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β-[^{3}H]Funaltrexamine-Labeled μ-Opioid Receptors: Species Variations in Molecular Mass and Glycosylation by Complex-Type, *N*-Linked Oligosaccharides

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SUMMARY

We previously showed that under defined conditions β-[^{3}H] funaltrexamine (β-[^{3}H]FNA) covalently labeled μ-opioid receptors with high specificity in bovine striatal membranes. β-[^{3}H]FNA-labeled μ-opioid receptors migrated as a broad band with a molecular mass range of 68–97 kDa. It is controversial whether β-FNA binds irreversibly to μ-opioid receptors in other species. In this study, we demonstrated that β-[^{3}H]FNA also labeled μ-opioid receptors with high specificity in brain membranes of the guinea pig, rat, and mouse. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography revealed that in each species β-[^{3}H]FNA specifically bound to a protein in which labeling was greatly reduced by naloxone. These labeled receptors had broad molecular mass ranges, and the molecular masses were different among these species, in the order of cow > guinea pig > rat > mouse. Membranes were subjected to solubilization with 2% Triton X-100 and wheat germ lectin (WGL) affinity chromatography. *N*-Acetylglucosamine eluted a peak of radioactivity. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography showed that in all four species the μ receptor was the only protein labeled with β-[^{3}H]FNA in the WGL eluate. The molecular masses of labeled μ-opioid receptors were 70–88 kDa (median, 77 kDa) for the cow, 66–80 kDa (median, 72 kDa) for the guinea pig, 60–75 kDa (median, 67 kDa) for the rat, and 60–72 kDa (median, 66 kDa) for the mouse. In addition, we investigated the nature of the carbohydrate moieties linked to the receptor protein and whether the species variation in the molecular mass was due to variable degrees of glycosylation. The bovine WGL eluate was treated with various glycosidases. Neuraminidase treatment decreased the receptor molecular mass by 6–7 kDa, whereas α-mannosidase had no effect. Removal of *N*-linked carbohydrates at asparagine residues by peptide-*N*^{4}-[*N*-acetyl-β-glucosaminyl]asparagine amidase (*N*-Glycanase) resulted in a much sharper specifically labeled protein band of 43 kDa. These results indicate that μ-opioid receptors are heavily glycosylated and the major carbohydrate moieties are of the complex type, *N*-linked to asparagine. After the WGL eluates for the four species were treated with *N*-Glycanase, the labeled receptors became much sharper bands with very similar molecular masses, i.e., 43 kDa for the cow and guinea pig, 39 kDa for the rat, and and 40 kDa for the mouse. These results indicate that the variation among species in the molecular mass of β-[^{3}H] FNA-labeled μ-opioid receptors is mainly the result of variable degrees of glycosylation.

β-FNA, the fumaramate methyl ester of naltrexamine, was synthesized by Portoghese *et al.* (1). It was found to have reversible κ agonist and irreversible μ antagonist activities in *in vitro* preparations of the guinea pig ileum (1–4) and the mouse vas deferens (4, 5) and in *in vivo* antinociceptive tests (6). The high selectivity and irreversible nature of its action make β-FNA a very useful pharmacological tool both *in vivo* and *in vitro* (for a review, see Ref. 7). Recently, we demonstrated that β-[^{3}H]FNA covalently labeled the binding site of the μ-opioid receptor with high specificity in bovine striatal membranes when the membranes were incubated with 5 nM β-[^{3}H] FNA at 37° for 60–90 min, in the presence of 100 mM NaCl (8). The specific irreversible binding of β-[^{3}H]FNA to opioid receptors, defined as that which could be inhibited by 1 μM naloxone, was saturable, was time and temperature dependent, and was linearly related to protein content. μ-Opioid ligands (sufentanil and morphine) were better inhibitors of specific irreversible binding than were δ [lsq6]D-Ala^2, D-Leu^5[rsq6]-enkephalin, ICI174,864) or κ (U50,488H) ligands, and levorphanol was 1000 times more potent than its inactive stereoisomer, dextrorphan. Na^+ and Li^+ greatly enhanced the specific binding of β-[^{3}H] FNA (8). In addition, autoradiographic analysis of irreversible

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ABBREVIATIONS: β-FNA, β-funaltrexamine; *N*-Glycanase, peptide-*N*^{4}-[*N*-acetyl-β-glucosaminyl]asparagine amidase; WGL, wheat germ lectin; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; CTAP, cyclic D-Phe-cys-tyr-D-Trp-arg-thr-pen-thr-NH_2.

binding of β-[^{3}H]FNA (5 or 10 nM) to forebrain sections showed that the distribution was very similar to that of [^{3}H][lsq6]D-Ala^2, N-Me-Phe^4, gly-ol[rsq6]-enkephalin and unlike that of [^{3}H][D-Pen^2,Pen^5]-enkephalin, indicating its specificity for the μ receptor (9). These results indicate that under appropriate conditions β-[^{3}H]FNA specifically and irreversibly binds to μ-opioid receptors. PAGE of the labeled membranes and subsequent fluorography showed that β-[^{3}H]FNA specifically bound to a protein that migrated as a broad diffuse band with an apparent molecular mass between 68 and 97 kDa. Such electrophoretic characteristics suggested that it was likely to be a glycoprotein, which was confirmed by its adsorption to WGL-Sepharose after solubilization with 2% Nonidet P-40 and its subsequent elution with *N*-acetyl-D-glucosamine. SDS-PAGE and fluorography of the WGL column eluate showed that in the total labeling preparation there was only one highly radioactively labeled protein band (8).

Because β-[^{3}H]FNA labels bovine μ-opioid receptors with high specificity, it may be used as a marker to study biochemical properties of the receptor without prior purification, which has been a difficult task for investigators in the field. In this study, we examined the labeling of μ-opioid receptors by β-[^{3}H]FNA in brain membranes of the guinea pig, rat, and mouse, possible species variation in the molecular mass of the μ-opioid receptor among these species, and the nature of carbohydrate moieties linked to the receptor protein.

There has not been a consensus on the molecular weight of the μ-opioid receptor. The molecular weight of the μ-opioid receptor was estimated to be 94,000, 44,000, and 35,000 (10), 65,000 (11–13), 58,000 (14, 15), 42,000, 35,000, and 23,000 (16), 68,000–97,000 (8), 108,000, 73,000, and 49,000 (17), or 61,000 (18). This discrepancy may be partially due to different species used for the purification or labeling of the receptor; some studies used rat brains (10, 13–18) and some used bovine striata (8, 11). In addition, our previous labeling study was performed with bovine striatal membranes. It is not clear whether β-[^{3}H]FNA at such a low concentration (5 nM) labels μ-opioid receptors in other species, such as the rat, guinea pig, and mouse. In several studies in which β-FNA was shown to bind irreversibly to opioid receptors in the rat and mouse, much higher concentrations of β-FNA (≥100 nM) were used (19–22). Corbett *et al.* (3) reported that, although β-FNA acted as an irreversible μ antagonist in guinea pig ileum, it did not bind irreversibly to the μ receptor in this preparation. On the other hand, β-[^{3}H]FNA (≤10 nM) bound irreversibly to μ-opioid receptors in rat brain sections in a receptor autoradiography study (9) and in slice preparations of mouse striatum and midbrain regions (23). Tam and Liu-Chen (24) found that pretreatment of guinea pig brain membranes with 1–5 nM β-FNA in the presence of Na^+ reduced μ-opioid receptor binding without affecting δ or κ binding. There is no direct evidence that β-[^{3}H]FNA labels μ-opioid receptors with high specificity in these species. Studies were thus conducted to determine whether β-[^{3}H]FNA specifically labeled the μ receptor in brain membranes of the guinea pig, rat, and mouse, three species widely used in opioid studies. We found that β-[^{3}H]FNA labeled μ-opioid receptors in these species with high specificity and that the molecular masses of these labeled μ receptors, determined by SDS-PAGE followed by fluorography, were different among these species.

μ-Opioid receptors, like many other membrane-bound receptors such as α_1- and β-adrenergic receptors, have been shown to be glycoproteins (8, 11, 14, 18, 25–27). The glycoprotein nature of this protein was demonstrated by absorption onto WGL-Sepharose beads and subsequent elution with *N*-acetyl-D-glucosamine (8, 11, 14, 18, 25). Although the notion that μ-opioid receptors are glycoproteins is widely accepted, the nature of their carbohydrate moieties has not been studied. In this study, we also investigated the nature of the carbohydrate moieties of the receptors by treating β-[^{3}H]FNA-labeled receptors with various exo- and endoglycosidases, such as neuraminidases (EC 3.2.1.18), α-mannosidase (EC 3.2.1.24), and *N*-Glycanase (EC 3.5.1.52). Changes in molecular masses were determined by SDS-PAGE and fluorography. In addition, we examined whether the species variation in the molecular mass of the receptor was due to variable degrees of glycosylation.

Experimental Procedures

Materials. β-[^{3}H]FNA (specific activity, 21.6 Ci/mmol) was supplied by the National Institute on Drug Abuse. Naloxone was a generous gift from DuPont/Merck Co. (Wilmington, DE). WGL-Sepharose 6MB was purchased from Pharmacia Fine Chemicals (Piscataway, NJ), *N*-Glycanase (EC 3.5.1.52) (from *Flavobacterium meningosepticum*) from Genzyme Co. (Boston, MA), and neuraminidase (EC 3.2.1.18) (from *Clostrium perfringens*) and α-mannosidase (EC 3.2.1.24) (from jack bean) from Sigma Chemical Co. (St. Louis, MO). Triton X-100 was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN); SDS, dithiothreitol, mercaptoethanol, urea, and chemicals for PAGE from Bio-Rad Laboratories (Richmond, CA); 2,5-diphenyloxazole from Kodak Co. (Rochester, NY); Omnifluor and Protosol from DuPont-NEN Research Products (Boston, MA); and tricine, leupeptin, bacitracin, soybean trypsin inhibitor, phenylmethylsulfonyl fluoride, 1,10-phenanthroline hydrate, dithioglycol, EGTA, and *N*-acetyl-D-glucosamine from Sigma. Prestained protein standards were purchased from Sigma, and ^{14}C-labeled standards were obtained from GIBCO-BRL (Gaithersburg, MD).

Preparation of bovine striatal membranes and guinea pig, rat, and mouse brain membranes. Fresh bovine striata were obtained from a slaughterhouse shortly after death of the animals, frozen immediately on dry ice, and stored at −70°. Male Hartley guinea pigs, Sprague-Dawley rats, and ICR:SKH albino mice were sacrificed by decapitation, and brains (minus cerebella) were removed immediately, frozen on dry ice, and stored at −70°. Membranes were prepared according to the procedure described previously (8) and were kept frozen at −70° until used.

Labeling of μ-opioid receptors with β-[^{3}H]FNA. Membranes suspended in 50 mM Tris·HCl buffer containing 1 mM EGTA and 10 μM leupeptin (TEL buffer) (2–4 mg of protein/ml) were incubated with 5 nM β-[^{3}H]FNA in the presence of 200 mM NaCl at 37° for 75–90 min, to label μ-opioid receptors (8). Nonspecific labeling was performed in the presence of 10 μM naloxone. Labeled membranes were washed once with 50 mM TEL buffer by centrifugation and resuspension.

Solubilization of labeled membranes and WGL affinity chromatography. Both solubilization and WGL affinity chromatography were carried at 4° according to a published procedure, with some modifications (8). Labeled membranes were resuspended in 2% Triton X-100 in buffer A (10 mM Tris·HCl, 1 mM EGTA, 4 μM leupeptin, 150 mM NaCl, 5 mM dithioglycol, 10 μM phenylmethylsulfonyl fluoride, 19 μg/ml soybean trypsin inhibitor, 50 μg/ml bacitracin, pH 7.5). The mixture was incubated at 4° with stirring, for periods from 2 hr to overnight, and then centrifuged at 100,000 × *g* for 1 hr. The clear supernatant was applied at 0.5 ml/min to a WGL-Sepharose 6MB column, which had been equilibrated with 20 bed volumes of buffer A containing 0.2% Triton X-100 (buffer B). The column was washed with the same buffer until 280-nm absorbance reached base-line levels. The retained glycoproteins were then eluted with 0.25 M *N*-acetyl-D-glucosamine in buffer B, 1-ml fractions were collected, and an aliquot of

each fraction was counted for radioactivity. Peak fractions were pooled and concentrated with Amicon concentrators.

Assay for irreversible binding of β-[³H]FNA. Membranes (0.8–1 mg of protein/ml), in 50 mM TEL buffer containing 200 mM NaCl, were pretreated at 37° for 20 min with a drug at the concentrations indicated, followed by incubation at 37° for 60 min with 5 nM β-[³H] FNA. The incubation volume was 1 ml. Irreversible binding of β-[³H] FNA in labeled membranes and solubilized preparations was assayed according the published procedure of Liu-Chen *et al.* (28).

Treatment of WGL eluate with neuraminidase or α-mannosidase. The WGL column eluate of the labeled bovine membrane preparation was treated with neuraminidase or α-mannosidase essentially according to the method described by Stiles *et al.* (26). For neuraminidase (*C. perfringens*) treatment, an aliquot of WGL eluate was buffer-exchanged into 100 mM sodium acetate, pH 5.0, containing 0.1% Nonidet P-40. Neuraminidase was added at a concentration of 2.5 units/ml and the mixture was incubated at 35° for 3 hr. For α-mannosidase treatment, WGL eluate was buffer-exchanged into 100 mM sodium citrate, pH 4.5, containing 0.01% Nonidet P-40. α-Mannosidase was added at a concentration of 5 units/ml and the mixture was incubated at 25° for 24 hr. Reaction was terminated by the addition of 1 volume of 2× Laemmli sample buffer (29) for SDS-PAGE.

Treatment of WGL column eluate with *N*-Glycanase. The WGL eluate of the total labeling preparation for each species was buffer-exchanged into 100 mM Tris·HCl buffer, 1.7% Nonidet P-40, 10 mM 1,10-phenanthroline hydrate, 10 mM mercaptoethanol, pH 7.5. The sample was incubated with *N*-Glycanase at an enzyme/substrate ratio of 5 units/10,000 dpm, in a volume of 75 μl (enzyme concentration, 67 units/ml), at 37° for 3 or 6 hr (30, 31). Reaction was terminated by the addition of 1 volume of 2× Laemmli sample buffer (29) for SDS-PAGE.

SDS-PAGE. SDS-PAGE was performed according to the method described by Schagger and von Jagow (32). In this system, tricine replaced glycine in the Laemmli SDS-PAGE system. The cathode buffer was 0.1 M Tris, 0.1 M tricine, 0.1% SDS, pH 8.25; the anode buffer was 0.2 M Tris·HCl buffer, pH 8.9; and the gel buffer was 0.1% SDS in 1.0 M Tris·HCl buffer, pH 8.45. Electrophoresis was carried out at 30 V for 60 min and then at 70 V overnight at 4°. Labeled membranes were solubilized in Laemmli sample buffer, and all solubilized preparations from either total or nonspecific labeling were treated with an equal volume of 2× Laemmli sample buffer before being applied to the gel. Separation was carried out with a 7% or 10% polyacrylamide gel with a 4% stacking gel. For protein molecular mass standards, prestained standards or ^{14}C-labeled standards were used.

Detection of radioactivity in the gel by fluorography or scintillation counting. Two methods were used to detect radioactivity in the gels, scintillation counting of gel slices and fluorography. Scintillation counting was performed in preliminary experiments to define optimal conditions. Once optimal conditions were established, fluorography was then performed.

For scintillation counting, gels were cut into 1-mm slices and each slice was incubated with 5% Protosol and 0.4% Omnifluor in toluene, at 37° overnight. Radioactivity was determined by scintillation counting after samples were cooled to room temperature.

For fluorography, gels were soaked in 25% 2-propanol/10% acetic acid for 3–4 hr, followed by soaking in dimethylsulfoxide for 2 × 30 min. The gels were immersed, with shaking, in 4 volumes of 22.2% (w/v) 2,5-diphenyloxazole in dimethylsulfoxide for 3 hr (33, 34), then in water for 15 min, and finally in 2% glycerol for 15 min. The gels were dried and exposed to Kodak X-Omat AR (XAR) film at −70° for 1 week to 1 month.

Protein determination. Protein contents of the membrane preparations were determined by the bicinchoninic acid method of Smith *et al.* (35), using bovine serum albumin as the standard. Solubilized proteins were estimated by a modified Lowry method (36) or the method of Smith *et al.* (35).

Results

Inhibition of irreversible β-[³H]FNA binding to opioid receptors in brain membranes of the rat, guinea pig, and mouse by μ (CTAP), δ (ICI174,864), and κ (U50,488H) ligands. It should be emphasized that it was the irreversible binding of β-[³H]FNA that was measured. Thus, the IC_{50} values of these reversible ligands would be much greater than their K_i values for the respective receptors, due to differences in binding kinetics. As shown in Fig. 1, in all three species CTAP was more potent than ICI174,864 or U50,488H in inhibiting irreversible binding of β-[³H]FNA to opioid receptors. ICI174,864 or U50,488H did not inhibit binding, except in rats when the concentration of either ligand exceeded 1 μM or in mice when the U50,488H concentration was greater than 1 μM. The potency of CTAP in these three species was in the order of rats > mice > guinea pigs. These results indicate that, in guinea pigs, rats, and mice, β-[³H]FNA preferentially binds irreversibly to μ-opioid receptors.

Labeling of μ-opioid receptors by β-[³H]FNA in guinea pig, rat, and mouse brain membranes and bovine striatal membranes. Guinea pig, rat, and mouse brain membranes and bovine striatal membranes were labeled with β-[³H]FNA as described in Experimental Procedures. SDS-PAGE under denaturing and reducing conditions and subsequent fluorography showed that, among several protein bands labeled by β-[³H] FNA in the total binding preparation of each species, there was one band in which labeling was greatly reduced by naloxone (Fig. 2). In all four species, this protein band appeared to be diffuse and broad. These apparent μ-opioid receptors migrated as proteins of different molecular masses in these species, in the order (high to low) of cow, guinea pig, rat, and mouse. The molecular masses were estimated, by taking the median of each band, to be 77, 72, 67, and 66 kDa for the cow, guinea pig, rat, and mouse, respectively.

Solubilization of the labeled membranes and WGL affinity chromatography. The detergent used in solubilization of the labeled membranes, Triton X-100, was chosen to be compatible with the subsequent lectin affinity chromatography. Solubilized preparations of labeled bovine membranes were applied to a WGL-Sepharose column and the bound glycoproteins were eluted with 0.25 M *N*-acetyl-D-glucosamine, concomitant with a peak of radioactivity (Fig. 3). This affinity chromatography purified the receptor approximately 30-fold, as determined by the amount of radioactivity per mg of protein in the pooled peak fractions versus that in the solubilized membrane preparation. Similar results were obtained with labeled preparations of guinea pig, rat, and mouse membranes.

SDS-PAGE and fluorography of WGL column eluates showed that in the total labeling preparation there was one broad and highly radioactively labeled protein band (Fig. 4), whereas in the nonspecific labeling preparation only a very faintly labeled band was observed in the same molecular mass range (data not shown). No other labeled protein band was observed in either preparation. In each species, the labeled protein migrated as a broad band. Molecular masses of labeled μ-opioid receptors were 70–88 kDa (median, 77 kDa), 66–80 kDa (median, 72 kDa), 60–75 kDa (median, 67 kDa), and 60–72 kDa (median, 66 kDa) for the cow, guinea pig, rat, and mouse, respectively.

Treatment of β-[³H]FNA-labeled bovine μ receptors with neuraminidase, α-mannosidase, or *N*-Glycanase. Neuraminidase treatment caused an increase in the mobility of labeled receptors on SDS-PAGE, compared with the untreated control. This increase in mobility corresponded to a decrease

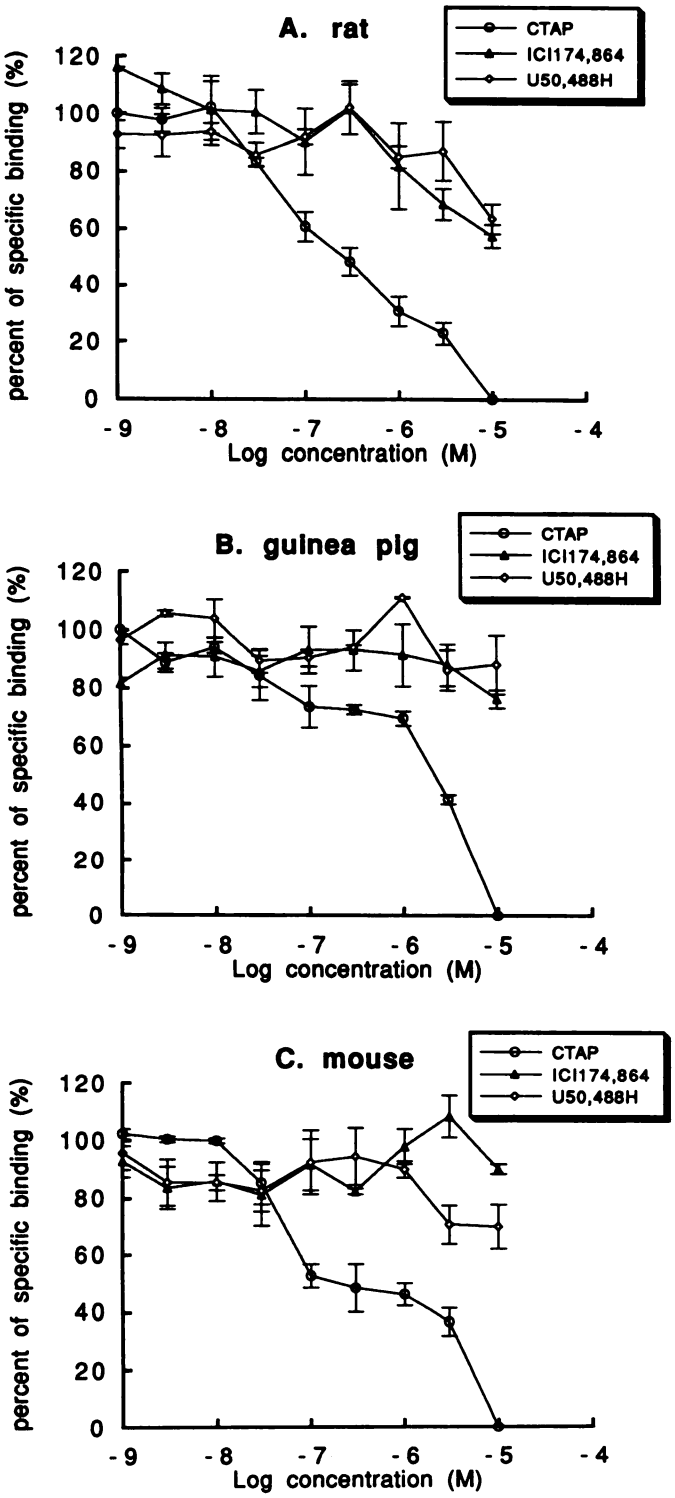

Fig. 1. Inhibition of irreversible binding of β-[^{3}H]FNA to opioid receptors by μ (CTAP), δ (ICI174,864), and κ (U50,488H) ligands in brain membranes of the rat (A), guinea pig (B), and mouse (C). Membranes (0.8–1 mg of proteins) were pretreated with various concentrations of CTAP, ICI174,864, or U50,488H for 20 min, followed by incubation with 5 nM β-[^{3}H]FNA for 60 min at 37°. Pretreatment with 10 μM naloxone was used to determine control specific irreversible binding to opioid receptors. Irreversible binding was determined, and data are expressed as percentage of the control specific irreversible binding. Each value represents mean ± standard error of three determinations in duplicate.

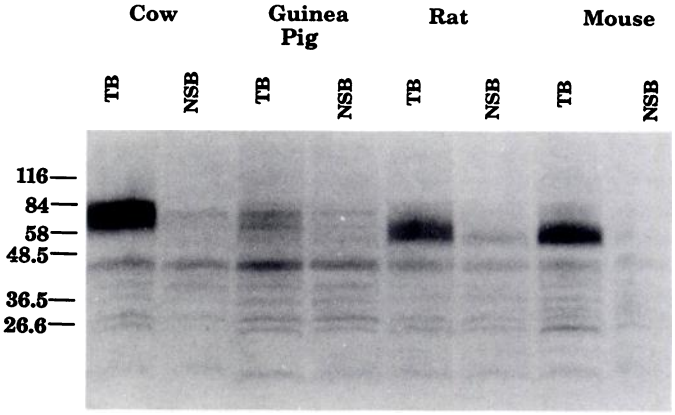

Fig. 2. Labeling with β-[^{3}H]FNA of bovine striatal membranes and guinea pig, rat, and mouse brain membranes. Labeling of membranes with β-[^{3}H]FNA, SDS-PAGE, and fluorography were performed as described in Experimental Procedures. Total binding (*TB*) and nonspecific binding (*NSB*) represent labeling in the absence and presence of 10 μM naloxone, respectively. Radioactivities loaded in each lane were 9900 dpm (cow, total binding), 4200 dpm (cow, nonspecific binding), 5700 dpm (guinea pig, total binding), 3300 dpm (guinea pig, nonspecific binding), 5400 dpm (rat, total binding), 3000 dpm (rat, nonspecific binding), 8400 dpm (mouse, total binding), and 3600 dpm (mouse, nonspecific binding). For each species, equal amounts of protein were loaded for total binding and nonspecific binding, i.e., 7.3 mg for the cow, 4.2 mg for the guinea pig, 3.9 mg for the rat, and 5.2 mg for the mouse. Molecular mass standards are in kDa. Note that for each species there was one radiolabeled protein band in which the labeling was greatly reduced by naloxone. This experiment was performed three times with similar results.

in molecular mass of 6–7 kDa (Fig. 5A). α-Mannosidase treatment had no effect on the mobility of the labeled receptor (Fig. 5B). After treatment with *N*-Glycanase, the broad band in the bovine WGL eluate became a sharp and highly radioactive band of 43 kDa (Fig. 6B, *cow C* and *NG*).

Deglycosylation with *N*-Glycanase of β-[^{3}H]FNA-labeled μ receptors of the cow, guinea pig, rat, and mouse. To determine whether the variation in the molecular mass of μ-opioid receptors among these four species was due to different degrees of glycosylation, WGL eluates were treated with *N*-Glycanase to remove *N*-linked sugars. With 3 hr of enzymatic treatment, there were two labeled protein bands between 40 and 50 kDa for each species (Fig. 6A). However, after 6 hr of incubation, the labeled protein band of higher molecular mass disappeared and there was one highly radioactively labeled band for each species. This band had similar molecular masses for all species, i.e, 43 kDa for the cow and guinea pig, 39 kDa for the rat, and 40 kDa for the mouse (Fig. 6B). In the cow and guinea pig, however, there was still a faintly labeled band of higher molecular mass (49 kDa). In bovine preparations, approximately 80% of the radioactivity associated with the 70–88-kDa protein band before *N*-Glycanase appeared in the 43-kDa band after the enzyme treatment, as estimated by scintillation counting of gel slices. These results indicate that the variation in the molecular mass of μ-opioid receptors appears to be largely due to different degrees of glycosylation.

Discussion

In this study we demonstrated that, under the conditions we defined previously for labeling of μ-opioid receptors in bovine striatal membranes, 5 nM β-[^{3}H]FNA labeled μ-opioid receptors with high specificity in brain membranes of the guinea pig, rat,

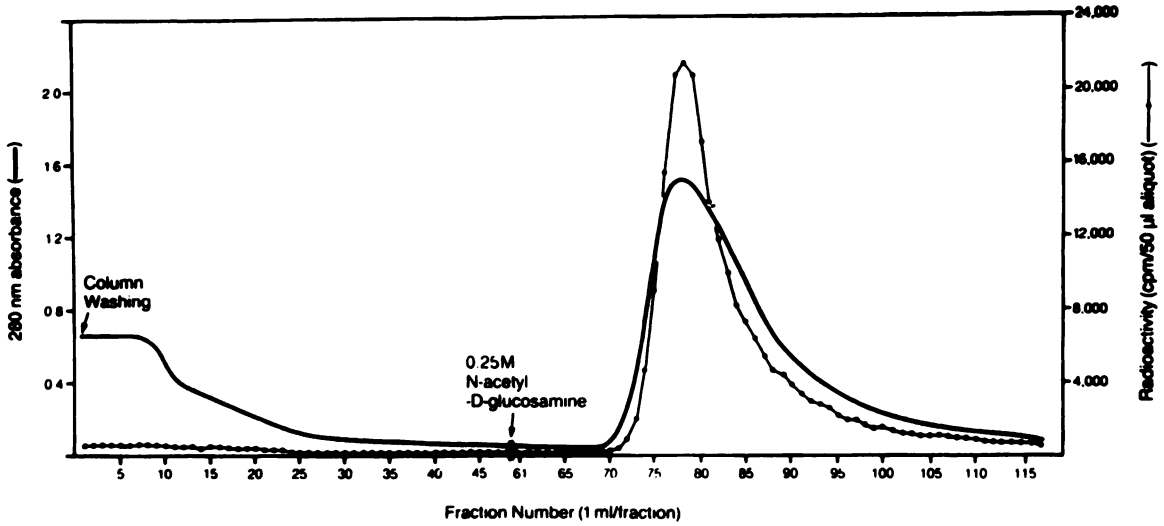

Fig. 3. WGL affinity chromatography of solubilized β-[³H]FNA-labeled preparation. Solubilization of β-[³H]FNA-labeled bovine striatal membranes and WGL affinity chromatography were performed as described in Experimental Procedures. After the column was washed until the 280-nm absorbance reached the base-line, *N*-acetylglucosamine eluted a peak of protein concomitant with a peak of radioactivity. The amount of protein loaded onto the column was approximately 1.8 g. The column was 25 mm in diameter and 45 mm in length and contained 20 ml of WGL-Sepharose 6MB. This experiment was performed 10 times with similar results.

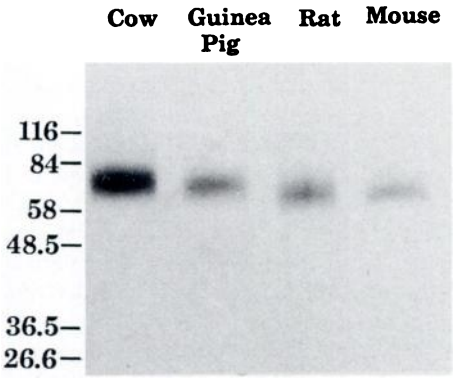

Fig. 4. SDS-PAGE and fluorography of the WGL eluates of total labeling preparations for the cow, guinea pig, rat, and mouse. SDS-PAGE and fluorography were performed on the WGL eluates for all four species, as described in Experimental Procedures. Radioactivities loaded in each lane were 15,000 dpm, 12,000 dpm, 12,700 dpm, and 10,000 dpm for the cow, guinea pig, rat, and mouse, respectively. Amounts of protein loaded in each lane were 300 μg for the cow, 240 μg for the guinea pig, 250 μg for the rat, and 200 μg for the mouse. Molecular mass standards are in kDa. The molecular masses of the labeled receptors were in the order (from high to low) of cow > guinea pig > rat > mouse. This experiment was performed three times with similar results.

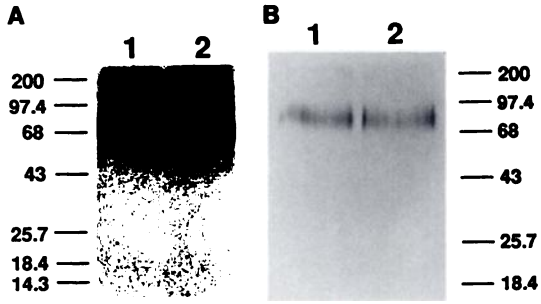

Fig. 5. Effect of neuraminidase (A) or α-mannosidase (B) treatment on β-[³H]FNA-labeled μ-opioid receptors. Treatment of the bovine WGL eluate with neuraminidase, α-mannosidase, or *N*-Glycanase, SDS-PAGE, and fluorography were performed as described in Experimental Procedures. A, *Lane 1*, untreated control; *lane 2*, treatment with neuraminidase (2.5 units/ml). The amounts of radioactivity and protein loaded in each lane were 7350 dpm and 150 μg, respectively. B, *Lane 1*, untreated control; *lane 2*, treatment with α-mannosidase. Amounts of radioactivities and proteins loaded in each lane were 3800 dpm and 80 μg, respectively. Molecular mass standards are in kDa. Both experiments were performed three times with similar results.

and mouse. Using this compound as a label, we found that the molecular masses of μ-opioid receptors were different in these four species and that the carbohydrate moieties associated with the receptor proteins were of the complex type, *N*-linked to asparagine. In addition, species differences in molecular masses were mainly due to variation in the degrees of glycosylation.

The observation that in brain membranes of the guinea pig, rat, and mouse irreversible binding of β-[³H]FNA occurs preferentially to μ- over δ- or κ-opioid receptors is similar to our finding in bovine striatal membranes. The finding that β-[³H]FNA labeled μ-opioid receptors with high specificity in guinea pig, rat, and mouse membrane preparations provides the first direct biochemical evidence that β-FNA binds covalently to μ-opioid receptors in these species. This finding is consistent with results of previous *in vitro* binding experiments showing that β-FNA binds irreversibly to μ-opioid receptors in the guinea pig (24), in the rat (9, 19), and in the mouse (22, 23). In contrast, these results are different from some observations (3, 21) that *in vitro* pretreatment with β-FNA had no effect or only a minor effect on μ-opioid receptor binding. The discrepancy is mainly due to differences in incubation conditions. Na^+ has been shown to be essential for irreversible binding of β-FNA to μ-opioid receptors (8, 24, 28). The apparently less intense labeling by β-[³H]FNA in guinea pig brain membranes may be due to the relatively low abundance of μ-opioid receptors in this species, compared with other species.

The findings that β-[³H]FNA-labeled μ-opioid receptors of four species can be adsorbed onto WGL-Sepharose beads and eluted with *N*-acetyl-D-glucosamine and that neuraminidase or *N*-Glycanase treatment reduces their molecular masses indicate that they are glycoproteins. This observation confirms our previous results (8) and is in accord with published reports (11, 14, 18, 25, 37).

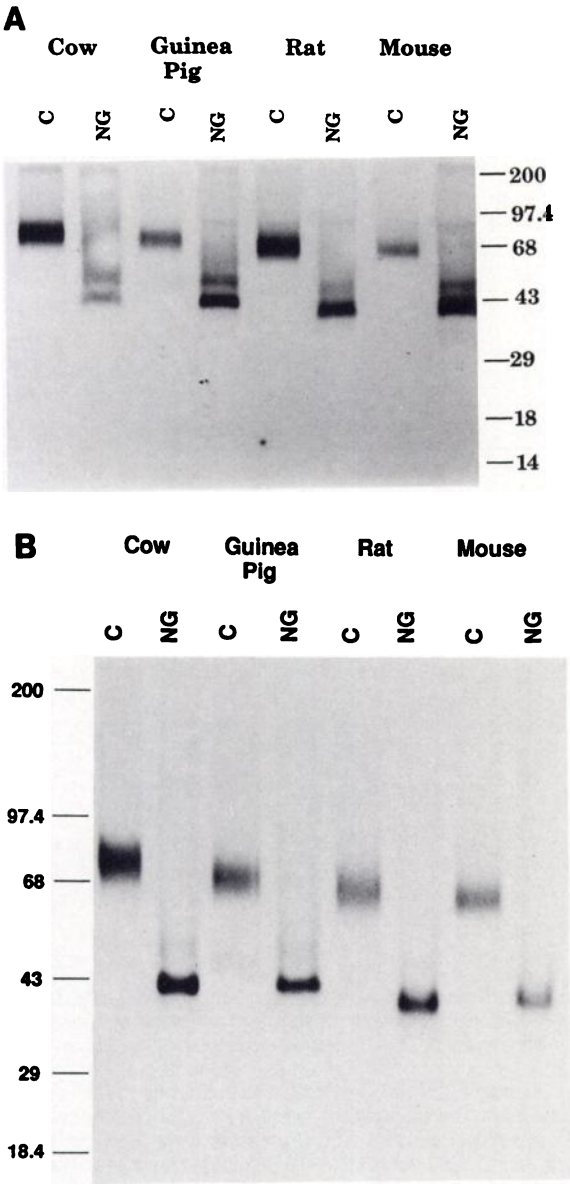

Fig. 6. Effect of *N*-Glycanase on β-[^{3}H]FNA-labeled μ-opioid receptors of the cow, guinea pig, rat, and mouse. WGL eluates of the four species (each containing ~10,000 dpm) were treated with *N*-Glycanase for 3 hr (A) or 6 hr (B) and subjected to SDS-PAGE and fluorography as described in Experimental Procedures. *C*, Control, incubated without enzyme; *NG*, treated with *N*-Glycanase. In both A and B, amounts of radioactivity and protein loaded in each lane were approximately 10,000 dpm and 200 μg, respectively, except in A for the guinea pig and mouse controls, where 5000 dpm and 100 μg were loaded, respectively. Molecular mass standards are in kDa. A, Experiment performed two times; B, experiment performed three times with similar results.

Neuraminidase cleaves terminal sialic acid residues from complex-type carbohydrate chains (38, 39). α-Mannosidase removes terminal mannose from hybrid and high mannose-type carbohydrate chains (38). The results showing that neuraminidase decreases the molecular mass of labeled μ receptors by 6–7 kDa but α-mannosidase has no effect indicate that the carbohydrate chains on the μ-opioid receptor are of the complex type. The finding that α-mannosidase does not reduce the molecular mass of labeled μ receptors is consistent with the finding that solubilized opioid receptors do not bind to concanavalin A affinity columns (25), which bind α-methyl-D-mannoside. *N*-Glycanase, which removes *N*-linked oligosaccharides from asparagine residues and leaves aspartic acid at the glycosylation sites (30, 31), causes the broad band of labeled bovine μ receptors of 70–88 kDa to become a sharp band of 43 kDa. These results indicate that the carbohydrate chains on the μ-opioid receptor are of the complex type and are *N*-linked to asparagine. To our knowledge, this is the first demonstration of the nature of the carbohydrate moieties in μ-opioid receptors. The presence of complex-type, *N*-linked oligosaccharides has been demonstrated for several membrane-bound receptors, including β-adrenergic receptors (26), α_1-adrenergic receptors (27), and insulin receptors (40).

The significant decrease in molecular mass produced by *N*-Glycanase treatment and the sharpness of the deglycosylated protein band indicate that μ receptors are heavily glycosylated and most, if not all, of the carbohydrate chains are *N*-linked at asparagine residues. It is possible that, after *N*-Glycanase treatment, some *O*-linked carbohydrate chains coupled to serine or threonine may remain. It is difficult to characterize *O*-linked oligosaccharides. Chemical cleavage of *O*-linked carbohydrates by the commonly used method of Iyer and Carlson (41) (with NaOH and $NaBH_4$) often leads to hydrolysis of peptide bonds. The enzyme endo-α-*N*-acetylgalactosaminidase (EC 3.2.1.97) catalyzes the hydrolysis of the Gal-β(1,3)GalNAc core disaccharides attached to serine or threonine residues of glycopeptides and glycoproteins. Substitutions on either galactose or *N*-acetylgalactosamine residues interfere with the enzyme activity. GlcNAc-β(1,3)-GalNAc and other structures linked to serine or threonine are not substrates (42). Due to the narrow range of substrates upon which this enzyme can act, we did not use it in our study. On the other hand, the sharpness of the labeled protein band after *N*-Glycanase treatment implies either that the receptor is completely deglycosylated or that there is little heterogeneity in *O*-linked sugars.

After deglycosylation with *N*-Glycanase, bovine β-[^{3}H]FNA-labeled μ-opioid receptors became a sharp and densely labeled band of 43 kDa. This result differs slightly from our previous findings (8). We showed that treatment of bovine β-[^{3}H]FNA-labeled μ-opioid receptors with *N*-Glycanase yielded two labeled bands, one densely labeled at 57 kDa and the other faintly labeled at 49 kDa. Two factors may account for this discrepancy. Firstly, the enzyme treatments were performed differently. In our present study, the *N*-Glycanase treatment was performed in 0.1 M Tris·HCl buffer at pH 7.5, whereas previously it was carried out in 0.2 M sodium phosphate buffer at pH 8.6. At pH 8.6 labeled receptors readily aggregated, whereas at pH 7.5 there was much less aggregation. In addition, there was higher *N*-Glycanase activity for labeled receptors at pH 7.5 than at pH 8.6. At pH 7.5, with *N*-Glycanase treatment for 3 hr, there were two labeled protein bands (Fig. 6A), which are similar to those seen after 18 hr of *N*-Glycanase treatment in 0.2 M sodium phosphate buffer, pH 8.6 (8). When *N*-Glycanase treatment was performed at pH 7.5 for 6 hr, there was only one labeled protein band, of the lower molecular mass (Fig. 6B). Thus, the two labeled protein bands shown in our previous report most likely represent products of incomplete deglycosylation of labeled receptors, due largely to receptor aggregation and less than optimal conditions for the enzyme activity. Secondly, SDS-PAGE was run quite differently in the two studies. Previously we used a discontinuous Laemmli gel in a mini-gel format with Tris-glycine buffer. In the present report, we

changed to a system described by Schagger and von Jagow (32), in a regular-size gel format with Tris-tricine buffer, because it had much larger protein capacity and gave better resolution. The deglycosylated receptor of 49 kDa in the former SDS-PAGE system appears as a 43-kDa band in the latter SDS-PAGE system. Thus, when SDS-PAGE is run differently, the estimation of molecular mass might vary slightly.

The carbohydrate moieties comprise a high percentage of the μ receptor molecular mass. The exact function of the carbohydrate moieties is not known. It was shown that neuraminidase treatment did not affect opioid receptor binding (43). NG108–15 cells cultured in the presence of tunicamycin, an inhibitor of protein *N*-glycosylation, showed a decrease in the number of δ-opioid receptors without a change in the affinity. Tunicamycin treatment did not produce measurable changes in the ability of etorphine to regulate intracellular cAMP production, nor did it potentiate the rate or magnitude of etorphine-induced down-regulation or desensitization of opioid receptors. However, this treatment inhibited the recovery of opioid receptor binding in chronically etorphine-treated cells after agonist removal (37). These findings suggest that glycosylation plays an important role in the structural maturation of δ-opioid receptors and their insertion into plasma membranes but not in ligand binding or activation of the second messenger system. Similarly, inhibition of complex-type carbohydrate chain biosynthesis in smooth muscle cells in culture does not alter the number or the affinity of α_1-adrenergic receptors (27). The carbohydrate moieties of both β_1- and β_2-adrenergic receptors are not important for ligand binding (44, 45) or for coupling to G_s proteins (46). The finding that μ-opioid receptors in the four species studied are glycosylated to different extents, yet the pharmacology of opioid agonist and antagonist binding is essentially the same, supports the notion that the carbohydrate moiety is not important in ligand binding.

Molecular masses of deglycosylated β-[³H]FNA-labeled μ-opioid receptors were estimated to be 43 kDa for the cow and guinea pig, 39 kDa for the rat, and 40 kDa for the mouse. These molecular masses are similar to those of many G protein-coupled receptors, as deduced from nucleotide sequences, including the δ-opioid receptor cloned recently (47, 48). However, it is surprising that the deglycosylated receptors have somewhat different molecular masses in these four species. It is possible that the receptors are not completely deglycosylated. As discussed above, there may still be carbohydrate chains *O*-linked to serine or threonine after *N*-Glycanase treatment. Alternatively, μ-opioid receptor proteins of different molecular masses may represent products of alternate splicing of the gene, similar to the long and short forms of dopamine D_2 receptors (49). In addition, there may be differences in post-translational modifications of μ-opioid receptor proteins among these species, including differences in phosphorylation, palmitoylation, or isoprenylation. Moreover, differential susceptibilities to proteolysis of μ-opioid receptors in these species cannot be ruled out, similarly to what was found for the β-adrenergic receptors (50).

In conclusion, under defined conditions β-[³H]FNA specifically labels μ-opioid receptors in the cow, guinea pig, rat, and mouse. There appears to be species variation in the molecular masses of the labeled μ receptors. The carbohydrate moieties in bovine μ-opioid receptors are of the complex-type, *N*-linked to asparagine. The species variations in molecular mass are mainly due to differences in glycosylation.

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