin (18-23) increases interleukin-2 and interleukin-4 production by CD4$^+$ T cells.

On the whole, our results presented in this paper tend to support the data mentioned above. We have shown

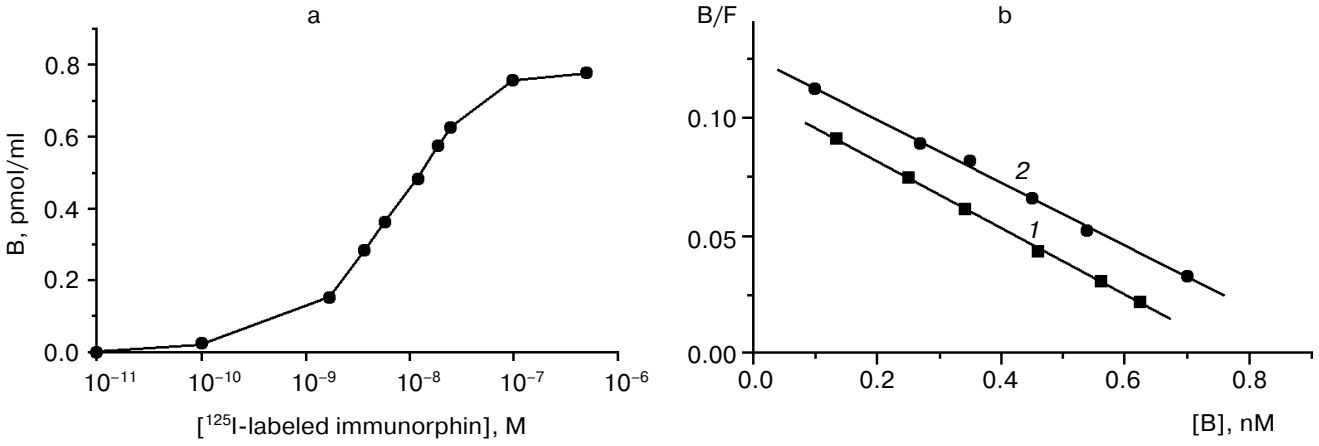

Fig. 4. ^{125}I-Labeled immunorphin binding to T lymphocytes from human blood: a) binding isotherm; b) Scatchard analysis of the specific binding in the presence (*2*) and in the absence (*1*) of 1 μM naloxone. B and F, molar concentrations of bound and free labeled immunorphin, respectively.

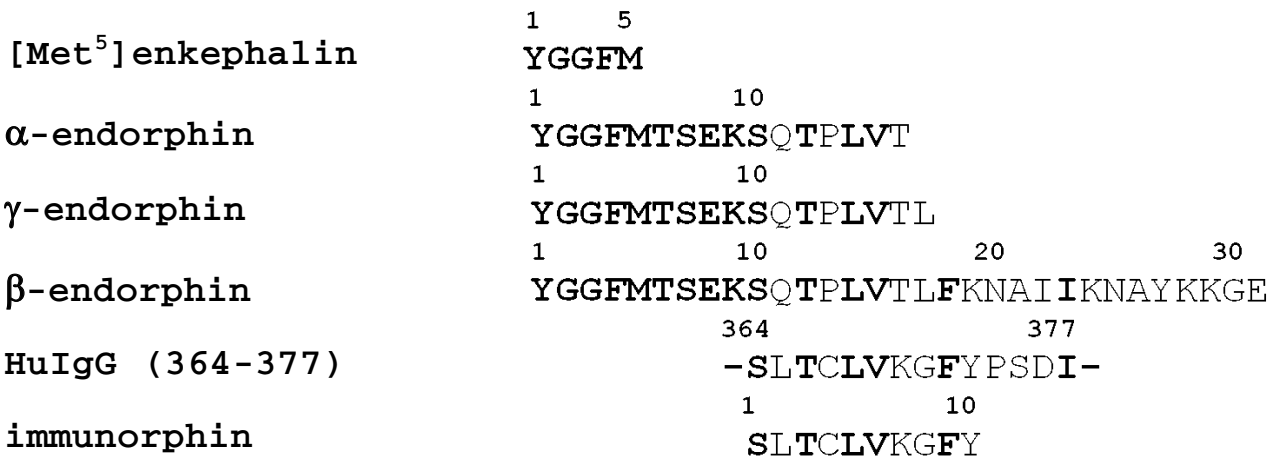

Fig. 5. Comparison of amino acid sequences of [Met^5]enkephalin, α-, γ-, β-endorphins, β-endorphin-like fragment from human IgG subclasses 1-4 heavy chain and immunorphin. Numbers of amino acid residues are specified by numerals. Coinciding residues are marked by bold letters.

that β-endorphin in the concentration range 10^{-11}-10^{-7} M significantly increased Con A-induced proliferation of T lymphocytes from donor blood, being the most active at the concentration of 10^{-10} M (Fig. 1, curve *2*). The effect of immunorphin on proliferating T lymphocytes was virtually the same: the increase in proliferation at doses ranging from 10^{-11} to 10^{-7} M with maximum at 10^{-10} M (Fig. 1, curve *1*). Naloxone (Fig. 2) and [Met^5]enkephalin (Fig. 1, curve *3*) had no influence on the level of T lymphocyte proliferative response. It should be pointed out that naloxone did not inhibit the activating effect of β-endorphin and immunorphin (Fig. 2).

The amino acid sequence of [Met^5]enkephalin is identical to that of β-endorphin fragment 1-5. It is precisely this sequence that affords binding of both peptides to opioid receptors. Immunorphin has 50% homology with β-endorphin fragment 10-19 (Fig. 5). The results presented in this paper indicate that immunorphin completely inhibits ^{125}I-labeled β-endorphin binding to T lymphocytes. Thus, we can assume that β-endorphin binding to the receptors on T lymphocytes is mediated by the fragment of the central part of the molecule, and not by the N-end pentapeptide.

Shahabi et al. have found and characterized naloxone-insensitive binding sites for β-endorphin on mouse splenocytes [17] and human U937 cell line [18]. The analysis of ^{125}I-labeled β-endorphin specific binding to mouse splenocytes revealed that these cells possess one type of receptors for the peptide with K_d = 4.1 nM. In studies of the specificity of the receptors found the following results have been obtained: N-Ac-β-endorphin completely (100%) inhibited ^{125}I-labeled β-endorphin binding to splenocytes; β-endorphin (6-31) and β-endorphin (28-31) were less active (10 and 1000 times, respectively); naloxone and β-endorphin (1-27) were not active at all. Mononuclear cells U937 were also shown to possess one type naloxone-insensitive receptors that bind β-endorphin and N-Ac-β-endorphin with equal affinity; β-endorphin (6-31) and β-endorphin (28-31) are bound with less affinity (5 and 100 times, respectively); β-endorphin (1-16), β-endorphin (1-27), morphine and endogenous opioid peptides are not bound at all. The K_d of ^{125}I-labeled β-endorphin–receptor complex was found to be 12 nM. Thus, in both cases in addition to β-endorphin, high affinity to naloxone-insensitive receptors was demonstrated for N-Ac-β-endorphin and β-endorphin (6-31) that are unable to bind to naloxone-sensitive opioid receptors. Consequently, β-endorphin fragment from the N-end of the molecule does not participate in binding to these receptors.

The data cited above together with the results presented in this paper suggest that, among all the natural opioid peptides, only β-endorphin molecule besides the N-end fragment necessary for interaction with opioid receptors, contains the sequence that provides its binding to fundamentally different receptors that are insensitive to naloxone and do not bind α-endorphin, γ-endorphin and enkephalins. It should be emphasized that, as immunorphin is not identical but only homologous (50%) to β-endorphin (10-19), one cannot say definitely that it is precisely this part of the hormone molecule that provides its binding to the receptors on T lymphocytes.

At present the nature of naloxone-insensitive receptor for β-endorphin is unknown. Hazum et al. have found naloxone-insensitive receptors for β-endorphin on human cultured lymphocytes and shown that these receptors do not bind β-lipotropin, adrenocorticotropic hormone, α-melanocyte-stimulating hormone, insulin, and glucagon [19]. Previously we have shown that ^{125}I-labeled β-endorphin and immunorphin binding to nalox-

Opioid receptor types

Receptor type	Agonist	Naloxone sensitivity	Presence on immunocompetent cells	References
μ		+	monocytes, granulocytes, lymphocytes, natural killers	[20, 21]
μ_1	morphine, enkephalins, β-endorphin			[20, 21]
				[20, 21]
μ_2	morphine, β-endorphin			[20, 22]
μ_3	only alkaloid opiates			
δ		+	monocytes, granulocytes, lymphocytes, natural killers	[20, 23]
δ_1	enkephalins, β-endorphin			[23, 25]
δ_2	[Met^5]enkephalin, β-endorphin			[23, 24]
κ		+	lymphocytes, natural killers, monocytes	[20, 26]
κ_1	ketocyclozocine, dinorphins, α-neoendorphin			[26-28]
				[26-29]
κ_2	bremazocine, β-endorphin, dinorphin A			[26]
κ_3	benzoyl-hydrazone			
ε	β-endorphin, ethylketocyclozocine, bremazocine	+	lymphocytes	[30, 31]
λ	4,5-epoxymorphines	+	not found	[32, 33]
ζ*	[Met^5]enkephalin	+	not found	[34]
σ		—	lymphocytes, granulocytes, natural killers	[35]
σ_1	(+)-N-allylnormetazocine, (+)-pentazocine and carbetapentane (high affinity); haloperidol, ditolylguanidine			[35-37]
σ_2	(+)-N-allylnormetazocine, (+)-pentazocine and carbetapentane (low affinity); haloperidol, ditolylguanidine			[35-37]

* This receptor type was found only on embryonic tissues. β-Endorphin binding to ζ-receptors was not studied.

one-insensitive receptors on mouse peritoneal macrophages did not interfere with human IgG H-chain and Fc fragment binding [3], that is, the receptors found are not the Fc receptors. We tried to answer the question if this receptor is one of the already known receptor types for β-endorphin. At present six types of opioid receptors are known—μ, δ, κ, ε, ζ, and λ. The main specificity characteristics of these receptors are shown in the table. It is evident that only μ, δ, κ, and ε binding sites bind β-endorphin with high affinity and are found on lymphocytes, but all of them are naloxone-sensitive. Therefore, we surmise that the receptors for β-endorphin that we have found on T lymphocytes do not belong to any known type of opioid receptors. The table also includes naloxone-insensitive σ receptors classified as non-opioid ones. The majority of synthetic ligands interacting with σ receptors, bind well to various opioid receptor types. For instance, (+)-N-allylnormetazocine is δ- and κ-agonist and μ-antagonist; haloperidol acts as μ- and δ-agonist, etc. [38]. Until recent times, it was believed that σ recep-

tors do not bind endogenous opioid peptides. However, recently Wollemann et al. [39] in studies of endogenous opioid heptapeptide Tyr-Gly-Gly-Phe-Met-Arg-Phe (MERF) interaction with membranes of frog and guinea pig brain revealed that tritium-labeled peptide binds to both opioid (κ_2 and δ) and naloxone-insensitive non-opioid receptors. The latter constitute a heterogeneous population consisting of σ_2-like receptors and σ-ligand-insensitive non-opioid sites, in which [^{3}H]MERF is displaced only by endogenous opioid peptides, such as β-endorphin, [Met^5]enkephalin and dinorphine (1-13). These results suggest that in the brain there are non-opioid receptors of presently unknown nature. In contrast to σ receptors, they bind β-endorphin and a number of other opioid peptides.

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