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Covalent Labeling of μ Opioid Binding Site by [^{3}H]β-Funaltrexamine

LEE-YUAN LIU-CHEN[1] and CATHERINE A. PHILLIPS[2]

Central Research and Development Department, E. I. Du Pont De Nemours and Co., Wilmington, Delaware 19898 (L.-Y. L.-C.) and Central Research and Development Department, E. I. Du Pont de Nemours and Co., Glenolden, Pennsylvania 19036 (C.A.P.)

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SUMMARY

[^{3}H]β-funaltrexamine ([^{3}H]β-FNA) bound irreversibly to bovine striatal membranes. Naloxone inhibited the irreversible binding of 5 nM [^{3}H]β-FNA in a dose-dependent manner and maximally inhibited this binding at approximately 1 μM. Thus, the specific irreversible binding of [^{3}H]β-FNA to opioid receptors was defined as that which could be inhibited by 1 μM naloxone. This specific irreversible binding of [^{3}H]β-FNA was characterized. Exclusion of Na^+ from the incubation medium reduced the specific binding of [^{3}H]β-FNA, and Na^+ could be substituted by Li^+ but not by K^+, Cs^+, Mg^{2+}, or guanylylimidodiphosphate. The specific irreversible binding was saturable, time- and temperature-dependent, and was linearly related to tissue mass. Several drugs were used to characterize this specific binding. Levorphanol was 1000 times more potent as an inhibitor than dextrorphan. μ Opioid ligands (sufentanil and morphine) were much better inhibitors than δ (ICI174,864) or κ (U50,488H) ligands. The potency of [D-Ala^2, D-Leu^5]enkephalin (DADLE) was between those of sufentanil and ICI174,864. These results demonstrated that under appropriate conditions [^{3}H]β-FNA specifically and irreversibly bound to the μ opioid binding site. Membrane preparations labeled with [^{3}H]β-FNA in the presence or absence of 1 μM naloxone or β-FNA were subjected to polyacrylamide gel electrophoresis under denaturing and reducing conditions. Fluorograms showed that [^{3}H]β-FNA specifically bound to a protein (most likely the μ opioid binding site) that migrated as a band with a molecular weight range of 68,000–97,000. Such electrophoretic behavior indicates that it is likely to be a glycoprotein. The glycoprotein nature was confirmed by its adsorption onto a wheat germ lectin-Sepharose column after solubilization and subsequent elution by *N*-acetyl-D-glucosamine. In this lectin column eluate, the μ opioid receptor was the only protein band labeled by [^{3}H]β-FNA in the total binding preparation, and no labeled protein was observed in the nonspecific binding preparation. When the wheat germ lectin column eluate of the total binding was treated with peptide:*N*-glycosidase F, the broad labeled band of M_r 68,000–97,000 became a sharp band of M_r 57,000 with high radioactivity and a faintly labeled band of M_r 49,000.

The existence of multiple opioid receptor types in the brain has been established by use of highly radioactively labeled ligands in binding sites. i.e., μ and δ receptors (1–3) and κ receptor (4–6). In addition, benzomorphan sites were shown to be recognition sites for the putative ε receptor (7–9). Questions arise: are these receptors different proteins derived from different genes? Or are they the same protein with different post-translational modification, such as glycosylation, sulfation, methylation, etc.? Or are the differences simply due to different membrane environments? Although it has been proposed that μ and δ receptors are interconvertible molecules (10) of allosteric relationship (11, 12), of the same molecular weight (13, 14), or distinctly different (15, 16), definitive answers to these questions must await the elucidation of complete amino acid sequences of these receptors. Purification and subsequent cloning of receptors is one approach to attain this goal.

Purification of the μ opioid receptor has been reported (14, 17–23). However, to date there is no consensus on its molecular weight(s). Nor has there been information available on its amino acid composition or partial amino acid sequence. One major problem encountered during purification of active opioid receptors is the loss or reduction of binding activities during solubilization and purification processes. There is also no tissue source that is particularly rich in μ opioid receptors, like *Torpedo* electrical fish for nicotinic acetylcholine receptors. One approach to circumvent the problem of unstable binding activities is to label the receptor specifically with an irreversible

[1] Present address: Department of Pharmacology, Temple University School of Medicine, MRB 321, 3420 N. Broad St., Philadelphia, PA 19140.
[2] Present address: Department of Internal Medicine, Texas Tech Health Science Center and Veteran's Administration Medical Center, 1400 Wallace Blvd, Amarillo, TX 79106.

ABBREVIATIONS: β-FNA, β-funaltrexamine; DADLE, [D-Ala^2, D-Leu^5]enkephalin; TEL, Tris-HCl buffer containing 1 mM EGTA and 10 μM leupeptin, the molar concentration refers to that of Tris-HCl buffer; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio] 1-propanesulfonate; NP-40, Nonidet P-40; EGTA, [ethylenebis-(oxyethylenenitrilo)]tetracetic acid; WGL, wheat germ lectin; $NaDodSO_4$, sodium dodecyl sulfate; Gpp(NH)p guanylylimidodiphosphate; PMSF, phenylmethyl sulfonyl flouride; PAGE, polyacrylamide gel electrophoresis.

ligand, which has been radioactively labeled, and proceed with purification by measuring radioactivity. By taking such an approach, Simonds *et al.* (24) purified the δ opioid receptor to homogeneity from NG108-15 cells. In this report, we examined the feasibility of using [^{3}H]β-FNA to specifically and irreversibly label the μ opioid receptor.

β-FNA, fumarate methyl ester of naltrexamine (Fig. 1), was synthesized by Portoghese *et al.* (25) and was found to possess reversible κ agonist and irreversible μ antagonist activities in *in vitro* preparations of the guinea pig ileum (25–28) and mouse vas deferens (27–29) and in *in vivo* antinociceptive tests (30). In binding studies using brain membranes, β-FNA inhibited binding of opioid ligands in a dose-dependent manner with K_i values of 2.2, 14, and 78 nM for μ, κ, and δ receptors, respectively (31). Some binding studies have also been performed on β-FNA-treated tissues with conflicting results. Pretreatment of brain membranes with high concentrations of β-FNA (1 μM) reduced μ binding with or without changing δ or κ binding (32–34). In myenteric plexus-longitudinal muscle of the guinea pig, pretreatment with 0.1 or 1 μM β-FNA did not alter μ, κ, or δ binding, whereas in guinea pig brain membranes β-FNA pretreatment reduced μ and δ binding when concentrations were greater than 250 and 62.5 nM, respectively, without changing κ binding (27). Recently it was demonstrated that incubation of brain membranes with low concentrations (1–5 nM) of β-FNA followed by extensive washing reduced μ opioid receptor binding without affecting δ or κ receptor binding (31). The reduction of μ binding by β-FNA appeared to be due to a decrease in B_{max} without a change in K_d, and this effect was more pronounced in the presence of 100 mM NaCl (31). In addition, while opioids added before [^{3}H]β-FNA could completely inhibit the binding of [^{3}H]β-FNA, opioids added after 1-hr incubation with [^{3}H]β-FNA could maximally displace only 70% of [^{3}H]β-FNA binding, even at high concentrations and after a long incubation time, suggesting irreversible binding of [^{3}H]β-FNA to opioid receptors (31).

In this study, we performed an assay to determine the irreversible binding of [^{3}H]β-FNA to bovine striatal membranes, defined conditions under which labeling of opioid receptors could be differentiated, and characterized this specific irreversible binding of [^{3}H]β-FNA.

Experimental Procedures

Materials. [^{3}H]β-FNA (26.7 Ci/mmol), ^{3}H at C_6, was custom-synthesized by Du Pont New England Nuclear Research Products (Boston, MA). β-FNA was synthesized by Dr. Chen-Yu Cheng of Du Pont. The following unlabeled drugs were used: naloxone from Du Pont, levorphanol and dextrophan from Hoffman-La Roche (Nutley, NJ), sufentanil from Janssen Pharmaceutical (Piscataway, NJ), morphine from Merck, Sharp, and Dohme (Rahway, NJ), DADLE from Peninsula Laboratories (San Carlos, CA), and U50,488H from Upjohn (Kalamazoo, MI). ICI174,864 was a gift from Dr. Alan Cowan, Department of Pharmacology, Temple University School of Medicine. $NaDodSO_4$, dithiothreitol, mercaptoethanol, urea, and chemicals for gel electrophoresis were purchased from Bio-Rad (Richmond, CA), prestained protein standards from Bethesda Research Laboratories, (Bethesda, MD), and 2,5-diphenyloxazole from Kodak (Rochester, NY). Gpp(NH)p was obtained from Boeringer Mannheim Biochemicals (Indianapolis, IN); leupeptin, soybean trypsin inhibitor, bacitracin, PMSF, *N*-acetyl-D-glucosamine, Lubrol PX, CHAPS, NP-40, and 1,10-phenanthroline monohydrate from Sigma (St. Louis, MO); EGTA from Fisher (Fair Lawn, NJ); WGL-Sepharose 6MB from Pharmacia Fine Chemicals (Piscataway, NJ); peptide:*N*-glycosidase F (*N*-glycanase) from Genzyme (Boston, MA). Other commonly used chemicals were from Sigma or Fisher.

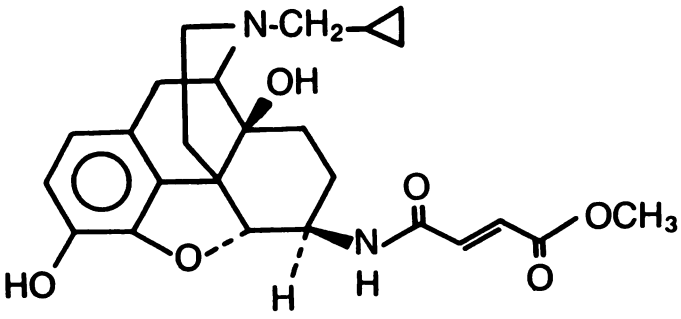

Fig. 1. Structure of β-funaltrexamine (25).

Calf striatal membrane preparation. Fresh calf brains were obtained from a slaughter house shortly after calves were killed. Striatal membranes were prepared at 4° according to a modification of the method of Chang and Cuatrecasas (2). Striata were cleaned and homogenized with approximately 10 vol 0.32 M sucrose in 10 mM Tris-HCl buffer (pH 7.5) containing 10 mM glucose, 1 mM EGTA, and 10 μM leupeptin, using Teflon pestle-glass tissue grinders. The homogenate was centrifuged at 920 × *g* for 10 min, the supernatant retained, and the pellet brought up with approximately 5 vol of the above sucrose solution and centrifuged again. The combined supernatant was centrifuged at 40,000 × *g* for 20 min, the crude membrane fraction (P_2) was brought up with approximately 5 vol (original weight) of 5 mM Tris-HCl buffer containing 1 mM EGTA and 10 μM leupeptin (pH 7.5), let swell for 25 min at 0°, homogenized to disrupt synaptosomes, and centrifuged at 40,000 × *g* for 20 min. The light brown pellet, which contained mostly mitochondria, was discarded, and the top white loose pellet was swollen, disrupted, and centrifuged again. The pellet was brought up in 5 vol 50 mM Tris-HCl buffer containing 1 mM EGTA and 10 μM leupeptin (TEL buffer, pH 7.5) and aliquots of membrane stored at −70° C until use.

Assay for irreversible binding of [^{3}H]β-FNA. Pretreatment of membranes (approximately 1 mg protein/ml) with drugs and incubation with [^{3}H]β-FNA were performed at 37° in 50 mM TEL buffer containing 100 mM NaCl, unless indicated otherwise. After incubation, membrane was denatured and precipitated with 10% trichloroacetic acid, centrifuged at 2,000 × *g* for 20 min at 4° and the supernatant aspirated. The precipitate was dissolved with 0.3 ml of 0.5% $NaDodSO_4$ by sonication, precipitated again with 1.5 ml 10% trichloroacetic acid, chilled on ice for at least 10 min, and filtered with Millipore HA 0.45-μm filters under reduced pressure. The filters were washed twice with 5 ml of ice-cold 10% trichloroacetic acid and the radioactivities on the filter determined by liquid scintillation counting. Experiments were conducted in duplicate or triplicate with variations less than 5% of the mean unless indicated otherwise.

Labeling of bovine striatal membranes with [^{3}H]β-FNA. Membranes were suspended in 50 mM TEL buffer containing 100 mM NaCl (1–2 mg protein/ml) and incubated with 5 nM [^{3}H]β-FNA in the presence and absence of 1 μM naloxone

or β-FNA for nonspecific and total labeling, respectively, at 37° for 60–90 min. Membranes were then washed once or twice by centrifugation and resuspension.

Solubilization of labeled membranes and WGL affinity chromatography. Both solubilization and column chromatography were conducted at 4° by a protocol similar to that of Simonds et al. (24). Labeled membranes were resuspended with 10 mM TEL buffer containing 150 mM NaCl (2 mg of protein/ml), and CHAPS and Lubrol PX were added to the final concentrations of 20 mM and 1%, respectively, and the mixture was stirred for 1 hr and centrifuged at 100,000 × g for 1 hr. The clear supernatant was filtered with Millipore GV filter (0.22 μm) and applied at 1 ml/min onto a WGL-Sepharose 6MB column, which has been equilibrated with 20 bed volumes of 5 mM CHAPS and 0.25% Lubrol PX in 10 mM TEL buffer containing 150 mM NaCl (buffer A), and the column was washed with the same buffer until 280 nm absorbance reached baseline level. The retained glycoproteins were then eluted by 0.25 M *N*-acetyl-D-glucosamine in buffer A, 1-ml fractions collected, aliquot of each fraction counted for radioactivity, and the peak fractions pooled.

Treatment of WGL column eluate with peptide:*N*-glycosidase F (*N*-glycanase). The eluate of the total binding preparation was precipitated with 60% cold acetone at 0° C for 30 min and centrifuged in a Beckman microfuge at setting 12 for 10 min at 4°. The pellet was resuspended in 0.2 M sodium phosphate buffer (pH 8.6) containing 1.5% NP-40 and 10 mM 1,10-phenanthroline (a zinc-dependent protease inhibitor). The samples were incubated with N-glycanase at 37° for 18 hr (35, 36).

PAGE and fluorography. Labeled membrane preparations were solubilized in Laemmli sample buffer, boiled for 5 min, and separated on a 10–20% gradient NaDodSO$_4$ PAGE (37). The WGL column eluates of both total and nonspecific labeling and the *N*-glycanase-treated WGL column eluate of total labeling were treated with an equal volume of 7% NaDodSO$_4$, 100 mM dithiothreitol, 5% 2-mercaptoethanol, and 5 M urea, incubated at room temperature for 30 min, and separated on a 10% NaDodSO$_4$-PAGE. The gels were fixed in Fairbank's stain without Commassie blue (38), prepared for fluorography by impregnating with 2,5-diphenyloxazole by the method of Bonner and Laskey (39). The gels were exposed to gas-hypersensitized Kodak X-Omat AR (XAR) film at −70° C by methods described in detail previously (40, 41). Briefly, before fluorographic exposure XAR film was baked at 65° C in an atmosphere of 8% hydrogen and 92% nitrogen for 5 hr. Film treated in this manner is 8–10 fold more sensitive in a fluorographic exposure than untreated film.

Protein determination. Protein contents of membrane preparations were determined by the method of Bradford (42) using bovine immunoglobulin G as the standard, or the method of Lowry *et al.* (43) using bovine serum albumin as the standard. Solubilized membrane proteins were estimated by a modified Lowry method (44).

Results

Control for irreversible binding assay. Three types of controls were performed: 1) binding of membranes with 5 nM [^{3}H]naloxone (reversible binding); 2) binding of boiled (denatured) membranes with 5 nM [^{3}H]β-FNA (no active opioid receptors); 3) binding with 5 nM [^{3}H]β-FNA in the absence of membrane (filter control). The irreversible binding was performed in the presence and absence of 1 μM naloxone. In the first two cases, the tissue was treated with 10% trichloroacetic acid, 0.5% NaDodSO$_4$, and 10% trichloroacetic acid, and filtered as described in Experimental Procedures. In the third case, the solution of [^{3}H]β-FNA with or without naloxone was filtered without additional treatment to examine its binding to filter. In all three cases, there were no differences in radioactivities bound to the filter whether naloxone was present or not. These results indicate that when the binding is reversible (the first control) or when there is no active opioid receptors (the second control), no irreversible binding to opioid receptors was seen with the assay method described. In addition, the filters used did not have naloxone-displaceable [^{3}H]β-FNA binding. Thus, the validity of the assay was established, measuring only the truly irreversible binding.

Inhibition of irreversible binding of [^{3}H]β-FNA by naloxone and definition of specific irreversible binding. Since it is the *irreversible* binding of [^{3}H]β-FNA that is examined, the binding kinetics are different from those of reversible ligands. The IC$_{50}$ of naloxone in inhibiting binding does not reflect its absolute affinity for the receptor. Naloxone inhibited the irreversible binding of 5 nM [^{3}H]β-FNA in the presence of 100 mM NaCl at 37° C in a dose-dependent manner (Fig. 2). The inhibition reached a plateau at approximately 1 μM naloxone. Further increase in naloxone concentration did not appreciably increase the inhibition. The specific irreversible binding to opioid receptors was thus defined as the binding of [^{3}H]β-FNA that could be inhibited by 1 μM naloxone.

Effect of monovalent cations, Mg^{2+}, and Gpp(NH)p. Exclusion of Na$^+$ reduced specific binding of [^{3}H]β-FNA and Na$^+$ could be substituted by Li$^+$ but not by K$^+$, Cs$^+$, Mg^{2+}, or

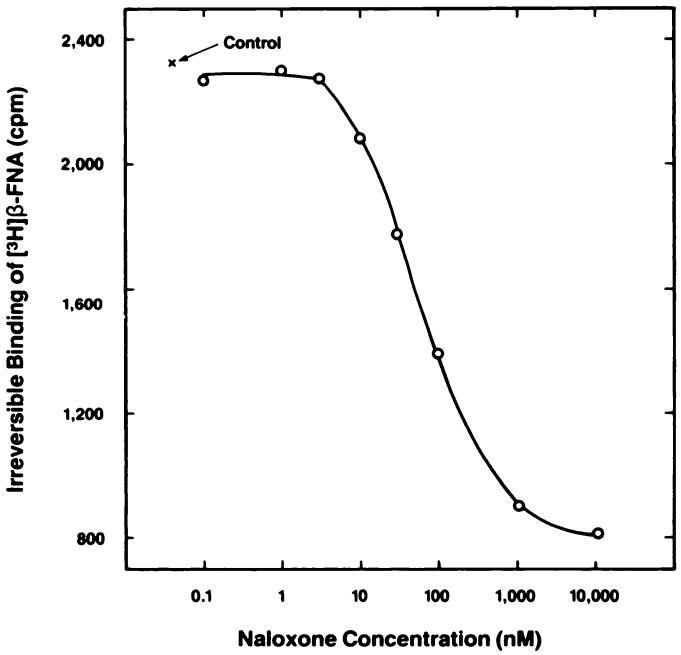

Fig. 2. Inhibition of naloxone of irreversible binding of [^{3}H]β-FNA to bovine striatal membranes at 37° C. Various concentrations of naloxone were added to bovine striatal membrane suspended in 50 mM TEL buffer containing 100 mM NaCL (approximately 0.40 mg protein/tube) for 20 min before incubation with 5 nM [^{3}H]β-FNA for 60 min. Controls were performed without naloxone. Values represent means of two determinations in triplicate.

Gpp(NH)p (Table 1). Binding increased with increasing Na^+ concentrations, although a strict dose-response relationship did not appear to exist (Table 1).

Effects of time and temperature on irreversible binding of [^{3}H]β-FNA. Fig. 3 shows that the nonspecific irreversible binding was linear with time, whereas the specific binding reached a plateau at approximately 60–90 min. In addition, the time course of binding of [^{3}H]β-FNA was performed at 0°, 25°, and 30°. At 25 and 30°, the results were similar to those at 37° except that the plateau specific binding was less than that at 37°. At 0° both specific and nonspecific binding occurred slowly, and the specific binding did not reach a plateau during the 4-hr incubation period. Thus, irreversible binding is both time- and temperature-dependent.

Effect of [^{3}H]β-FNA concentration on its irreversible binding. Fig. 4 shows that the nonspecific binding was linear with [^{3}H]β-FNA concentration; however, specific binding reached a plateau at 5 nM [^{3}H]β-FNA. Further increase in [^{3}H]β-FNA concentration did not increase specific binding, thus demonstrating that the irreversible binding of [^{3}H]β-FNA to opioid receptors in bovine striatal membrane was saturable. The specific binding at 5 nM [^{3}H]β-FNA represented 50–70% of the total irreversible binding or 20–30% of specific binding (reversible and irreversible) to membrane opioid receptors.

Relationship of irreversible binding to the amount of tissue. All total, nonspecific, and specific bindings were linearly related to the amount of tissue from 100 to 400 μg of membrane proteins. Larger amounts of tissue clogged the filter.

Inhibition of specific irreversible binding of [^{3}H]β-FNA by levorphanol or dextrorphan and sufentanil, DADLE, ICI174,864, or U50,488H. The IC_{50} values of these reversible ligands in inhibiting the *irreversible* binding of [^{3}H]β-FNA are comparative and do not represent the true affinities of these ligands. Dextrorphan was approximately 1,000 times less potent than levorphanol in inhibiting specific binding of [^{3}H]β-FNA (Fig. 5A). The stereospecific inhibition suggests that [^{3}H]β-FNA irreversibly binds to the opioid receptor. The order of potency among selective ligands for different types of opioid receptors was sufentanil > DADLE ≫ U50,488H = ICI174,864 (Fig. 5B). Both sufentanil and DADLE inhibited the binding in a dose-dependent manner, whereas U50,488H and ICI174,864 were inactive below 1 and 3 μM, respectively. Sufentanil was approximately 25, 1,000, and 3,000 times more potent than DADLE, U50,488H, and ICI174,684, respectively.

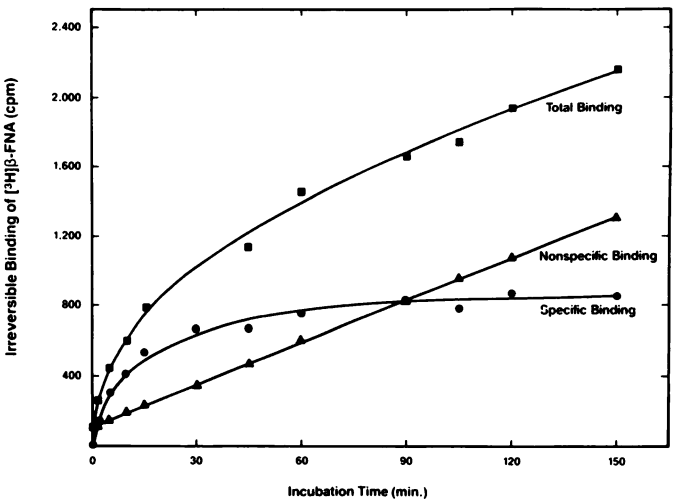

Fig. 3. Time of irreversible binding of 5 nM [^{3}H]β-FNA at 37° C. Membranes were pretreated with or without 1 μM naloxone for 20 min and then incubated with 5 nM of [^{3}H]β-FNA. At various intervals, aliquots of incubation mixture containing approximately 0.3 mg protein were taken into tubes containing 10% trichloroacetic acid and the irreversible binding of [^{3}H]β-FNA determined. Each value represents the mean of two determinations in triplicate.

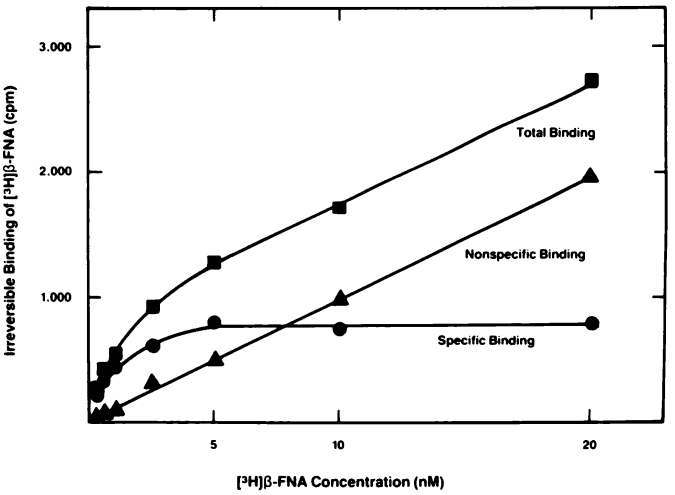

Fig. 4. Effect of [^{3}H]β-FNA concentration on its irreversible binding. Membranes (0.35 mg protein/tube) were pretreated with or without 1 μM naloxone for 20 min followed by incubation with various concentrations of [^{3}H]β-FNA for 60 min. Irreversible binding of [^{3}H]β-FNA was then determined. Each value represents the mean of two determinations in triplicate.

TABLE 1

Effect of monovalent cations, Mg^{2+}, and Gpp(NH)p and concentration of NaCl on irreversible binding of [^{3}H]β-FNA to opioid receptor.

A solution of Gpp(NH)p or ion (all Cl^- salts) was added to bovine striatal membrane suspension (0.35 mg/tube) to a final concentration indicated. Membranes were pretreated with or without 1 μM naloxone at 37° for 20 min and then incubated with 5 nM [^{3}H]β-FNA for 60 min. Irreversible binding of [^{3}H]β-FNA was determined. Data are expressed as percentage of control (in the presence 100 mM NaCl), and each value represents mean ± standard error; *n*, number of determinations in duplicate.

	Control (100 mM NaCl)	*n*
	%	
Monovalent cations		
None	34.3 ± 6.0	8
50 mM Na^+	73.4	2
100 mM Li^+	89.4	2
50 mM Li^+	63.0	2
50 mM K^+	40.0	2
50 mM Cs^+	41.6	2
5 mM Mg^{2+}	29.3	2
10 μM Gpp(NH)p	27.5	2
NaCl concentration (mM)		
0	34.3 ± 6.0	8
25	74.3	2
50	73.4	2
200	129.2 ± 7.9	5
300	105.3 ± 15.9	4
400	113.4 ± 16.3	4
500	161.3 ± 23.5	4

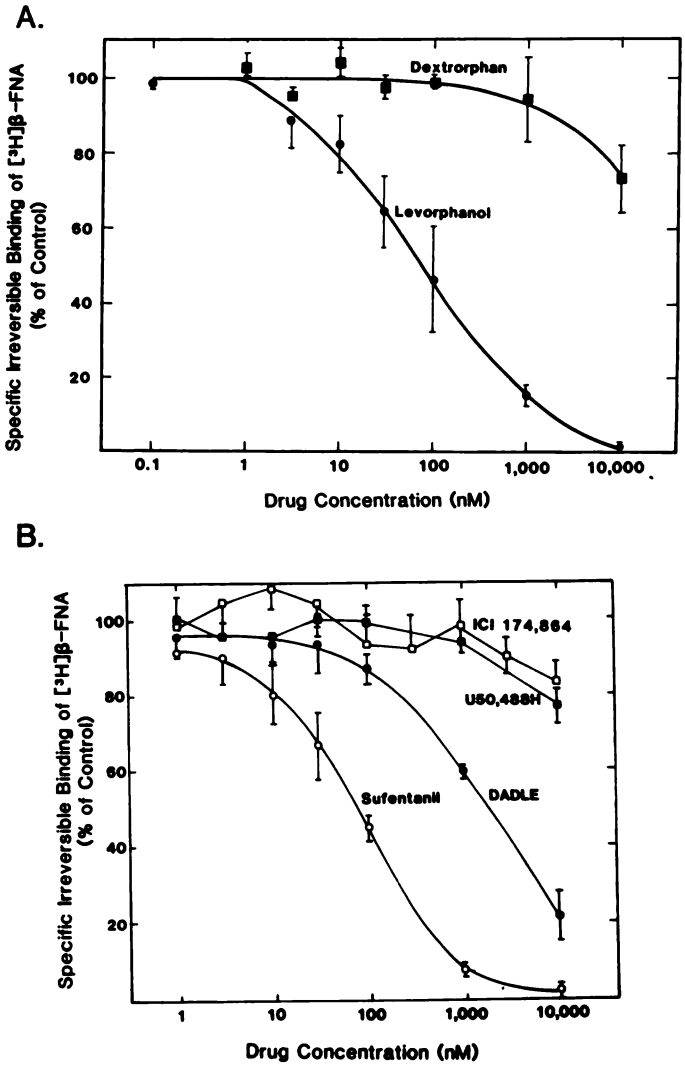

Fig. 5. Inhibition of irreversible binding of [³H]β-FNA to opioid receptors by (*A*) levorphanol or dextrophan or (B) μ (sufentanil), δ (DADLE, ICI174,864), or κ (U50,488H) ligands. Membranes (approximately 0.35 mg protein) were pretreated with various concentrations of levorphanol, dextrorphan, sufentanil, DADLE, ICI174,864, or U50,488H for 20 min followed by incubation with 5 nM [³H]β-FNA for 60 min. Pretreatment with 1 μM naloxone was used to determine control specific irreversible binding to opioid receptors. Irreversible binding was determined, and data were expressed as percentage of the control specific irreversible binding. Each value represents mean ± standard error of three determinations in duplicate.

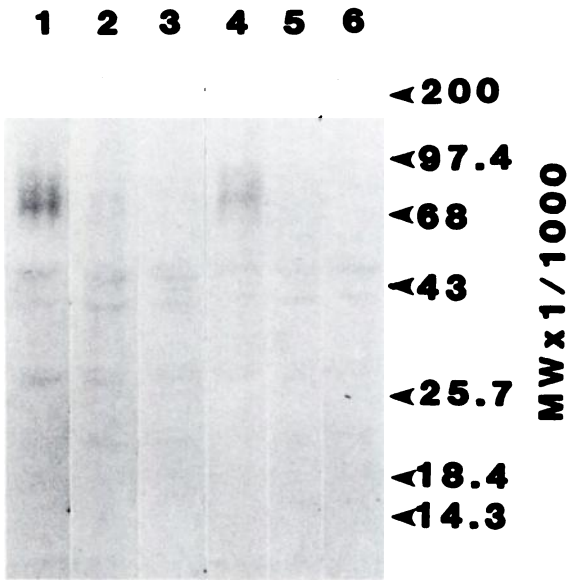

Fig. 6. PAGE and fluorography of [³H]β-FNA-labeled bovine striatal membrane proteins. Membranes were incubated with or without 1 μM naloxone or β-FNA in the presence of 100 mM NaCl at 37° C for 90 min. Membranes were prepared and separated by $NaDodSO_4$-PAGE at 4° C and subjected to fluorography. Lanes 1, 2, and 3 were loaded with 1800 cpm/lane; lanes 4, 5, and 6 were loaded with 900 cpm/lane. Lanes 1 and 4 represent total [³H]β-FNA-labeled proteins (in the absence of either β-FNA or naloxone). Proteins nonspecifically labeled by [³H]β-FNA are presented in lanes 2 and 5 (in the presence of a 1 μM naloxone) and in lanes 3 and 6 (in the presence of 1 μM β-FNA).

Morphine also inhibited the specific irreversible [³H]β-FNA binding dose-dependently with a slightly less potency than sufentanil (not shown). These results indicate that [³H]β-FNA preferentially binds to μ opioid receptors irreversibly.

Polyacrylamide gel electrophoresis (PAGE) and fluorography of labeled membranes. $NaDodSO_4$-PAGE results demonstrated that [³H]β-FNA specifically bound a protein(s) that electrophoretically migrated as a band having a broad molecular weight range of 68,000 to 97,000 (Fig. 6). [³H]β-FNA binding to this band could be inhibited and virtually eliminated by naloxone and completely eliminated with nonradioactive β-FNA, whereas nonspecifically labeled proteins remained unchanged. In some experiments, one protein band of M_r 35,000 appeared to be specifically labeled (not shown). However, when the labeling process was performed in the presence of various protease inhibitors (1 mM EGTA, 10 μM leupeptin, 10 μM PMSF, 19 μg/ml soybean trypsin inhibitor, and 50 μg/ml bacitracin) or the gel electrophoresis was performed at 4° C, this band did not appear. It was thus concluded that this labeled protein of lower molecular weight was a proteolytic product of the labeled receptor.

Solubilization of the labeled membranes and wheat germ lectin (WGL) affinity chromatography. With 20 mM CHAPS and 1% Lubrol PX, the solubilization yield was approximately 60%. After application of solubilized labeled membranes onto a WGL-Sepharose column and washing of the column, 0.25 M *N*-acetyl-D-glucosamine eluted a peak of radioactivity. When the membranes were labeled with [³H]β-FNA in the presence of 1 μM naloxone, the radioactivity in this peak was reduced by 85–90%. This affinity chromatography purified the receptor approximately 17-fold as determined by the amount of irreversibly bound radioactivity in the pooled eluate (1.0×10^5 dpm/mg protein) versus that in the labeled membrane preparation (6.0×10^3 dpm/mg protein).

$NaDodSO_4$-PAGE and fluorography of WGL column eluates showed that in the total labeling preparation there was one highly radioactively labeled protein band, whereas in the nonspecific labeling preparation only a very faintly labeled band was observed in the same molecular weight range (Fig. 7). No other labeled protein band was observed in either preparation. The labeled protein migrated as a broad band with M_r of 68,000–97,000, identical to the results obtained from the labeled membrane preparation. This result indicates that in the WGL

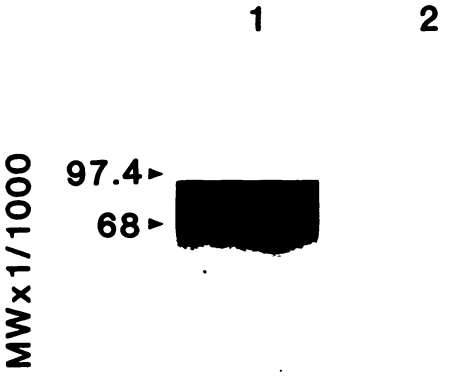

Fig. 7. PAGE and fluorography of WGL affinity column eluate. The pooled fractions of WGL affinity chromatography of both total and nonspecific labelings were treated and subjected to NaDodSO$_4$-PAGE in a 10% gel and fluorography as described in Materials and Methods. Both lanes contain equal amounts of protein (100 μg), and the total labeling has 12,000 cpm (lane 1) and the nonspecific labeling 1,500 cpm (lane 2).

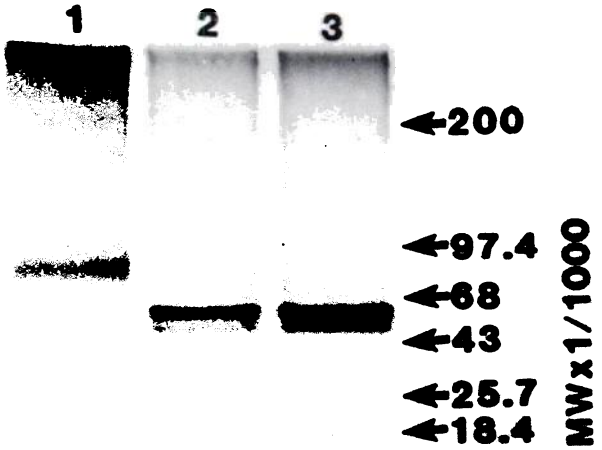

Fig. 8. NaDodSO$_4$-PAGE and fluorography of peptide:*N*-glycosidase F-treated WGL column eluate. The eluate of the total labeling preparation was precipitated with 60% acetone at 0° C, centrifuged, and resuspended in buffer as described. The resultant suspension was divided into three equal samples and incubated with 0, 3, and 6, U peptide:*N*-glycosidase F (N-glycanase) at 37° C for 18 hr. The reaction mixtures were treated as described in Experimental Procedures. All samples were subjected to NaDodSO$_4$-PAGE in a 10% gel and fluorography. Lanes 1, 2, and 3 represent eluate treated with 0, 3, and 6 U N-glycanase, respectively, and each contains 3,010 cpm. MW, molecular weight.

column eluate there is only one labeled protein band whose labeling can be inhibited by naloxone.

Deglycosylation of the WGL column eluate of the total labeling preparation with peptide:*N*-glycosidase F (*N*-glycanase). NaDodSO$_4$-PAGE and fluorography of the *N*-glycanase-treated eluate showed that the broad band of M_r 68,000–97,000 became a sharp and highly radioactive band of M_r 57,000 and a faintly labeled band of M_r 49,000 (Fig. 8). In the higher molecular weight range, there appeared to be some labeled protein bands that did not appear in the original WGL column eluate (Fig. 7). These are most likely the result of aggregation of labeled proteins in the acetone precipitation process. In the absence of exogenous glycosidase (Fig. 8, lane 1), there was a band of 57,000 present, probably due to the action of endogenous glycosidases and/or proteases. Thus, the labeled bands shown in lanes 2 and 3 of Fig. 8 might be the results of the actions of both endogenous and exogenous enzymes.

Discussion

In this report, we demonstrated that [^{3}H]β-FNA covalently labeled opioid binding sites, most likely the μ type, in bovine striatal membranes with high specificity when membranes were incubated with 5 nM [^{3}H]β-FNA in the presence of 100 mM NaCl at 37° C for 60–90 min. The [^{3}H]β-FNA-labeled μ opioid binding site migrated in NaDodSO$_4$-PAGE as a broad protein band of M_r 68,000–97,000, which, after treatment with peptide:*N*-glycosidase F to remove N-linked oligosaccharide, became a sharp and highly radioactively labeled band of M_r 57,000 and a weakly labeled band of M_r 49,000.

The irreversible specific binding of [^{3}H]β-FNA to the μ opioid binding site was greatly enhanced by Na^+. The finding is consistent with the result that pretreatment of brain membranes with β-FNA in the presence of Na^+ caused a greater extent of irreversible inhibition on μ binding than in the absence of it (31). This Na^+ effect is most likely due to a conformational change of the receptor induced by Na^+ (46).

The irreversible binding to the opioid receptor at 37° C reached a plateau at 5 nM [^{3}H]β-FNA and at approximately 60–90 min. The observation that at the plateau the specific irreversible binding of [^{3}H]β-FNA represented approximately 30% of specific membrane binding (both reversible and irreversible) agrees with the findings that 30% of [^{3}H]β-FNA binding to membrane opioid receptors was not displaceable and was likely to be irreversibly bound (31). It has been demonstrated that when [^{3}H]β-FNA-treated (5 nM) membranes were solubilized with NaDodSO$_4$ and applied to a Sephadex G-50 column, all of the specifically bound radioactivity to opioid receptors appeared in the void volume with proteins and represented approximately 30–40% of specific membrane binding (45).

The results of competition experiments indicate sterospecific labeling of the μ opioid binding site by [^{3}H]β-FNA. DADLE was 25 times less potent than sufentanil, and its potency was most likely due to interaction with the μ opioid receptor.

Selective agonists for μ or δ receptors such as Tyr-Pro-NMePhe-D-Pro-NH_2 (PL017), [D-Ala^2, MePhe^4, Gly(OH)^5]enkephalin (DAGO), and [D-Pen^2, D-Pen^5]enkephalin (Pen represents penicillamine) (DPDPE) were not used in these experiment because 100 mM NaCl was included in the incubation medium to obtain high levels of [^{3}H]β-FNA irreversible binding, a condition that would render them much less potent. The finding that 5 nM [^{3}H]β-FNA covalently labeled the μ opioid binding site is consistent with the published result that pretreatment of brain membranes with low concentrations of β-FNA (1–5 nM) reduced the binding of μ opioids ligands but not that of δ or κ ligands (31). This observation also agrees with the original finding that β-FNA acted as an irreversible μ antagonist (25, 26, 29, 30) and the reports that β-FNA pretreatment decreased the μ opioid binding (31, 32, 34) but not with the finding that β-FNA pretreatment did not reduce μ binding (27). The reason for this discrepancy is unclear. It may be due partially to differences in incubation conditions.

That [^{3}H]β-FNA did not irreversibly bind to the κ receptor is in accord with published results (25–34). The effect of β-FNA on the δ receptor has been controversial. β-FNA treatment did or did not irreversibly block the δ receptor (25–34). Under the incubation condition described in this report, it is unlikely that [^{3}H]β-FNA covalently binds to a significant number of δ receptors based on the following reasons. ICI174,864, a selective δ antagonist with IC_{50} of approximately 10 nM in the presence of NaCl (47), did not inhibit the irreversible binding of [^{3}H]β-FNA below 3 μM. In addition, since K_i for β-FNA in competing with binding of the δ receptor was 78 nM (31) and a reversible β-FNA-receptor complex was thought to form before the formation of a covalent bond (48), it seemed unlikely that 5 nM [^{3}H]β-FNA, with less than 10% receptor occupancy, would covalently bind to a substantial portion of the δ receptor. Thus, the reactivity of β-FNA with the δ receptor very much depends on the concentration and the incubation condition. Indeed, pretreatment of brain membranes with β-FNA did not reduce the δ binding below 7 nM; however, when the concentration exceeded 10 nM, it decreased the δ binding in a dose-dependent manner with an ED_{50} of approximately 100 nM (31).

The amount of specific [^{3}H]β-FNA labeling to the μ opioid binding site was approximately 6,000 dpm/mg protein. Calculated on the basis of 250 fmol/mg protein of μ opioid receptors, this represented labeling of approximately 50% of the receptors. This might reflect partially the receptor occupancy at 5 nM, which was approximately 70% since the K_i of β-FNA for the μ receptor binding was 2.2 nM (31). In addition, it might also reflect the percentage of receptors assuming the conformational state that could be labeled by [^{3}H]β-FNA.

The findings that the μ opioid receptor was a glycoprotein and could be partially purified by WGL affinity chromatography agree with published observations (14, 20, 22). The labeled protein appeared to be different from bovine serum albumin (M_r 66,000) since they behaved differently in mono-Q ion exchange chromatography (L.-Y. Liu-Chen, unpublished observations).

The labeled protein band in the WGL column eluate was very broad, even for a glycoprotein. A similar phenomenon was observed in the β_2-adrenergic receptor (49). This is most likely due to microheterogeneity in varying degrees of glycosylation (49). When the eluate of the total binding preparation was treated with peptide:*N*-glycosidase F (*N*-Glycanase) to remove *N*-linked oligosaccharides and leave aspartic acid at glycosylation site (35, 36), the labeled receptor became a major band of high radioactivity with M_r 57,000 and a minor band of M_r 49,000. A few possibilities may account for the minor band. It may be a proteolytic product of the major band since it is not uncommon to have protease contamination in glycosidase preparations. Alternatively, different degrees of post-translational modifications may still exist between the two bands, similar to that found in the β_2-adrenergic receptor (49). In addition, these two bands may represent plasma membrane form and smooth microsomal form of the μ receptor (50, 51) or the two subtypes of μ receptors (52). Further investigation is needed.

The molecular weight of the μ receptor reported here is not consistent with published results. The molecular weight of the μ opioid receptor has been estimated to be 94,000, 44,000, and 35,000 (23), 58,000 (13, 14), 65,000 (15, 16), or 45,000, 35,000, and 23,000 (17). A few possible reasons may explain these discrepancies. First, every laboratory runs $NaDodSO_4$-PAGE differently from others, which may result in some differences in the estimation of molecular weight, particularly for glycoproteins. Second, other proteins tightly coupled to the receptor, such as G protein(s), may be coeluated with the receptor in purification steps (23). Third, there may be species variation in the molecular weight of the μ receptor. Preliminary results showed that μ opioid binding sites in brain membranes of the guinea pig, rat, and mouse could also be labeled by [^{3}H]β-FNA under the same condition and the molecular weights were different from that of the cow and also varied among these species (Liu-Chen and Phillips, unpublished observations). In addition, the presence of protease inhibitors in the process of preparing membranes in this report may be an important difference, as is shown in the labeling of β_2-adrenergic receptor (53).

Although there was only one labeled protein band in the WGL column eluate, it was by no means a homogenous preparation. Based on the [^{3}H]β-FNA specific activity of 26.7 Ci/mmol and the assumption that the molecular weight of the core protein of the μ opioid receptor is 57,000, the receptor will have 1.04×10^9 dpm/mg protein when it is purified to homogeneity. After the WGL column chromatography, the eluate has approximately 1.0×10^5 dpm/mg protein and still needs 10,000-fold purification to reach homogeneity.

Purification of opioid receptors could be greatly facilitated by use of radiolabeled irreversible and specific ligands. After labeling of the receptor, one could monitor the purification by radioactivity, thus avoiding the problem of unstable binding activity. Although this approach yields inactive receptors, the information obtained will complement the studies of those purifying active receptors. A few radiolabeled irreversible ligands and photoaffinity ligands for μ opioid receptors have been developed. For example, [^{3}H]Tyr-D-Ala-Gly-Phe-Leu-CH_2Cl ([^{3}H]DALECK) identified the μ opioid receptor as a 58,000-dalton protein (13, 54). In addition, [^{125}I]^{27} Tyr-human β-endorphin was cross-linked to μ opioid receptors in the presence of a selective δ ligand, DPDPE (15, 16). Being a nonpeptide ligand, [^{3}H]β-FNA has additional advantages over peptide affinity ligands. The partial amino acid sequence and the amino acid composition of the purified receptor can be determined without interference from [^{3}H]β-FNA. In addition, the labeled receptor can undergo proteolytic digestion without losing the

radioactive label, since the reaction between [^{3}H]β-FNA and the μ receptor is probably a Michael addition (25, 31, 48). Thus, this approach will yield information on binding domain of the receptor. This partial amino acid sequence of the binding domain will, in turn, allow molecular cloning of the receptor and generation of antibodies for further characterization of the receptor.

In conclusion, [^{3}H]β-FNA is a highly specific affinity label for the μ opioid binding site. This observation enables an alternative approach toward complete purification and biochemical characterization of the protein. More importantly, this method will allow one to obtain information on the ligand binding domain of the receptor.

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Send reprint requests to: Dr. L.-Y. Liu-Chen, Department of Pharmacology, Temple University School of Medicine, 3420 N. Broad Street, Philadelphia, PA 19140.