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Biochemistry

β-Endorphin: Characteristics of binding sites in a neuroblastoma–glioma hybrid cell

(opiate receptor/opioid peptide/morphine/naloxone)

R. GLENN HAMMONDS, JR., PASCUAL FERRARA, AND CHOH HAO LI

Hormone Research Laboratory, University of California, San Francisco, California 94143

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ABSTRACT **Specific binding of human β-endorphin to NG108-15 cells is described; human β-[*Tyr*^{27}-$^{3}H_2$] endorphin was used as the ligand. The binding is time dependent and saturable; $K_d = 0.3$ nM and $k_a = 1.8 \times 10^8$ M^{-1} min^{-1}. Under the conditions optimal for β-endorphin binding, leucine-enkephalin has one-fourth to one-third as many binding sites as β-endorphin and its affinity is 7–10% that of β-endorphin. Monovalent and divalent cations potently inhibit binding. Trypsin, phospholipase A, and *N*-ethylmaleimide reduce the ability of NG108-15 cells to bind β-endorphin. β-Endorphin analogs are able to fully inhibit the binding of β-[*Tyr*^{27}-$^{3}H_2$]endorphin, although enkephalins, morphine, and naloxone inhibit only 50–80%.**

β-Endorphin (1) is the most analgesically active naturally occurring peptide when injected directly into the brain (2); its binding to rat brain membrane preparations has recently been described (3). Brain membrane preparations contain opiate receptor populations heterogeneous with respect to ligand preference (4, 5). Also, the interaction of ligands with brain membranes has not been linked to any biological action. The neuroblastoma–glioma hybrid cell line NG108-15 has high levels of opiate receptors (6) that are both functionally linked to adenylate cyclase (7, 8) and homogeneous with respect to ligand preference (9, 10), showing high affinity for enkephalin-like compounds. The ability of β-endorphin to act on the adenylate cyclase system in NG108-15 cells has been demonstrated (11). The affinity of β-endorphin has been measured indirectly by inhibition of enkephalin binding (8, 12). This communication describes the binding of human β-[*Tyr*^{27}-$^{3}H_2$]endorphin (^{3}H-β_h-EP) to NG108-15 cells and presents data comparing the displacement behavior of several opiate compounds toward this primary ligand.

MATERIALS AND METHODS

^{3}H-β-EP (50 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) and [*Tyr*^{1}-$^{3}H_2$]leucine-enkephalin (^{3}H-[Leu]EK) (50 Ci/mmol) were prepared as described (13, 14). β_h-Endorphin and [Leu]EK were synthesized by the solid-phase method as described (15). [^{3}H]Dihydromorphine (50 Ci/mol) was purchased from New England Nuclear. Bovine serum albumin was from Schwarz/Mann. Bacitracin was purchased from Aldrich. Trypsin and lima bean trypsin inhibitor were from Worthington. Phospholipase A, phospholipase C, phospholipase D, and Tris base were from Sigma. Myelin basic protein was isolated from bovine brains as described (16). Naloxone was purchased from Endo Laboratories (Garden City, NY) and morphine sulfate from Mallinckrodt. Tissue culture supplies were obtained from the Tissue Culture Center of this university.

NG108-15 cells were a gift from Sydney Udenfriend of Roche Institute for Molecular Biology. Cells were grown in Falcon T75 flasks in Dulbecco's modified Eagle's medium/10% fetal calf serum/penicillin at 100 units per ml/streptomycin at 100 units per ml/2 mM glutamine/0.1 mM hypoxanthine/1 μM aminopterin/16 μM thymidine at 37°C in a 90% air/10% CO_2 atmosphere. Cells were subcultured either with 0.5% trypsin/0.5% EDTA in phosphate-buffered saline or with Ca^{2+}-, Mg^{2+}-free phosphate-buffered saline plus glucose (D1 medium) as described (12). Cells from passages 19–35 were used.

The cell layer was washed with warm D1 medium and incubated with D1 medium for 5 min; the cells were removed from the flasks by gently rocking them. The cell suspension was centrifuged for 5 min at 500 × *g*, and the pellet was washed 2 times and suspended in assay buffer (290 mM sucrose/25 mM Tris·HCl, pH 7.4/bovine serum albumin at 1 mg per ml/bacitracin at 0.1 mg per ml). Cells were counted in a hemocytometer after the first resuspension in D1 medium. Binding was measured in suspensions of $0.2–1.0 \times 10^6$ cells per 2 ml in plastic tubes. Two sets of tubes, one with and one without a 100- to 400-fold excess of unlabeled ligand were preincubated for 5 min. Binding was initiated by addition of the labeled ligand, and the tubes were incubated at a constant temperature for the desired time period. The binding reaction was terminated by rapid vacuum filtration through myelin basic protein coated Whatman glass fiber filters (GF/B) (3). The filters were washed three times with 4 ml of cold (4°C) 50 mM Tris·HCl, pH 7.4/0.1% bovine serum albumin/0.1% Triton X-100 and transferred to vials containing 5 ml of PCS scintillation fluid (Amersham). The vials were capped, the mixtures were incubated overnight at room temperature, and radioactivity was measured by liquid scintillation spectrometry. Specific binding was considered to be the difference in radioactivity trapped on the filters in the presence and absence of excess unlabeled ligand.

^{3}H-β_h-EP was stored in 10% EtOH/5% HOAc at 4°C and diluted into buffer just prior to addition to the assay mixture. Recovery of labeled ^{3}H-β_h-EP at the end of the incubation was 70–80% of that added. The loss was most likely due to absorption by the assay tubes, as it varied among tubes of different composition. In experiments demonstrating saturation of binding, total ^{3}H-β_h-EP was measured by assaying an aliquot taken from each tube just prior to filtration.

RESULTS AND DISCUSSION

Scatchard analysis of replicate saturation binding experiments suggests a difference for β_h-EP and [Leu]EK in the maximum binding capacity (B_{max}) (Table 1). This difference was not abol-

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Abbreviations: β_h-EP and β_c-EP, human and camel β-endorphin, respectively; ^{3}H-β_h-EP, human β-[*Tyr*^{27}-$^{3}H_2$]endorphin; [Leu]EK, [Leu]enkephalin; ^{3}H-[Leu]EK, [*Tyr*^{1}-$^{3}H_2$]leucine-enkephalin; B_{max}, maximum binding capacity.

Table 1. K_ds and binding capacities of NG108-15 cells with ^{3}H-β_h-EP and ^{3}H-[Leu]EK

Temperature, °C	Ligand	K_d, nM	B_{max}, receptors × 10^5 per cell	n
24	^{3}H-β_h-EP	0.35 ± 0.06	4.0 ± 0.3	5
	^{3}H-[Leu]EK	4.80 ± 0.90	1.2 ± 0.2	5
0	^{3}H-β_h-EP	0.29 ± 0.05	2.0 ± 0.3	5
	^{3}H-[Leu]EK	4.70 ± 0.30	0.46 ± 0.05	3

Experiments at 24°C were incubated for 25 min prior to filtration; those at 0°C were incubated for 90 min.

ished by incubation at lower temperatures, although the value of B_{max} for both β-EP and [Leu]EK is lower at lower temperatures. As noted by Blume *et al.* (10), the B_{max} varies among cell preparations, but the proportion of β_h-EP sites to [Leu]EK sites remains in 3–4 range. The question of possible heterogeneity of β_h-EP binding was examined by combining data from several saturation experiments (Fig. 1.) Although there is considerably more scatter in the data for β_h-EP than in that for [Leu]EK, it is clear that a single affinity-class model is sufficient to characterize the interaction of β_h-EP with the cell. K_d for β-EP, calculated from these data, is 0.3 nM, and K_d for [Leu]EK is 5.0 nM. These compare well with the values reported for opiate receptors in NG108-15 (6, 12), but the B_{max} for [Leu]EK is considerably less.

The kinetics of binding of β_h-EP and [Leu]EK is shown in Fig. 2. The data are consistent with single k_a for β_h-EP and [Leu]EK, with a 20-fold difference in magnitude. The k_a of β_h-EP, 1.8×10^8 M^{-1} min^{-1}, is similar to that previously reported using rat brain membranes (3).

The kinetics of dissociation of β_h-EP from NG108-15 cells initiated by addition of excess unlabeled β_h-EP after a 20-min incubation with ^{3}H-β_h-EP at 24°C is best described as a double exponential decay process (data not shown). Nonlinear least-squares minimization yields estimates of the two decay constants and the relative proportion of bound material initially found in each pool: $k_{-1} = 0.007$ min^{-1}; $k_{-2} = 0.41$ min^{-1}; $a_1 = 0.42$; $a_2 = 0.58$.

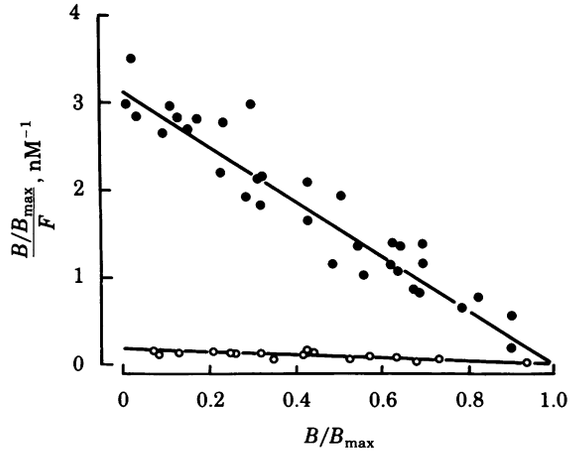

FIG. 1. Saturation analysis of binding of ^{3}H-β_h-EP (●) and ^{3}H-[Leu]EK (○) to NG108-15 cells (24°C, 20 min). To combine separate experiments performed at different total receptor concentrations, separate least-squares estimates of B_{max} were made and both axes were scaled by that quantity yielding a plot of B/B_{max} vs. $(B/B_{max})/F$ with −slope = y − intercept = $1/K_d$. The combined estimates are $K_d = 0.3$ nM for β_h-EP and $K_d = 5.0$ nM for [Leu]EK. The difference in B_{max} values of β_h-EP and [Leu]EK is not apparent in this figure.

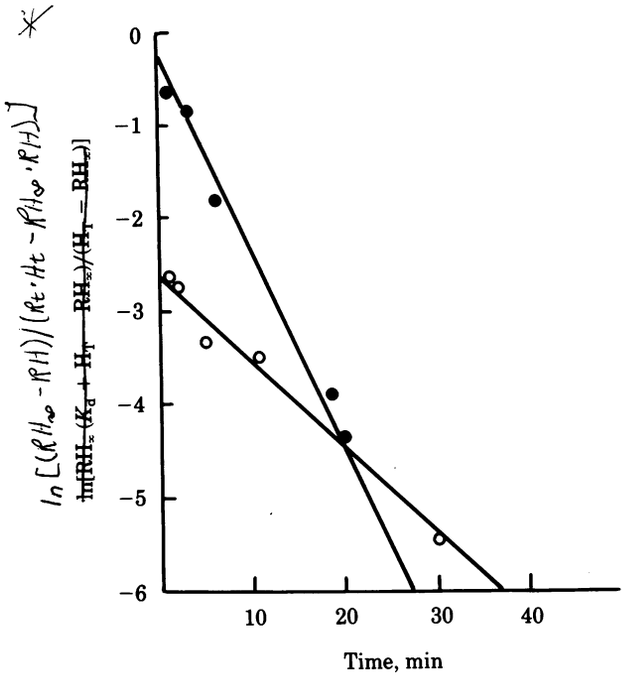

FIG. 2. Kinetics of association of β_h-EP and [Leu]EK with NG108-15 at 24°C. Cells were incubated with ^{3}H-[Leu]EK or ^{3}H-β_h-EP in the presence or absence of a 400-fold excess of unlabeled compound, and specific binding was determined at the times indicated after addition of the labeled compound. ●, 1 nM ^{3}H-β_h-EP, 1×10^5 cells per ml; ○, 5 nM ^{3}H-[Leu]EK, 2×10^5 cells per ml. The data were analyzed according to the equation given by Maelicke *et al.* (17). RH_∞, equilibrium concentration of bound hormone–receptor complex: H_T, total β-EP added. For β-EP, $k_a = 1.8 \times 10^8$ M^{-1} min^{-1}; for [Leu]EK $k_a = 9.1 \times 10^6$ M^{-1} min^{-1}.

The sensitivity of ^{3}H-β_h-EP binding to a variety of treatments is shown in Table 2, demonstrating the similarity of these receptor sites to those in the brain (18). *N*-Ethylmaleimide, trypsin, and phospholipase A all reduce binding, as do both monovalent and divalent cations. The reduction of β_h-EP binding by divalent cations in both brain (18) and NG108-15 cells may indicate a difference in the interactions of enkephalins and endorphins with opiate receptors; divalent cations enhance binding of enkephalins and opiate agonists in both brain and NG108-15 cells (19–23).

Although the ability of Mg^{2+} and Mn^{2+} to enhance binding

Table 2. Effect of various treatments on binding of ^{3}H-β_h-EP to NG108-15 cells

Treatment	Conditions	Binding remaining, %
None	Control	100
Na^+	50 mM	15
Mn^{2+}	2 mM	17
Mg^{2+}	2 mM	33
Trypsin	0.1 μg/ml	44
N-Ethylmaleimide	0.1 mM	50
Dithiothreitol	5 mM	99
Phospholipase A	1 μg/ml	41
Phospholipase C	10 μg/ml	95
Phospholipase D	10 μg/ml	130

Cells were preincubated for 30 min at 24°C in the absence of bovine serum albumin or bacitracin and then assayed for binding activity remaining. The reactions of trypsin and *N*-ethylmaleimide were stopped by adding equal concentrations of lima bean trypsin inhibitor or dithiothreitol.

* Correction: Proc. Natl. Acad. Sci. USA 78 (1981) - Vol. 78 (10)

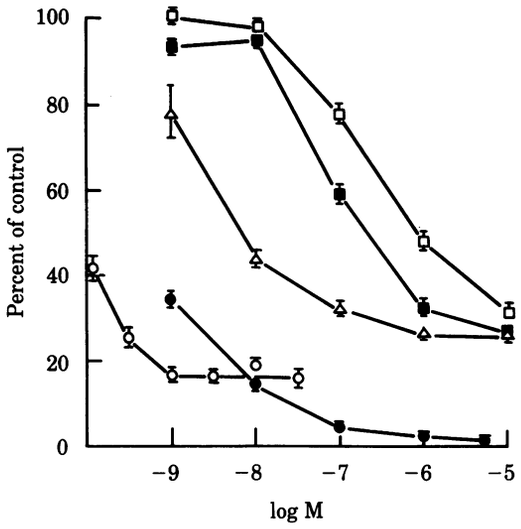

FIG. 3. Competitive inhibition of ^{3}H-β_h-EP binding to NG108-15 cells by morphine (□), naloxone (■), [Leu]EK (△), β_h-EP (●), and β_c-EP (○). The behavior of [Met]EK was identical to that of [Leu]EK (data not shown).

of enkephalins to NG108-15 cells has been reported previously (22), we also report their effect on β-EP binding.

Competitive inhibition of β_h-EP binding by a number of opiates shows a striking phenomenon: the inability of [Leu]EK, [Met]EK, morphine, or naloxone to fully displace specific β_h-EP binding (Fig. 3). The range of ligand concentrations was chosen to demonstrate this rather than to yield 50% potency estimates; however, it is clear that β_h-EP has substantially higher affinity for NG108-15 sites than [Leu]EK, naloxone, or morphine.

Several EP analogs have also been examined for their ability to inhibit binding of ^{3}H-β_h-EP (unpublished results). The behavior of two analogs, β_h-[Leu^5]endorphin (24) and β_c-EP (25), is reported here. The former is approximately equipotent with β_h-EP and capable of displacing all specific binding (data not shown), and β_c-EP displaces only 90% of the specific binding, yet is more potent than β_h-EP in displacing the remainder (see Fig. 3).

In contrast, the displacement of [^{3}H]dihydromorphine (Fig. 4*A*) and of ^{3}H-[Leu]EK (Fig. 4*B*) by naloxone, morphine, [Leu]EK, or β_h-EP does not show any nondisplaceable component. In each case, β_h-EP is more potent than [Leu]EK, in agreement with the data of Chang *et al.* (9) for experiments performed in a Krebs Ringer buffer containing both Na^+ and Mg^{2+} but not in agreement with those of Gerber *et al.* (13), who

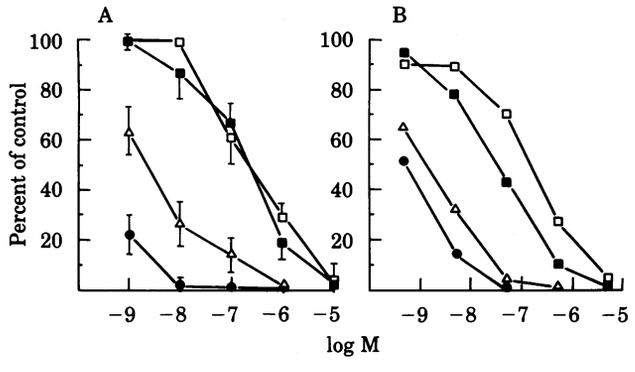

FIG. 4. Competitive inhibition of ^{3}H-dihydromorphine (*A*) and ^{3}H-[Leu]EK (*B*) to NG108-15 cells by morphine (□), naloxone (■), [Leu]EK (△), and β_h-EP (●).

used a Tris/sucrose buffer with Mg^{2+}. We have confirmed the observation of homogeneity of ligand preference in NG108-15 opiate receptors: [Leu]EK is a more potent displacer of [^{3}H]dihydromorphine than morphine or naloxone.

Optimal binding of ^{3}H-β_h-EP to NG108-15 cells is obtained by omitting both Na^+ and divalent cations from the incubation medium and including bovine and serum albumin and bacitracin to prevent adsorptive and degradative loss of the peptides. Under these conditions β_h-EP not only shows much higher affinity for binding than [Leu]EK but also binds to more sites. Although both β_h-EP and [Leu]EK appear to bind to a single affinity component, the difference in binding capacities, the multiple dissociation rates, and the inability of [Leu]EK, naloxone, morphine, and β_c-EP to fully displace bound ^{3}H-β_h-EP suggest a heterogeneous population of endorphin binding sites in the NG108-15 cell. It is possible that nonopiate sites for β_h-EP binding are present or that the conditions chosen for optimal binding of β_h-EP render a portion of the opiate receptors available only to β_h-EP. At any rate, the binding of ^{3}H-β_h-EP to NG108-15 cells provides a sensitive radioassay for opioid peptides.

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