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β-Endorphin: Characterization of binding sites specific for the human hormone in human glioblastoma SF126 cells

(camel β-endorphin/synthetic analogs/tritiated β-endorphin)

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ABSTRACT **The established human glioblastoma cell line SF126 was found to bind tritiated human β-endorphin (β_h-EP) in a saturable fashion. From displacement studies, the ED_{50} was estimated to be about 2.5 nM. The K_d was estimated as 1.9×10^{-9} M and Scatchard analysis showed a biphasic pattern with a predominant low-affinity component. Binding reached a maximum at about 90 min at 22°C and was instantaneously reversible. Tritiated [D-Ala^2,D-Leu^5]enkephalin and tritiated dihydromorphine did not bind to the cells. Sodium at a concentration of 150 mM decreased the specific binding by 80%. The interaction with the cellular binding site appeared to be mediated by the COOH-terminal segment of β_h-EP, as β_h-EP-(6–31) retained a high potency for displacing tritiated β_h-EP, and β_h-EP-(1–27) has no activity. Camel β-EP was only about 1% as active as the human hormone.**

β-Endorphin (β-EP) (1, 2), an opioid peptide derived from proopiomelanocortin, has been characterized in membrane preparations from brains of rat (3–5), mouse (6), rabbit (7), and neuroblastoma–glioma hybrid NG108-15 cells (8, 9). The nonopioid COOH-terminal segment of β-EP was found to mediate actions of β-EP on human complement (10) and to displace tritiated human β-EP (β_h-EP) binding in neuroblastoma N18TG2 cells (11). Human brain tumors were recently shown to contain β_h-EP immunoreactivity (12). Since NG108-15 cells not only possess opiate receptors but also secrete opioid peptides (13), we decided to investigate a human glioblastoma cell line for the β_h-EP binding, using tritiated β_h-EP as the primary ligand.

MATERIALS AND METHODS

β_h-EP (14), camel β-EP (β_c-EP) (15), ostrich β-EP (β_{os}-EP) (16), turkey β-EP (β_{tu}-EP) (17), β_h-N^{α}-AcEP (14), β_h-EP-(6–31) (18), β_h-EP-(2–31) (18), β_h-EP-(21–31) (18), β_h-EP-(28–31) (18), β_h-EP-(1–27) (19), β_h-[Gly^{31}]EP (20), β_h-[Gln^8,Gly^{31}]EP-Gly-Gly-NH_2 (21) were synthetic products. Tritiated β_h-EP (β_h-[1,27-*tyrosyl*-$^3H_2$]EP, 100 Ci/mmol; 1 Ci = 37 GBq) was prepared as described (22). Tritiated [D-Ala^1, D-Leu^5]enkephalin and tritiated dihydromorphine were purchased from New England Nuclear.

Human glioblastoma cell line (SF126) at passage 16 was a gift of Mark Rosenblum of the Nafziger Laboratory of Brain Tumor Research Center at the University of California, San Francisco. Passages 17 to 30 were used in this study. Cells were maintained in Dulbecco's modified Eagle Medium with Earle's balanced salt solutions supplemented with 20% fetal calf serum and garamycin (50 μg/l) in a humidified (10% CO_2) atmosphere. Corning T75 tissue culture flasks were used throughout the study. For the binding assay, 8–10 flasks were used per 200 ml of binding buffer (25 mM Tris·HCl, pH 7.4/0.1% bovine serum albumin/0.01% bacitracin/10% sucrose). After the culture medium was aspirated and replaced by Ca^{2+}/Mg^{2+}-free phosphate-buffered saline containing 0.04% EDTA, the cultures were kept in the incubator for another 15 min. Then the cells were removed with mechanical agitation, and the suspension then was centrifuged at 800 rpm for 10 min in a desk-top centrifuge (500 × *g*). The pellet was resuspended by repeated trituration and then added to 200 ml of binding buffer. This usually resulted in a concentration of 400,000 cells per ml. Two milliliters of cell suspension was incubated in Falcon 2052 plastic tubes with 50 μl of cold peptide; then 60,000 cpm of tritiated β_h-EP was added and left at room temperature for 70–80 min with occasional resuspensions. The incubated cells were then filtered through myelin basic protein precoated glass fiber filters (Whatman GF/B) and counted after a 10-hr incubation in Hydrofluor on a Packard liquid scintillation counter with 33% efficiency. Specific binding was defined as the difference of radioactivity bound (cpm) in the presence and absence of a 100-fold excess of unlabeled peptide.

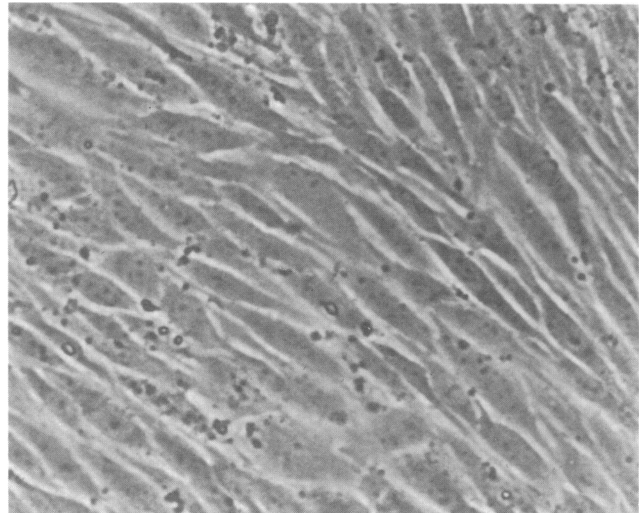

FIG. 1. Photomicrograph of SF126 glioblastoma cells in culture at the point of use in binding experiments. (×150).

Data analysis was performed with an HP85 desk-top computer. Displacement curves from competition binding assay were analyzed by using nonlinear least-squares analysis from published methods (23, 24).

RESULTS

When cells from confluent cultures (Fig. 1) were incubated with 1 pmol of tritiated β_h-EP per tube in the presence and absence of excess unlabeled ligand, the time course of binding of the tritiated β_h-EP as shown in Fig. 2 was obtained. The binding reached a maximum between 60 and 90 min.

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Abbreviations: β-EP, β-endorphin; β_h-EP, human β-EP; β_c-EP, camel β-EP; β_{os}-EP, ostrich β-EP; β_{tu}-EP, turkey β-EP.

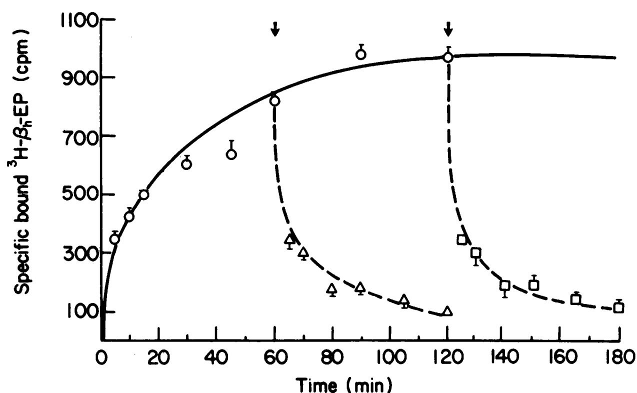

FIG. 2. Time course of tritiated β_h-EP binding. At times indicated with arrows, a 100-fold excess of unlabeled ligand was added to the tubes.

When an excess of cold peptide was added at 60 min or 120 min, a rapid dissociation with parallel curves at both points was observed. Fig. 3 presents the saturation curves for tritiated β_h-EP in the presence and absence of 150 mM NaCl as well as for tritiated dihydromorphine and tritiated [D-Ala[1],D-Leu[5]]enkephalin. Whereas the μ and δ opiate ligands exhibited no specific binding at all, tritiated β_h-EP saturated at about 10 pM with a K_d of 1.9×10^{-9} M. The specific binding was reduced by 80% in the presence of 150 mM NaCl. Scatchard analysis shows that the binding has two components, a small high-affinity and a major low-affinity component (Fig. 3 *Inset*). Based on DNA measurements and parallel cell counts of trypsinized cultures in a Coulter Counter, the number of binding sites was roughly estimated to about 300 sites per cell for the high-affinity component and 120,000 sites per cell for the low-affinity component.

To further characterize the binding site, different synthetic fragments of β_h-EP were used in the equilibrium binding assay to compete with tritiated β_h-EP. β_h-EP-(1–27) was inactive in displacing the radioligand but NH_2-terminally shortened fragments still retained significant potency, depending on chain length (Fig. 4, Table 1). Although β_h-EP-(28–31) had only very low activity, it could still fully displace tritiated β_h-EP. Modifications of the NH_2 terminus as in β_h-N^{α}-AcEP or the COOH terminus as in β_h-[Gly[31]]EP showed that residue position 31 is very important in the interaction with the binding site. Of particular interest is an omission analogue, β_h-EP-(1–5)-(16–31), which was as active as the intact molecule.

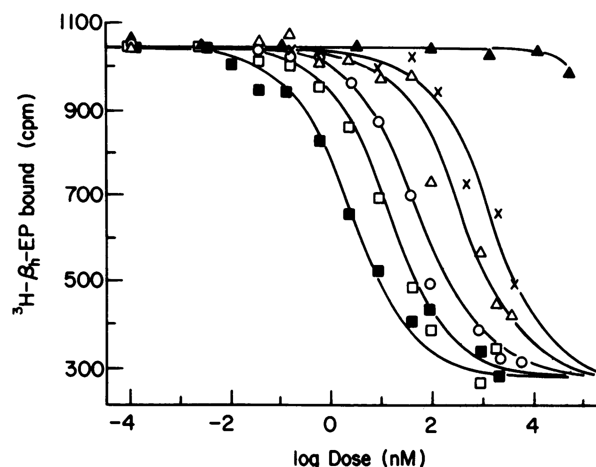

FIG. 4. Computer-assisted curve analysis from several competition binding experiments. Increasing concentrations of unlabeled ligands were allowed to compete with tritiated β_h-EP (1.5 mM) for 70 min. β_h-EP (■), β_h-EP-(6–31) (□), β_c-EP (○), β_h-EP-(21–31) (△), β_h-[Gln[8],Gly[31]]EP-Gly-Gly-NH_2 (×), and β_h-EP-(1–27) (▲).

Three synthetic naturally occurring homologs were included in this study. As summarized in Table 1, β_c-EP, β_{tu}-EP, and β_{os}-EP were approximately 100-fold less active in their displacing potency relative to the human hormone. When the conditioned media from SF126 cultures were assayed for β_h-EP immunoreactivity, no activity could be detected.

DISCUSSION

Glioblastomas are an important group of malignant brain tumors (25) and have been extensively characterized in their response to growth and differentiation factors (26) and susceptibility to chemotherapeutic agents (27). We show here a binding site specific for β_h-EP in an established human glioblastoma cell line (SF126). From the results obtained with various fragments of β_h-EP, it appears that the binding is mediated through the COOH terminus. Further evidence for the nonopioid character of this binding site comes from the lack of any specific binding for tritiated dihydromorphine or [D-Ala[1],D-Leu[5]]enkephalin. Furthermore, replacement of the COOH-terminal glutamic acid (position 31) with glycine or glutamine in the natural homologs indicates that the glutamic acid in position 31, which is unique to the human hormone, is crucial in the recognition of the binding site.

Two modes of inactivation have been described for the

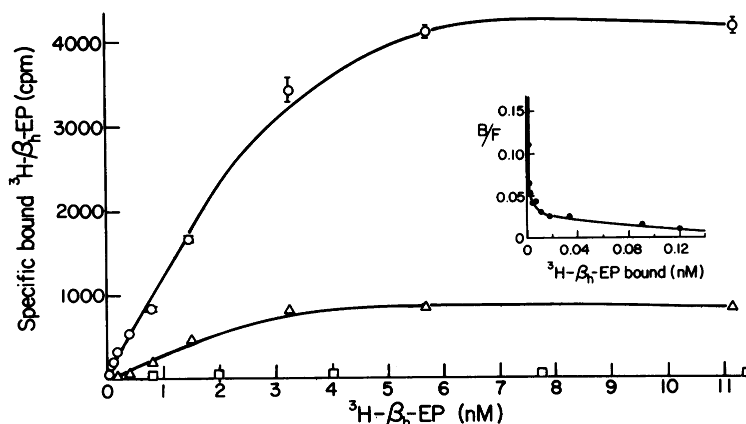

FIG. 3. Saturation analysis of tritiated β_h-EP binding to SF126 in the presence (△) and absence (○) of 150 mM NaCl. Specific binding was determined in the presence and absence of 100-fold excess of unlabeled ligand. Saturation analysis of tritiated [D-Ala[1],D-Leu[5]]enkephalin and tritiated dihydromorphine binding is also shown (□). (*Inset*) Scatchard analysis of the saturation data, using total tritiated β_h-EP bound for both axes.

Table 1. Relative potency of β-endorphin and analogs in displacing β_h-[1,27-*tyrosyl*-$^3H_2$]endorphin from human glioblastoma cells SF126

Synthetic peptide	IC_{50},* nM	Relative potency
β_h-EP	2.5 (1.4–4.6)	100
β_c-EP	197 (106–187)	1
β_{os}-EP	380 (280–600)	0.7
β_{tu}-EP	355 (250–500)	0.7
β_h-EP-(1–5)-(15–31)	1.9 (1.4–2.5)	132
β_h-EP-(2–31)	6.2 (4.2–9)	40
β_h-$N\alpha$-AcEP	8.7 (3.4–40)	29
β_h-EP-(6–31)	12.8 (11.7–14)	20
β_h-EP-(21–31)	331 (241–442)	0.8
β_h-EP-(28–31)	866 (407–1806)	0.3
β_h-[Gly^{31}]EP	1250†	0.2
β_h-[Gln^8,Gly^{31}]EP-Gly-Gly-NH_2	1100 (740–1680)	0.2
β_h-EP-(1–27)	>5000	<0.1

*95% confidence limit.
†Computer estimate.

opioid actions of β-EP, NH_2-terminal acetylation and COOH-terminal cleavage between tyrosine and lysine at positions 27 and 28, respectively (28). As shown in Table 1, NH_2-terminal acetylation has very little effect on tritiated β_h-EP binding on the glioblastoma cells, but the COOH-terminal cleavage [β_h-EP-(1–27)] totally inactivates the molecule. Recently, several reports presented evidence for actions of β-EP that are mediated through the COOH terminus (10, 11, 29). The results presented here have some similarities with the binding site found on human complement using ^{125}I-labeled β_h-EP as the primary ligand (29) with respect to its selectivity but have dissimilarities with respect to its binding characteristics. We found that β_c-EP has 1% of the displacing activity of β_h-EP. When ^{125}I-labeled β_h-EP was used as the primary radioligand under the described assay conditions in our hands, no specific binding in the glioblastoma cells could be obtained (data not shown). This may indicate either that there are different classes of nonopioid binding sites for β-EP or that the structural requirements for binding sites are different. From these studies, it is evident that β-EP may turn out to be a molecule with two different biologically active domains. For both binding activities, however, the entire amino acid sequence is required, which is in agreement with findings on its analgesic potency (30) and the tertiary structure (31) of the molecule.

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