Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 67–71, January 1986
Biochemistry

Characterization of solubilized human and rat brain β-endorphin–receptor complex

(opiate receptor/size-exclusion chromatography/[methionine]enkephalin/3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate)

DAIGA M. HELMESTE AND CHOH HAO LI

Laboratory of Molecular Endocrinology, University of California, San Francisco, CA 94143

Contributed by Choh Hao Li, September 3, 1985

ABSTRACT **Opioid receptors have been solubilized from human striatal and rat whole-brain membranes by use of 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS). Tritiated human β-endorphin (^{3}H-β_h-EP) binding revealed high-affinity competition by morphine, naloxone, and various β-EP analogues, suggesting predominantly μ-type binding. Lack of high-affinity competition by (±)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide methanesulfonate (U50-488, Upjohn) indicated that κ sites were not labeled by ^{3}H-β_h-EP under these conditions. Affinities were similar in both soluble and membrane preparations except for [Met]enkephalin, which appears to be rapidly degraded by the solubilized extract. Size differences between human and rat solubilized ^{3}H-β_h-EP–receptor complexes were revealed by exclusion chromatography.**

Over the past decade, pharmacological and physiological studies have suggested the presence of multiple opioid receptors. Whether opioid receptor sites are physically associated or represent distinct proteins remains unclear, with data available for both possibilities (1, 2). The answers to these questions have tremendous implications for the design of new opioid drugs and the understanding of drug action at the molecular level. Ultimately this requires information such as amino acid sequence, subunit structure, and function of reconstituted receptor proteins. For this purpose, we have been interested in solubilizing opioid receptors from rat whole brain and human striatum, using tritiated human β-endorphin (^{3}H-β_h-EP) as the label. Receptors were solubilized from brain membranes by using the zwitterionic detergent 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS). Previous studies from this laboratory have demonstrated displaceable binding only when the tissue was prelabeled with ^{3}H-β_h-EP before solubilization (3). Using a polyethylene glycol (PEG)-precipitation method, we now have found that the solubilized receptor from both rat and human tissues binds ^{3}H-β_h-EP in a displaceable manner. Drug affinities are similar to those seen in nonsolubilized membranes except that affinity for [5-methionine]enkephalin [Met]EK is much lower after solubilization.

MATERIALS AND METHODS

Materials. β_h-EP was prepared by solid-phase synthesis as described (4). ^{3}H-β_h-EP (50 Ci/mmol; 1 Ci = 37 GBq) was the product of catalytic reduction of a synthetic diiodotyrosine derivative of β_h-EP (5). CHAPS was synthesized as described by Hjelmeland (6). Bacitracin was from Aldrich; phenylmethylsulfonyl fluoride was from Calbiochem; PEG 6000 was from Koch-Light Laboratories, Bucks, England; dithiothreitol, Tris, aprotinin, and polyethylenimine (PEI) were from Sigma; morphine was from Mallinckrodt; naloxone was from Endo Laboratories (Garden City, NY); and (±)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide methanesulfonate hydrate (U50-488) was from Upjohn. All other chemicals were reagent grade.

Tissue Preparation. Male Sprague–Dawley rats (120–180 g; Simonsen Laboratories, Gilroy, CA) were decapitated, and the brains rapidly were removed and placed on ice. The whole brain (minus cerebellum) was homogenized in 50 mM Tris Cl buffer (pH 7.4) with a Brinkmann Polytron (setting 6) and then centrifuged (44,000 × *g*, 15 min) and resuspended in buffer. The centrifugation and resuspension were repeated twice. The final pellet was resuspended in buffer to make the final volume 10 ml per original whole brain (≈1.5 g of original wet weight). The tissue was stored at −70°C until use.

Human striatal tissue (caudate and/or putamen) was obtained from the Canadian Brain Tissue Bank (Banting Institute, Toronto) and the Multiple Sclerosis Human Neurospecimen Bank (Veterans Administration Wadsworth Medical Center, Los Angeles). The tissue was processed in the same way as for the rat, except that the centrifugation and resuspension steps was done only once. The final volume was 10 ml per gram of original wet weight.

Homogenates were diluted 1:10 with 50 mM Tris Cl, pH 7.4/0.1% bovine serum albumin/0.01% bacitracin for the membrane binding assay. Incubations were at 22°C for 1 hr (1.5 nM ^{3}H-β_h-EP; nonspecific binding measured in the presence of 1 μM β_h-EP) as described (7).

For solubilization, rat or human brain homogenates in 50 mM Tris Cl (pH 7.4) were centrifuged at 44,000 × *g* for 15 min. The pellet was resuspended (at the same concentration) in solubilization buffer [10 mM Tris Cl (pH 7.4)/10 mM CHAPS/5 mM dithiothreitol/0.1% bacitracin/0.1 mM phenylmethylsulfonyl fluoride/aprotinin (200 kallikrein-inhibitor units/ml)]. Samples were incubated on ice for 10 min before ultracentrifugation (105,000 × *g*, 1 hr). The clear supernatant was removed and adjusted to pH 5.6 with potassium acetate buffer (100 mM, pH 5.6). PEG 6000 was added to a final concentration of 10% (wt/vol). The receptor preparation then was centrifuged (44,000 × *g*, 10 min), and the pellet was washed in modified solubilization buffer (10 mM Tris Cl, pH 7.5/1 mM CHAPS/0.5 mM dithiothreitol). The preparation was centrifuged once more and resuspended in the same buffer (to a volume equal to that of the original homogenate). The suspended receptor preparation was used directly for binding assays (1 hr, 22°C; 1.5 nM ^{3}H-β_h-EP, alone or with various concentrations of nonradioactive competitor). The incubation mixture was filtered on PEI (0.5%)-pretreated Whatman GF/B glass fiber filters. It was found in

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: β_h-EP, human β-endorphin; ^{3}H-β_h-EP, [$^3H_2$-Tyr^{27}]-β_h-EP; [Met]EK, [5-methionine]enkephalin; U50-488, (±)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide methanesulfonate hydrate; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.

Table 1. Inhibition of specific binding of ^{3}H-β_h-EP to rat brain membrane preparations

Compound	Membrane		Soluble	
	IC_{50},* nM	Hill coefficient	IC_{50},* nM	Hill coefficient
β_h-EP-(1–31)	3.5 ± 0.2	1.03	1.8 ± 0.2	0.93
β_h-EP-(1–27)	6.0 ± 0.7	0.86	4.3 ± 0.7	1.05
β_h-EP-(6–31)	(No inhibition at 1 μM)			
Ostrich β-EP	1.4 ± 0.1	0.92	1.1 ± 0.3	0.52
Morphine	2.8 ± 0.4	0.66	1.8 ± 0.4	0.50
[Met]EK	30.1 ± 4.9	0.79	387 ± 167	0.79
Naloxone	8.0 ± 2.4	0.80	6.4 ± 1.5	0.71
U50-488	2394 ± 353	0.79	ND†	

*Mean ± SEM (n = 3–9 independent experiments).
†Not done.

initial experiments that addition of PEG before filtration did not increase specific radioactivity trapped by the filter. Therefore this step was omitted in subsequent assays. Filters were washed rapidly with 10 ml of 10 mM Tris Cl (pH 7.4) containing 10% PEG and were assayed for radioactivity by liquid scintillation counting (33% counting efficiency).

Molecular-Exclusion Chromatography. Sizes of prelabeled ^{3}H-β_h-EP receptor complexes were estimated by gel-filtration chromatography. Brain homogenates (rat or human) were incubated with 1.5 nM (rat) or 3.2 nM (human) ^{3}H-β_h-EP for 1 hr at 22°C (as described above for membrane binding assays). A parallel set of tubes contained 1 μM β_h-EP to define specific binding. After incubation, samples were centrifuged in an Eppendorf microcentrifuge (at 4°C) and the pellets were resuspended in solubilization buffer. Solubilization and ultracentrifugation were performed as described above. A 3-ml sample of the supernatant was applied to a Sepharose 6B column (2 × 45.5 cm) at 4°C and eluted with 0.32 M sucrose/1 mM CHAPS/10 mM Tris Cl, pH 7.4; 3-ml fractions were collected, and 0.8-ml aliquots of each fraction were taken for liquid scintillation counting.

Protein concentrations were measured by the Bradford method (8), with bovine gamma globulin as the standard and with Bio-Rad Protein Assay reagents.

High Performance Liquid Chromatography. HPLC analysis of [Met]EK was performed on a Vydac C_{18} column (cat. no. 218TP104, 10-μm particle size, 250 × 4.6 mm). Elution was with a linear gradient (10–50%, 60 min) of 2-propanol in 0.1% trifluoroacetic acid. [Met]EK (10 μM) plus ^{3}H-labeled [Met]EK (3.5 × 10^6 cpm/ml; 34.4 Ci/mmol, New England Nuclear) was incubated with CHAPS-solubilized rat brain extract for 1 hr at 22°C. Samples were then lyophilized overnight, reconstituted in 0.1% trifluoroacetic acid, and injected for HPLC. Eluate fractions (0.5 ml, 1 min per fraction) were monitored for radioactivity and/or absorbance (210 nm).

RESULTS

Rat Brain ^{3}H-β_h-EP-Labeled Receptors. As shown in Table 1, β_h-EP and its analogues compete for membrane binding with affinities similar to those previously reported (9–11). High-affinity competition by morphine and naloxone are consistent with a predominantly "μ-like" profile (12). However, since the Hill coefficients for these drugs were significantly less than one, it is likely that multiple sites are labeled by ^{3}H-β_h-EP under these assay conditions. U50-488 did not compete significantly with ^{3}H-β_h-EP in the nanomolar range. The solubilized receptor extract showed affinities similar to those seen in membrane preparations except for [Met]EK, whose affinity decreased by a factor of 13.

Scatchard analysis of ^{3}H-β_h-EP binding (0.005–6 nM) to the solubilized rat preparation revealed a site with K_d = 1.8 ± 0.5 nM and B_{max} = 31 ± 7 fmol/mg of protein. This represented a yield of 21% compared to rat brain membranes (K_d = 0.9 ± 0.1; B_{max} = 146 ± 16). A super-high-affinity, low-capacity component also appeared to be present, in both membrane and soluble preparations, but was not quantitated (Fig. 1).

HPLC analysis showed that [Met]EK is extensively degraded in the CHAPS-solubilized extract. Tritiated [Met]EK preincubated with solubilized rat brain extract (1 hr, 22°C)

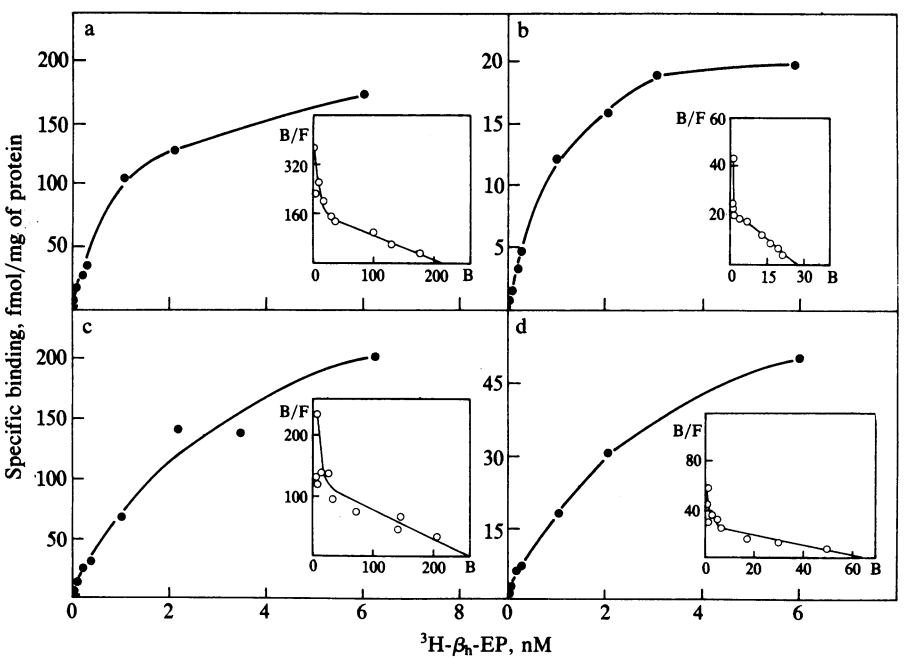

FIG. 1. Representative saturation experiments for rat whole brain membrane (a) and soluble (b) preparations and human caudate-plus-putamen membrane (c) and soluble (d) preparations. (*Insets*) Scatchard analyses: ordinate (B/F) = (fmol/mg of protein)/nM; abscissa (B) = fmol/mg of protein.

Table 2. Inhibition of specific binding of ^{3}H-β_h-EP to human striatal membrane preparations

Compound	Membrane		Soluble	
	IC_{50},* nM	Hill coefficient	IC_{50},* nM	Hill coefficient
β_h-EP-(1–31)	1.6 ± 0.3	0.99	2.3 ± 0.4	0.81
β_h-EP-(6–31)	(No displacement at 1 μM)			
[Met]EK	22 ± 4	0.71	>1000	
Morphine	24 ± 1	0.59	ND	
Naloxone	17 ± 4	0.67	ND	
U50-488	2112 ± 144	0.87	ND	

ND, not done.
*Mean ± SEM (n = 3–5 independent experiments).

was eluted as three distinct peaks with retention times (t_R) of 8, 16, and 21 min. These peaks represented 97, 1, and 2% of total radioactivity, respectively. The first peak (t_R = 8 min) corresponded to tyrosine, and the second and third peaks with the methionine sulfoxide form of [Met]EK and with unoxidized [Met]EK, respectively. This contrasts with the elution profile of tritiated [Met]EK standard (not incubated with biological sample), which showed peaks of radioactivity only at 21 min (83% of total radioactivity) and 16 min (17% of total radioactivity).

Human Striatal ^{3}H-β_h-EP-Labeled Receptors. Caudate and/or putamen samples of 1 g wet weight or more were obtained from 24 different individuals (11 females, 13 males). Of the 24 different individuals studied, 9 had died of cancer, 4 of heart disease, 4 of multiple sclerosis, and 7 of accident or other causes. Since the membrane binding in the four multiple sclerosis patients was not significantly different compared to the other samples, these tissues were included in all subsequent correlations. Death-to-freezing interval ranged from 3.5 to 24 hr (mean ± SEM, 13.8 ± 1.2 hr). Mean age (±SEM) was 59.3 ± 3.7 yr (range, 17–89); mean female age was 61.4 ± 4.5 yr; mean male age was 57.6 ± 2.1 yr. Mean caudate specific binding (1.5 nM ^{3}H-β_h-EP) was 40.1 ± 3.9 fmol/mg of protein (n = 13); mean putamen specific binding was 52.1 ± 4.5 (n = 18). There was no correlation between binding (caudate and putamen) and death-to-freezing interval (r = −0.193, n = 37 separate tissues), between age and putamen binding (r = −0.234, n = 18), or between age and caudate binding (r = −0.422, n = 13). In the 13 pairs of samples (caudate and putamen) from the same individuals, there was a significant correlation between membrane binding in the caudate and in the putamen [t = 2.05, $P > 0.05$ (two-tailed paired t-test, for hypothesis that a difference exists)].

Table 2 shows IC_{50} values generated from competition experiments in membrane and soluble preparations. For the competition experiments, equal amounts of caudate and putamen were pooled, rather than analyzed separately. In the membrane assays, β_h-EP, [Met]EK, morphine, and naloxone all competed with high affinity in the nanomolar range. However, since Hill coefficients for [Met]EK, morphine, and naloxone curves were significantly less than one, it is likely that multiple binding components are present. U50-488 did not compete for any portion of binding sites when present at nanomolar concentrations but inhibited binding only in the micromolar range.

The solubilized receptor preparation retained high affinity for β_h-EP. β_h-EP-(6–31) showed no displacement at 1 μM in either the membrane or the soluble receptor preparations. Affinity for [Met]EK was lost in the soluble preparation.

Scatchard analysis of ^{3}H-β_h-EP binding (0.005–6 nM) to the solubilized human caudate-plus-putamen preparation revealed a site with K_d = 1.6 ± 0.3 nM and B_{max} = 48 ± 5 fmol/mg of protein. This represented a yield of 24% compared to human membrane preparations (K_d = 2.6 ± 0.3; B_{max} = 199 ± 21). As for the rat, a super-high-affinity, very-low-capacity component to the Scatchard plot was observed but not quantitated (Fig. 1).

Molecular-Exclusion Chromatography. Prelabeled ^{3}H-β_h-EP–rat receptor complex was eluted as two broad peaks (Fig. 2). The first occurred at a position corresponding to a protein species of Stokes radius 64 Å (n = 3) by comparison with protein standards of known sizes. The second peak appeared at the position expected for free ^{3}H-β_h-EP. Only the first peak represented displaceable binding as defined with 1 μM β_h-EP.

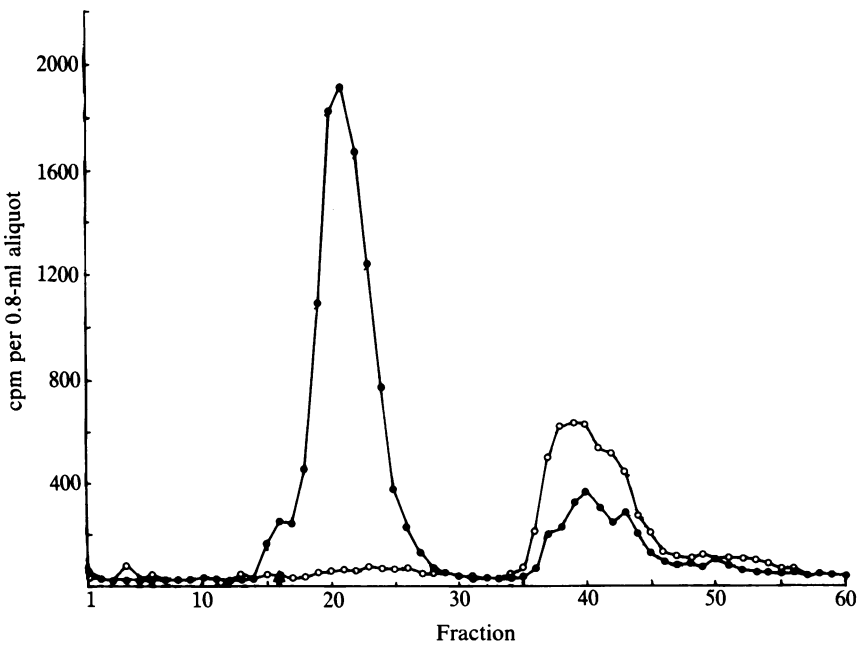

FIG. 2. Sepharose 6B chromatography of ^{3}H-β_h-EP–receptor complex solubilized from rat whole brain membranes prelabeled by incubation with 1.5 nM ^{3}H-β_h-EP in the absence (●) or the presence (○) of 1 μM nonradioactive β_h-EP. Three-milliliter fractions were collected; fraction 16 (small arrow) corresponded to the void volume.

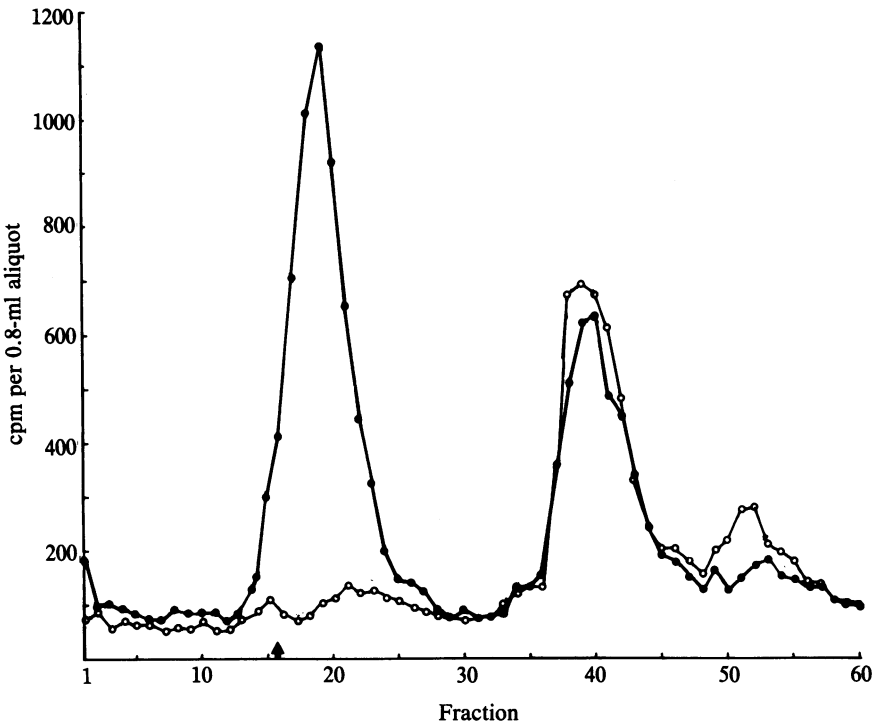

FIG. 3. Sepharose 6B chromatography of ^{3}H-β_h-EP–receptor complex solubilized from human putamen membranes prelabeled by incubation with 3.2 nM ^{3}H-β_h-EP in the absence (●) or the presence (○) of 1 μM nonradioactive β_h-EP. Fractions were 3 ml each; fraction 16 (small arrow) corresponded to the void volume.

Prelabeled ^{3}H-β_h-EP–receptor complexes from human caudate and putamen were eluted at positions corresponding to Stokes radii of 80 and 72 Å, respectively (Fig. 3). These assays were performed on five pairs of samples where each pair represented caudate and putamen obtained from the same individual. The difference in Stokes radii between caudate and putamen was statistically significant (sign test, $P < 0.05$). There was no obvious correlation between Stokes radius and death-to-freezing interval (data not shown).

DISCUSSION

Our data show that ^{3}H-β_h-EP can be used to label CHAPS-solubilized opioid receptors from both rat and human brain. Under these experimental conditions, drug affinities were very close to those seen in membrane binding experiments. This suggests that the opioid–receptor complex was solubilized in an active form. The binding profile is "μ-like," as shown by high-affinity competition for morphine, β-EP, and naloxone. However, multiple components may exist in both membrane and soluble preparations, as suggested by Hill coefficients less than one for some drugs. This is reasonable when one considers that β_h-EP is known to have high affinity for μ, δ, and ε sites (12). At nanomolar concentrations, U50-488 did not compete for any portion of ^{3}H-β_h-EP binding. This suggests that possible κ components of binding are negligible at this concentration of ^{3}H-β_h-EP (1.5 nM). The low-affinity competition by U50-488 (micromolar range) is consistent with a predominantly μ-type binding profile (13). One-micromolar β_h-EP-(6–31) did not compete for binding in either rat whole brain or human striatal membranes. This suggests either that the "nonopiate" binding site that exhibits high affinity for the carboxyl-terminal portion of β_h-EP (14–16) is not present in these tissues or that negligible binding to this site occurs at a ^{3}H-β_h-EP concentration of 1.5 nM.

[Met]EK was the only opioid peptide to lose affinity in the soluble-receptor assay. HPLC analysis indicated that [Met]EK was converted to a form where 97% of the radioactivity corresponded to free tyrosine. This suggests extensive aminopeptidase activity. An aminopeptidase has been reported to be solubilized from membrane fractions of rat brain (17), making it likely that it is present in our soluble extract as well. β_h-EP and its analogues did not appear to show preferential degradation in the soluble assay, as shown by IC_{50} values not significantly different from those in membrane assays. This is consistent with the greater metabolic stability of β-EP compared to [Met]EK (18). Other investigators have shown that δ-selective ligands ([D-Ala^2, D-Leu^5]enkephalin) that are resistant to metabolic inactivation exhibit much lower affinity for solubilized brain opiate receptors than for membrane-bound receptors unless assayed in the presence of manganese chloride, sodium chloride, and GTP (19). In this case, low-affinity binding is more likely due to conformational changes in the solubilized extract, rather than metabolic inactivation.

Scatchard analysis of rat and human tissues revealed a major component [K_d = 0.9 nM (rat) or 2.6 nM (human); B_{max} = 146 (rat) or 199 (human) fmol/mg of protein]. For the rat, K_d and B_{max} are similar to previously described values (7, 10, 11, 20, 21). Yields of 21–24% are close to those previously described for [^{3}H]etorphine–receptor complexes (19) and suggest that active ^{3}H-β_h-EP–receptor complexes are not preferentially solubilized in this assay protocol. Further comparison of affinity and density differences for ^{3}H-β_h-EP binding in rat whole brain and human striatum must await computer dissection of the binding data and/or the use of drugs to selectively block receptor subtypes. Molecular-exclusion chromatography (Sepharose 6B) of prelabeled, CHAPS-solubilized extract gave a Stokes radius of 64 Å for rat whole brain samples. This agrees well with values reported elsewhere (19). CHAPS-solubilized extract from human caudate and putamen gave Stokes radii of 80 and 72 Å, respectively. The larger size in the caudate is probably not due to major glycosylation differences, since preliminary data (not shown) indicate that CHAPS-solubilized extract from either human caudate or human putamen is retained on wheat germ agglutinin-agarose and is eluted specifically with

N-acetylglucosamine. Whether the larger size of the caudate receptor represents a different receptor subpopulation or coelution of accessory binding proteins (e.g., guanine nucleotide-binding regulatory protein) remains for future investigation.

We thank Dr. C. Bergeron of the Canadian Brain Tissue bank and Dr. W. W. Tourtellotte of the Multiple Sclerosis Human Neurospecimen Bank (Los Angeles) for generous donation of human brain samples. We thank D. Chung and R. Hammonds for helpful discussions. This work was supported in part by grants from the National Institute on Drug Abuse (DA03434) and the National Institutes of Health (GM2907). D.M.H. is a recipient of a fellowship from the Medical Research Council of Canada.

1. Morley, J. S. (1983) *Trends Pharmacol. Sci.* **4,** 370–371.
2. Loh, H. H., Lee, N. M. & Smith, A. P. (1983) *Trends Pharmacol. Sci.* **4,** 371–372.
3. Hammonds, R. G., Nicolas, P. & Li, C. H. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 6494–6496.
4. Li, C. H., Yamashiro, D., Tseng, L. F. & Loh, H. H. (1977) *J. Med. Chem.* **20,** 325–328.
5. Houghten, R. A. & Li, C. H. (1978) *Int. J. Pept. Protein Res.* **12,** 325–326.
6. Hjelmeland, L. M. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 6368–6370.
7. Nicholas, P., Hammonds, R. G., Gomez, S. & Li, C. H. (1982) *Arch. Biochem. Biophys.* **217,** 80–86.
8. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254.
9. Yamashiro, D. & Li, C. H. (1984) in *The Peptides*, eds. Udenfriend, S. & Meienhofer, J. (Academic, New York), Vol. 6, pp. 191–217.
10. Ferrara, P., Houghten, R. & Li, C. H. (1979) *Biochem. Biophys. Res. Commun.* **89,** 786–792.
11. Houghten, R. A., Johnson, N. & Pasternak, G. W. (1984) *J. Neurosci.* **4,** 2460–2465.
12. Paterson, S. J., Robson, L. E. & Kosterlitz, H. W. (1984) in *The Peptides*, eds. Udenfriend, S. & Meienhofer, J. (Academic, New York), Vol. 6, pp. 147–189.
13. James, I. F. & Goldstein, A. (1984) *Mol. Pharmacol.* **25,** 337–342.
14. Westphal, M. & Li, C. H. (1984) *Biochem. Biophys. Res. Commun.* **120,** 873–878.
15. Westphal, M. & Li, C. H. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 2921–2923.
16. Schweigerer, L., Bhakdi, S. & Teschemacher, H. (1982) *Nature (London)* **296,** 572–574.
17. Gorenstein, C. & Snyder, S. H. (1979) *Life Sci.* **25,** 2065–2070.
18. Marks, N., Grynbaum, A. & Neidle, A. (1977) *Biochem. Biophys. Res. Commun.* **74,** 1552–1559.
19. Chow, T. & Zukin, R. S. (1983) *Mol. Pharmacol.* **24,** 203–212.
20. Law, P. Y., Loh, H. H. & Li, C. H. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 5455–5459.
21. Akil, H., Hewlett, W. A., Barchas, J. D. & Li, C. H. (1980) *Eur. J. Pharmacol.* **64,** 1–8.