A Photoactivatable Naltrexone Derivative Labels Glycoproteins of Different Molecular Weight Corresponding to the μ- and κ-Opioid Receptors†

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ABSTRACT: The synthesis and characterization of a novel opioid receptor photoaffinity probe [^{3}H]naltrexyl urea phenylazido derivative ([^{3}H]NUPA) is described. In the absence of light, [^{3}H]NUPA binds with high affinity in a reversible and saturable manner to rat brain and guinea pig cerebellum membranes. Dissociation constants and binding capacities (Scatchard plots) are 0.11 nM and 250 fmol/mg of protein for rat brain and 0.24 nM and 135 fmol/mg of protein for guinea pig cerebellum. Competition experiments indicate that this ligand interacts with high affinity at both μ- and κ-opioid binding sites while exhibiting low affinity at δ sites (K_i = 21 nM). On irradiation, [^{3}H]NUPA incorporates irreversibly into rat brain and guinea pig cerebellum membranes. SDS gel electrophoresis of rat brain membranes reveals specific photolabeling of a 67-kDa molecular mass band. Conversely, a major component of 58 kDa and a minor component of 36 kDa are obtained from [^{3}H]NUPA-labeled guinea pig cerebellum membranes. Different photolabeling patterns are obtained in rat brain ($\mu/\delta/\kappa$, 4/5/1) and guinea pig cerebellum ($\mu + \delta/\kappa$, 1,5/8,5) membranes in the presence of selective opioid ligands indicating labeling of μ and κ sites, respectively. Thus, [^{3}H]NUPA behaves as an efficient photoaffinity probe of μ- and κ-opioid receptors, which are probably represented by distinct glycoproteins of 67 and 58 kDa, respectively.

Soon after the identification of stereospecific opiate binding sites in mammalian brain in the early 1970s (Pert & Snyder, 1973; Simon et al., 1973; Terenius, 1973), the pharmacological experiments described by Martin et al. (1976) and the discovery of the endogenous opioids enkephalins (Lord et al., 1977) provided the first convincing evidence for the presence of multiple opiate receptors. Analysis of the binding profiles of selective ligands [reviewed in Robson et al. (1983) and Leslie (1987)] and of the regional distribution of opioid binding sites in brain and peripheral nervous tissues [reviewed in Mansour et al. (1988)] further established the existence of three major receptor types termed μ, δ, and κ. However, despite the clear antinociceptive effects of their specific ligands, the functional relevance of each opioid receptor type still remains to be elucidated.

Different experimental approaches were employed to characterize individual receptor types. Purification of native receptors by affinity chromatography (Simonds et al., 1985; Cho et al., 1986; Bidlack et al., 1981; Gioannini et al., 1985; Maneckjee et al., 1985; Ueda et al., 1987; Simon et al., 1990) led to substantial enrichment in polypeptides of 40–70 kDa molecular mass. These studies, however, were complicated by the difficulty in identifying the purified species, largely because significant alterations in binding activities occurred after the solubilization and purification processes (Simon et al., 1973; Hasegawa et al., 1987; Simonds et al., 1980; Ruegg et al., 1981; Howells et al., 1982).

Another approach consisted of specifically labeling the target receptor with irreversible ligands prior to or after purification. This technique has been successfully used to identify the previously purified proteins corresponding to the glycine receptor as well as several dopaminergic, adrenergic, and serotonergic receptor subtypes (Wouters et al., 1987; Lomasney et al., 1986; Shorr et al., 1982; Senogles et al., 1988; Regan et al., 1986).

In the case of the opioid receptors, Simonds et al. (1985) covalently labeled a 58-kDa protein in crude membrane preparations from NG 108-15 neuroblastoma–glioma hybrid cells, known to contain only the δ-opioid receptor, with tritiated superFIT,[1] which served as a marker in subsequent purification steps. In rat brain membranes, the δ-specific photoaffinity ligand [^{125}I]azido-DTLET (Bochet et al., 1988) labeled two bands of 44 and 34 kDa, and a 33-kDa band in NG 108-15 cells. Newman and Barnard (1984) showed that a 58-kDa protein from rat brain membranes was affinity labeled by [^{3}H]DALECK at alkaline pH and presumably represented μ-receptor sites. Liu-Chen and Phillips (1987) using [^{3}H]β-FNA as an irreversible probe in bovine striatal membranes obtained on SDS–PAGE a broad-labeled band of molecular mass 68–97 kDa corresponding to μ sites. Ueda et al. (1987) applied a cross-linking technique in rat brain membranes using the κ-specific agonist [^{3}H]dynorphin 1–17 as ligand. The material labeled had molecular masses of 19 and 36 kDa. Such dispersion in molecular weight estimates, which may be due to the source of tissues, to the molecular structure and properties of the probes, or to different analytical systems, precludes any unambiguous identification of the proteins associated with the μ- δ-, and κ-receptors. In one case, however, Howard et al. (1985) demonstrated by covalent cross-linking that [^{125}I]β-endorphin labels both μ- and δ-receptors and that these receptors could be distinguished by molecular weight

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[1] Abbreviations: DAGO, [D-Ala^2,(Me)Phe^4,Gly(ol)^5]enkephalin; DP-DPE, [D-penicillamine^2,D-penicillamine^5]enkephalin; DALECK, Tyr-D-Ala-Gly-Phe-Leu-CH_2Cl; azido-DTLET, Tyr-D-Thr-Gly-pN_3Phe-Leu-Thr; superFIT, (+)-*cis*-3-methylfentanyl isothiocyanate; β-FNA, β-funaltrexamine; EKC, ethylketocyclazocine.

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criteria. The μ- and δ-receptors were associated with 65- and 53-kDa bands, respectively. Considering the range of molecular weight values published for the opioid receptor types, it is not yet possible to conclude whether or not they represent different proteins.

Here we describe the synthesis and characterization of a novel photoactivatable opioid ligand: naltrexyl urea phenylazido derivative (NUPA, **4**). NUPA was also synthesized in a tritiated form with a high specific radioactivity (>70 Ci/mmol). In the absence of light, both compounds reversibly bind with high affinity to the μ- and κ-receptor types. Upon irradiation, [^{3}H]NUPA covalently photoaffinity labels distinct bands corresponding to the μ and κ binding sites, therefore strongly suggesting that these receptor types correspond to different glycoproteic entities.

MATERIALS AND METHODS

N-[^{3}H]Methyl-*N*-(chloroformyl)-*N'*-(*tert*-butoxycarbonyl)-*p*-phenylenediamine was synthesized and radiolabeled by Dr. B. Rousseau at the Commissariat à l'énergie atomique (Saclay, France) as described (Klotz et al., 1991). Its specific radioactivity (77 Ci/mmol) was determined by mass spectrometry. [^{3}H]DTLET [(tyrosyl-[3,5-^{3}H]-D-Thr-Gly-Phe-Leu-Thr) enkephalin derivative, 61 Ci/mmol] was from CEN (Saclay, France), and (–)naloxone was from du Pont de Nemours (Glenolden). Naltrexone hydrochloride was a gift of Francopia (Paris, France), etonitazene was a gift of Ciba-Geigy (Basel, Switzerland), U 50,488 was a gift of Upjohn Co. (Kalamazoo, MI), DTLET was a gift of Dr. B. Roques (Faculté de Pharmacie, Paris, France), (+)naloxone was a gift of Dr. E. J. Simon (New York, NY), and levorphanol tartrate was a gift of Hoffman-La Roche (Basel, Switzerland). Glass-fiber filters (type GF/B) were from Whatman. Electrophoresis chemicals were from Bio-Rad, and premixed electrophoresis molecular weight standards were from Pharmacia (Uppsala, Sweden). All other chemicals were of analytical grade from Prolabo (Paris, France), E. Merck (Darmstadt, FRG), or Fluka (Buchs, Switzerland).

Synthesis of [^{3}H]NUPA

The synthesis of [^{3}H]NUPA is illustrated in Figure 1. The 6β-naltrexamine (**1**) was prepared according to a previously described procedure (Sayre & Portoghese, 1980) with an overall yield of 28%.

*Preparation of [^{3}H]Naltrexone Derivative ([^{3}H]**3**).* *N*-[^{3}H]-Methyl-*N*-(chloroformyl)-*N'*-(*tert*-butoxycarbonyl)-*p*-pheny lenediamine ([^{3}H]**2**, 0.65 μmol, 50 mCi) was concentrated in a Microfuge vial, and the residue was taken up in 80 μL of acetonitrile. 6β-Naltrexamine (**1**, 1.3 μmol) in tetrahydrofuran (THF) (250 μL) was then added, followed by 1.95 μmol of triethylamine in THF (27 μL) at room temperature. After 12-h incubation under nitrogen, the mixture was purified by reverse-phase HPLC using a Waters μBondapak C_{18} column (3.9 × 300 mm) eluted in 0.3% trifluoroacetic acid with a linear gradient of 0–40% acetonitrile at 2 mL/min for 40 min to yield a [^{3}H]naltrexone derivative ([^{3}H]**3**) (R_t = 26 min, yield 30%).

The nonradioactive compound **3** was fully characterized: mp oxalate = 164 °C; mass spectrum *m/e* 589 (M^+). Anal. $C_{35}H_{44}N_4O_{10}$ (oxalate)/$1H_2O$: C, H, N.

*Conversion of [^{3}H]Naltrexone Derivative ([^{3}H]**3**) to [^{3}H]-NUPA.* [^{3}H]Naltrexone derivative ([^{3}H]**3**) (1155 μCi, 15 nmol) was concentrated to dryness in a polypropylene microfuge tube and dissolved in 50 μL of a mixture of trifluoroacetic acid/concentrated HCl (1:1). The solution

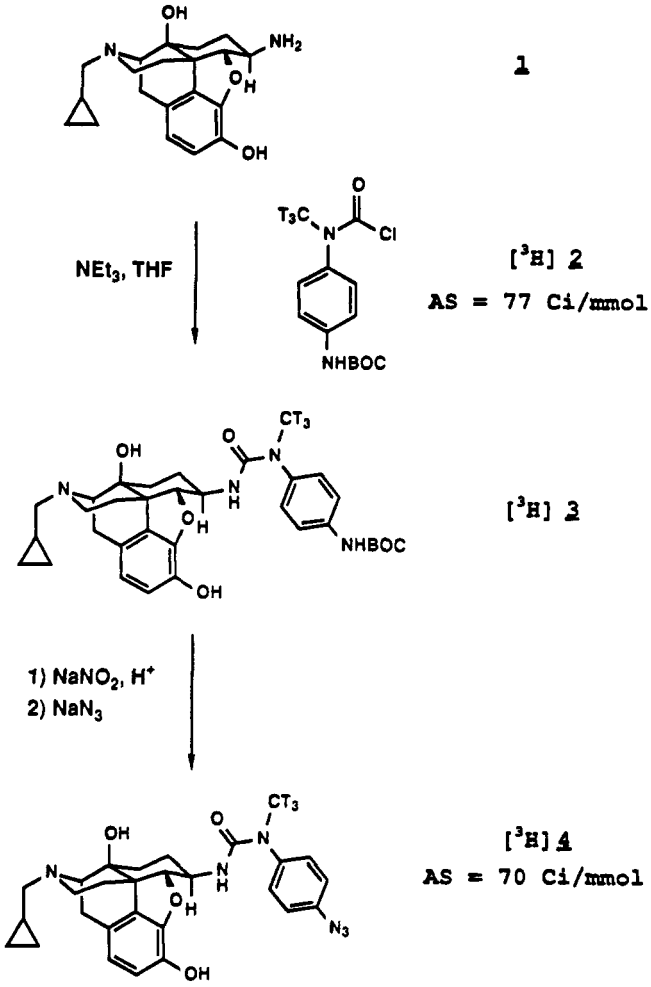

FIGURE 1: Radiochemical synthesis of [^{3}H]NUPA ([^{3}H]**4**).

was allowed to stand for 30 min at room temperature and then cooled to –10 °C. Sodium nitrite (23 nmol) in water (23 μL) was added in small amounts over a period of 20 min. After being stirred for an additional 5 min in the dark, the solution was evaporated to dryness, and the residue was taken up in 50 μL of H_2O/acetonitrile (1:1). Sodium azide (150 nmol in 15 μL of water) was added, and the reaction was stirred at room temperature for 5 min. The azido derivative was purified on a Waters μBondapak C_{18} (3.9 × 300 mm) column and eluted in 0.3% trifluoroacetic acid with a linear gradient of 0–30% acetonitrile at a flow rate of 2 mL/min for 40 min. [^{3}H]NUPA ([^{3}H]**4**) (R_t = 25 min) was shown to comigrate with nonradioactive compound **4** both in the crude reaction mixture and as a purified product. The collected fractions were stored at 4 °C in the dark and were found to be stable for at least 6 months. The overall yield was 30%, and the specific activity of [^{3}H]NUPA ([^{3}H]**4**) was 70 Ci/mmol.

The nonradioactive NUPA was characterized: UV absorption spectrum (λ_{max} 262 nm, ϵ = 19 000); mass spectrum *m/e* (M^+).

Membrane Preparation

Crude membrane fractions were prepared from rat brain and guinea pig cerebellum tissue as described (Galzi et al., 1990a) and stored at a concentration of 9–11 mg of protein/mL (rat brain) or 5 mg/mL (guinea pig cerebellum) in 32 M sucrose at –80 °C.

The NG 108-15 hybrid cell line was cultured in Dulbecco's modified Eagle's medium at 37 °C. Cells grown to confluency

were detached by mechanical agitation, centrifuged at 1000*g* for 10 min, and washed three times in ice-cold culture medium. The resulting packed cell pellets were stored at −80 °C. NG 108-15 membranes were prepared by thawing the frozen cells in 50 mM Tris-HCl/1 mM EDTA buffer (pH 7.4) and homogenizing with a Duall homogenizer. After two successive centrifugations at 1100*g* for 10 min at 4 °C, supernatants were pooled and centrifuged at 54000*g* for 10 min to yield a pellet which was promptly resuspended in binding buffer at a protein concentration of 1–2 mg/mL and stored at −80 °C until use.

Ligand Binding Assays

Membrane preparations were thawed and diluted in 50 mM Tris-HCl/1 mM EDTA buffer (pH 7.4) to obtain a final protein concentration of 0.3 mg/mL (rat brain binding assay), 0.2 mg/mL (guinea pig cerebellum binding assay), or 0.12 mg/mL (NG 108-15 cell binding assay). For saturation experiments, membranes were incubated with [^{3}H]NUPA in the dark for 1 h at 37 °C in a total volume of 1 mL. The nonspecific binding level was determined in the presence of 10^{-6} M naloxone (rat brain binding assay) or 10^{-6} M U 50,488H (guinea pig cerebellum binding assay). For competition experiments, samples (1 mL final volume) were incubated at 37 °C for 30 min with [^{3}H]NUPA (0.5 nM) or [^{3}H]DTLET (1 nM) and various concentrations of unlabeled drugs. Incubation was stopped by filtering the assay mixture over GF/B filters, which were rapidly rinsed three times with 3 mL of ice-cold binding buffer and counted for radioactivity in Biofluor (New England Nuclear) scintillation cocktail. The filters were presoaked in 0.05% polyethylenimine to reduce the nonspecific retention of [^{3}H]NUPA.

Apparent binding affinity constants (K_i values) of drugs were calculated from IC_{50} values, determined in competition experiments, according to the Cheng and Prusoff (1973) relation:

$$K_i = IC_{50}/(1 + L/K_D)$$

where L and K_D are the radioligand concentration and its equilibrium dissociation constant respectively. K_Ds were estimated from Scatchard plots: K_D [^{3}H]NUPA = 0.11 nM (rat brain) or 0.24 nM (guinea pig cerebellum); K_D [^{3}H]-DTLET = 1.1 nM (rat brain) or 1.4 nM (NG 108-15 cells).

Reversible and Irreversible Binding of Nonradioactive NUPA

Reversible Binding. Competition experiments with rat brain membranes were performed using [^{3}H]DAGO (1 nM, 0.24 mg of protein/mL) and [^{3}H]DTLET (1 nM, 0.17 mg of protein/mL) as described in Galzi et al. (1990b).

The reversibility of the binding of the probe was assayed using a filter dissociation procedure: after incubation of rat brain membranes (0.8 mg of protein/mL) for 20 min at 37 °C with NUPA (10 nM), drug dissociation started with a 4-fold dilution of the membrane sample. Aliquots (2 mL) of diluted membranes (0.35 mg of protein) were layered on GF/B filters, and the filter-adsorbed material was repetitively washed at 37 °C for 40 min with Tris–EDTA buffer supplemented with 1 M NaCl prior to the measure of free receptor sites by a [^{3}H]sufentanil binding assay (Ilien et al., 1988).

Irreversible Binding. Aliquots of rat brain membranes (0.8 mg of protein/mL) in 50 mM Tris-HCl/1 mM EDTA buffer (pH 7.4) were incubated for 20 min at 37 °C with 10 nM NUPA. Incubation mixtures (3 mL) were irradiated at 10 °C for 20 min with gentle magnetic stirring in a 1-cm path-length quartz cell. A monochromatic light beam of wavelength 255 nm was focused on the cell to form a 10-mm-high and 2-mm-wide spot. The light intensity was measured (in volts) with a thermopile (Kipp and Zohnen) and adjusted using an iris diaphragm to an energy of 40 μV. The irradiated membrane samples were then repetitively rinsed using the filter dissociation procedure described above in order to remove the noncovalently bound ligand and photoproducts and then were assayed for specific [^{3}H]sufentanil binding. The entire procedure has been detailed elsewhere (Galzi et al., 1990a).

Photoaffinity Labeling with [^{3}H]NUPA

Filter Assay. Rat brain membranes (0.45 mg of protein/mL) were incubated for 30 min at 37 °C with 1 or 3 nM of [^{3}H]NUPA in the presence or absence of naloxone (10^{-6} M) in 50 mM Tris-HCl, pH 7.4/1 mM EDTA buffer containing 1 mM reduced glutathione (GSH) as scavenger and 100 mM NaCl. Following incubation, membrane suspensions (3.3 mL) were photolabeled as described above. After irradiation, all suspensions were incubated with naloxone (10^{-6} M) for 30 min at 37 °C, adsorbed on GF/B filters (0.45 mg of protein/filter), and rinsed 10 times with 3 mL of 37 °C binding buffer supplemented with 100 mM NaCl and 1 μM naloxone. The radioactivity retained by the membranes was measured by scintillation counting.

SDS–PAGE. Membranes (1 mg/mL) were incubated in the dark with 3 nM [^{3}H]NUPA in a total volume of 3.3 mL for 30 min at 37 °C in the presence or absence of opioid agents as indicated. Following incubation, samples were centrifuged at 75000*g* for 20 min. Membrane pellets were resuspended in 2.7 mL of Tris–EDTA buffer supplemented or not with GSH (1 mM) and the protecting agent and were irradiated as described above. After irradiation, [^{3}H]NUPA-labeled membranes were sedimented for 30 min at 15000*g* and prepared for SDS–PAGE.

Electrophoresis was performed according to the method of Laemmli (1970). Samples of 1 mg of protein were incubated for 1.5 h at room temperature with 0.0625 M Tris (pH 6.8), 0.001% bromophenol blue, 4% SDS, 10% glycerol, and 1% β-mercaptoethanol. Aliquots (300 μg of protein) were loaded on slab gels containing a 10% acrylamide separating gel and a 5% stacking gel and were electrophoresed overnight. Protein staining was performed using 0.15% Coomassie brilliant blue in 45% (v/v) methanol/10% (v/v) acetic acid for 1 h and was destained overnight. For quantitative determination of radioactivity, the gel was cut in 2-mm slices. Each slice was placed in a glass scintillation vial containing 400 μL of 30% hydrogen peroxide; the tightly capped vials were heated to 75 °C for 12 h; then 500 μL of 4 M urea and 1% SDS was added. After being shaken the samples were counted following addition of 10 mL of scintillation cocktail. The molecular weights corresponding to the labeled bands were estimated by determining the R_f (from the center of the band) and interpolating this value on a standard curve: log of the molecular weight of known proteins versus the R_f of these proteins.

RESULTS

Synthesis of [^{3}H]NUPA

Figure 1 describes the synthesis of a novel analogue of the potent nonselective opioid receptor antagonist naltrexone. The compound, [^{3}H]NUPA ([^{3}H]**4**), possesses an arylazido group, which has been widely used for photoaffinity labeling (Bayley,

Table I: Reversible and Irreversible Binding of Unlabeled NUPA

assay	recovery of specific [^{3}H]sufentanil binding (%)	
	$-h\nu$[a]	$+h\nu$[b]
membranes	100	98
membranes + 1 μM naloxone	111	105
membranes + 3 nM NUPA		64
membranes + 6 nM NUPA		53
membranes + 10 nM NUPA	97	43
membranes + 10 nM NUPA + 1 μM naloxone		70

[a] Following incubation for 20 min at 37 °C with 10 nM NUPA, nonirradiated membranes (0,2 mg of protein/mL; 2 mL) were layered on filters and washed for 40 min, and the remaining [^{3}H]sufentanil binding sites were determined. The filter procedure and the recovery in free μ-opioid receptor sites, monitored by specific binding of [^{3}H]sufentanil were performed as described previously (Ilien et al., 1988). [b] Membrane aliquots (0,8 mg of protein/mL; 2 mL), preincubated at 37 °C with various concentrations of NUPA (10, 6, and 3 nM) in the absence or presence of naloxone (1 μM), were irradiated at 255 nm (40 μV) for 20 min. Thereafter, irradiated membranes were submitted to the same procedure described above for nonirradiated samples ($-h\nu$).

1983), and a high specific radioactivity (70 Ci/mmol) essential for the detection of receptors present in very low concentration.

The synthesis of the tritiated precursor ([^{3}H]**3**) took advantage of a tritritiated heterobifunctional reagent ([^{3}H]-**2**) (Klotz et al., 1991) possessing a carbamoyl chloride group as an electrophilic center and a protected amine as a precursor of the photoactivatable group. The coupling reaction with the nucleophilic primary amine of 6β-naltrexamine (**1**) afforded only the required compound ([^{3}H]**3**) (30% yield), which upon purification coeluted with the nonradioactive compound **3** in several chromatographic systems. Conversion of the protected 4-aminophenyl group to the corresponding 4-azidophenyl moiety, via an intermediate diazonium salt, has been achieved in a "one pot" procedure with an overall yield of 30%. The radioactive compound [^{3}H]**4** was purified by HPLC and its specific activity (70 Ci/mmol) determined. [^{3}H]NUPA ([^{3}H]**4**) was shown to comigrate on HPLC with the characterized nonradioactive NUPA (**4**).

Reversible and Irreversible Binding of NUPA to Opioid Receptors

Reversible Binding of NUPA. Binding affinity constants of NUPA (**4**) were determined from competition experiments, in the absence of light, using a μ-selective ([^{3}H]DAGO) and a δ-selective ([^{3}H]DTLET) radioligand. The modification at position 6 of naltrexone led to a slight increase in affinity of NUPA as compared to naltrexone for μ sites since the estimated K_i value using [^{3}H]DAGO was 0.25 nM (K_i = 1.8 nM for naltrexone), whereas NUPA showed a much lower potency in competing for [^{3}H]DTLET binding to the δ sites of rat brain membranes (K_i = 21 nM; naltrexone K_i = 6.7 nM).

Reversibility of the photosensitive drug binding from the μ sites was quantitatively assessed by a filter dissociation technique at high ionic strength, coupled to a filter [^{3}H]-sufentanil binding assay (Ilien et al., 1988). As shown in Table I, the binding of the probe **4** (10 nM) was fully reversible in the absence of light after a 40-min dissociation step on filters. This property allows its use in further photoinactivation experiments.

Irreversible Binding of NUPA. After incubation of rat brain membranes with NUPA (**4**), irradiation of the reaction mixture followed by washing of the membranes (Table I) led to a decrease in μ-opioid binding sites. In the absence of the probe, all of the μ-receptor sites were recovered. NUPA (**4**) produced a concentration-dependent μ-sites inactivation, reaching 57% alkylation in the presence of the probe at 10 μM. This irreversible inactivation of μ-opioid binding sites by NUPA was partially prevented by the presence of an excess (10^{-6} M) of naloxone. Such experiments make NUPA a suitable tool, available under radioactive form, to investigate opioid receptor types.

Reversible Binding of [^{3}H]NUPA

The reversible binding properties of [^{3}H]NUPA ([^{3}H]**4**) were assessed on membrane preparations of rat brain, NG 108-15 cells, and guinea pig cerebellum.

Rat Brain Membranes. As shown in Figure 2A, [^{3}H]NUPA bound to rat brain membranes in a saturable manner with a dissociation constant of 0.11 ± 0.02 nM to an apparently homogeneous population of binding sites. This K_D value is consistent with the K_i value deduced from [^{3}H]DAGO competition experiments using unlabeled NUPA (**4**) in rat brain membranes (K_i = 0.25 nM). The B_{max} (250 ± 20 fmol/mg of protein) is in good agreement with the amount of binding sites determined with μ-selective radioligands (Leysen et al., 1983; Ilien et al., 1988) in similar membrane preparations. When the saturation experiment was conducted in the presence of a δ-selective ligand (50 nM DPDPE), the dissociation constant and B_{max} remained unchanged. We checked that, under these conditions, the binding of [^{3}H]NUPA to rat brain membranes was completely reversible and insensitive to the addition of sodium chloride to the binding buffer up to 200 nM (data not shown).

In order to analyze in more detail the receptor-type specificity of [^{3}H]NUPA, competition experiments (Figure 3A) were conducted. In fact, receptor-bound [^{3}H]NUPA (0.5 nM) is displaced by several agents with a representative opioid receptor selectivity. All agents tested yielded steep, monophasic competition curves, indicating the presence of an apparently homogeneous population of binding sites. The estimated K_i values for fentanyl (4.6 nM), etonitazene (1.3 nM), and naloxone (1.8 nM) are in good agreement with those obtained using μ-selective radioligand [^{3}H]DAGO (Gillan & Kosterlitz, 1983; Ilien et al., 1988). The δ-selective ligand DTLET was also found to displace [^{3}H]NUPA binding with a K_i value of 14.3 nM. This value indicates that the competition occurred rather on μ sites (K_D DTLET μ = 25 nM) rather than on δ sites (K_D DTLET δ = 1.3 nM, Zajac et al., 1983). Thus, saturation and competition experiments suggest that, at concentration below 10 nM, [^{3}H]NUPA does not significantly occupy δ sites.

Guinea Pig Cerebellum Membranes. In the absence of light, [^{3}H]NUPA binds with high affinity in a saturable and specific manner to opioid receptors present in guinea pig cerebellum membranes (Figure 2B), which are enriched in κ-receptors (Kosterlitz et al., 1981). The dissociation constant and binding capacity derived from Scatchard analysis were K_D = 0.24 ± 0.03 nM and B_{max} = 135 ± 20 fmol/mg of protein.

Competition experiments between [^{3}H]NUPA and unlabeled opioids were conducted (Figure 2B). U 50,488H, a κ-selective ligand (Vonvoigtlander et al., 1983) was 3-fold more potent than levorphanol, which was approximately equipotent with naloxone. The rank order of potency of these drugs in competing for [^{3}H]NUPA binding agrees well with that for the binding of the κ-selective ligand [^{3}H]EKC in the guinea pig cerebellum membranes (Frances et al., 1985; James & Goldstein, 1984).

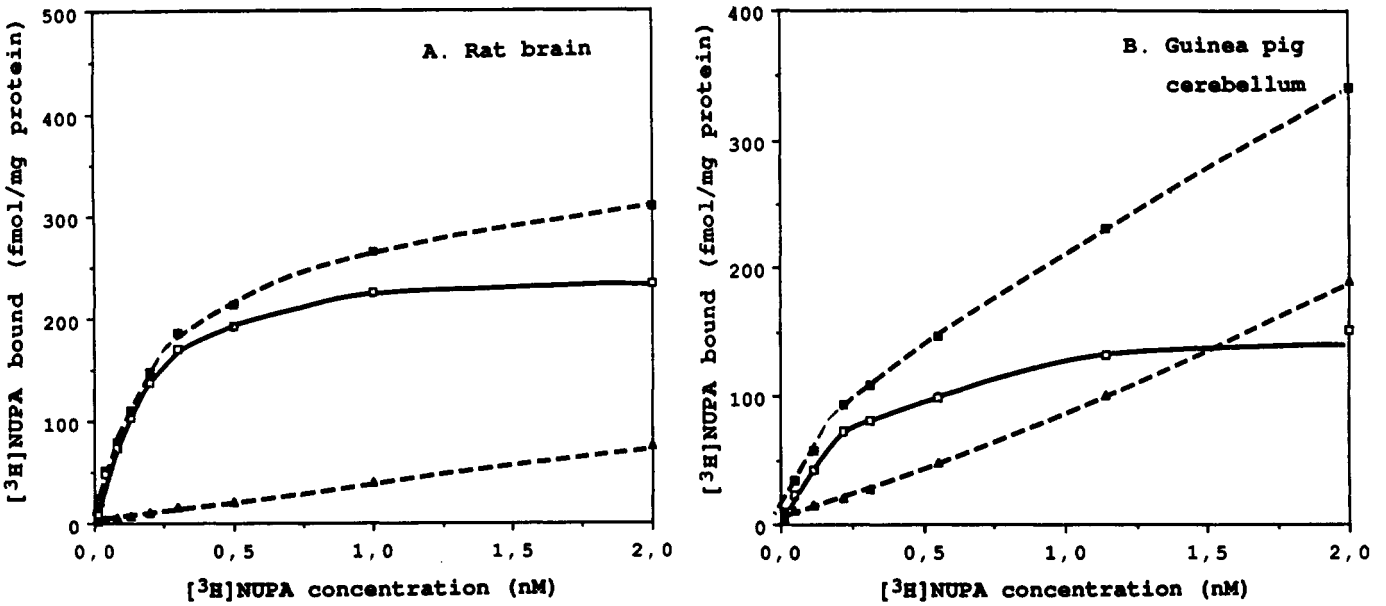

FIGURE 2: Saturation curves and Scatchard analyses for the binding of [^{3}H]NUPA to rat brain (A) and guinea pig cerebellum (B) membranes. Each assay tube (1 mL) contained 300 μg (A) or 200 μg (B) of protein and the indicated concentrations of [^{3}H]NUPA in the absence or presence of 10^{-6} M naloxone (A) or 10^{-6} M U 50,488H (B). Binding assays were performed as described under Materials and Methods. Total binding (■), nonspecific binding (▲), and specific binding (□) are shown. Scatchard analysis of specific binding yielded K_D = 0.11 nM and B_{max} = 250 fmol/mg of protein in rat brain membranes (A) and K_D = 0.24 nM and B_{max} = 135 fmol/mg of protein in guinea pig cerebellum membranes (B). The data are consistent with a single class of binding sites in each tissue. Each point is the average of triplicate determinations. Experiments were repeated two times with comparable results.

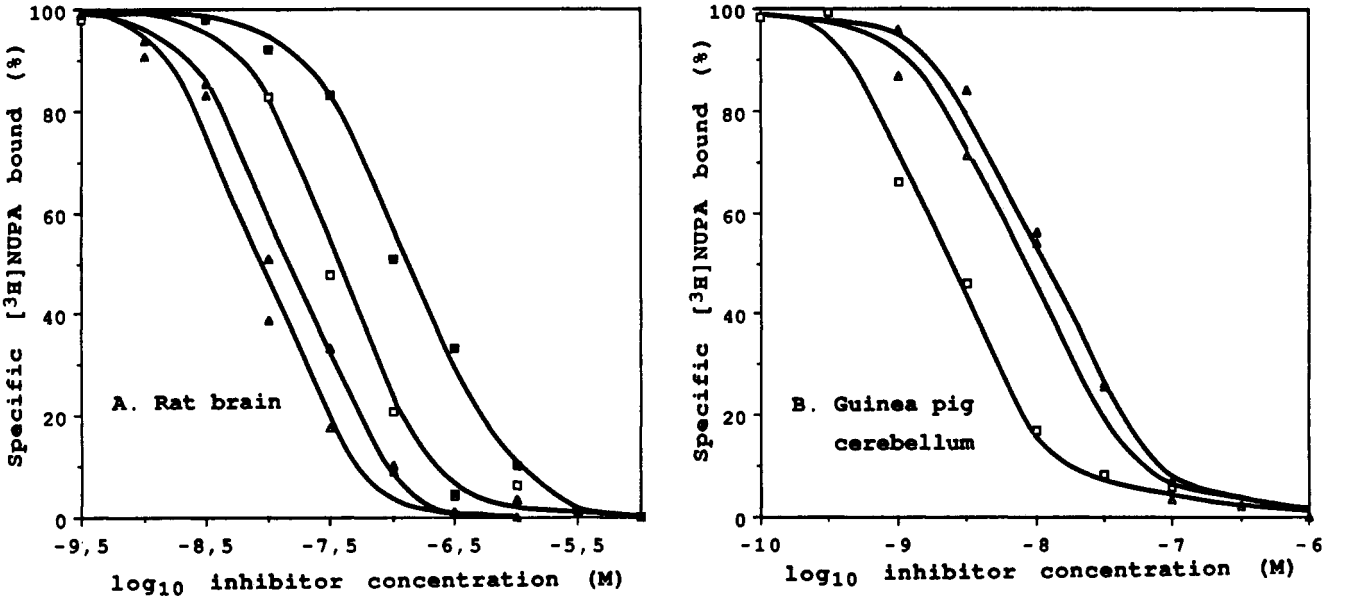

FIGURE 3: Competition by unlabeled opioids for the binding of [^{3}H]NUPA to rat brain (A) and guinea pig cerebellum (B) membranes. Experiments were performed as described under Materials and Methods in the presence of 0.5 nM [^{3}H]NUPA and various concentrations of fentanyl (□), etonitazene (△), naloxone (▲), and DTLET (■) for (A) and U 50,488H (□), levorphanol (△), and naloxone (▲) for (B). Results are expressed as the percentage of the specific [^{3}H]NUPA binding. Nonspecific binding was determined in the presence of 10^{-6} M naloxone for (A) and 10^{-6} M U 50,488H for (B). 100% specific binding represents approximately 150 fmol/mg of protein for (A) and 92 fmol/mg of protein for (B). Data are representative of two to three independent experiments.

NG 108-15 Membranes. In our hands, no significant specific binding of [^{3}H]NUPA could be detected in membranes prepared from NG 108-15 cells. However, nonradioactive NUPA (4) competes for the binding of the radioligand [^{3}H]-DTLET to the δ-opioid receptor of NG 108-15 cells membranes (Figure 4). For comparison, the K_i values found in NG 108-15 membranes for naloxone (73 nM) and DTLET (1.2 nM) are in good agreement with the K_D values ([^{3}H]naloxone = 73 nM and [^{3}H]DTLET = 1.04 nM) derived from saturation experiments with the corresponding radiolabeled ligands in the same system (Akiyama, 1985; Bochet et al., 1988).

In summary, in the absence of light, [^{3}H]NUPA binds in a reversible manner with high affinity to the μ- and κ-opioid receptor types, and a preferential occupation of these sites can be achieved depending on the tissue used.

Photoaffinity Labeling of Opioid Receptors

To assess the usefulness of the radiolabeled probe in identifying opioid receptors through photolabeling experiments, we investigated labeling of tissues rich in a particular type of opioid binding site: rat brain ($\mu/\delta/\kappa$ 4/5/1, Mansour

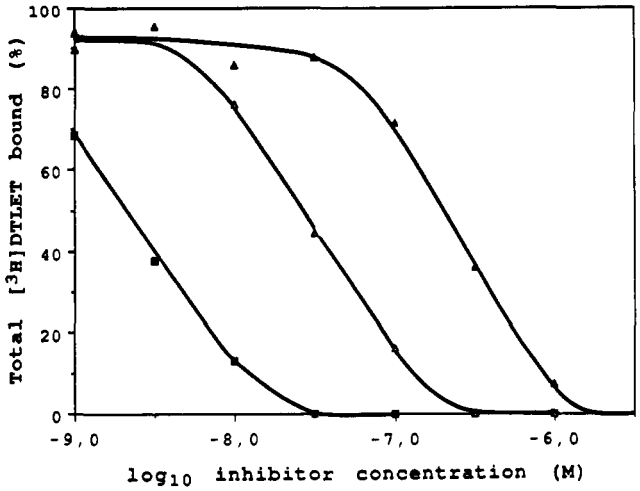

FIGURE 4: Inhibition of [^{3}H]DTLET binding to NG 108-15 membranes by unlabeled NUPA and other opioid compounds. Competition of NUPA for [^{3}H]DTLET binding was assessed as described under Materials and Methods in the presence of 1 nM [^{3}H]DTLET and various concentrations of naloxone (▲), DTLET (■), and **4** (△). Results are expressed as the percentage of the total [^{3}H]DTLET binding and K_is were calculated using a K_D of 1.04 nM for [^{3}H]DTLET. Results shown are from a typical experiment performed in duplicate where 100% represents 2100 dpm ± 100/assay.

Table II: Concentration Response of Photolabeling by [^{3}H]NUPA As Detected by Filter Method[a]

[^{3}H]NUPA concn (nM)	[^{3}H]NUPA radioactivity remaining on filters (dpm/0.45 mg of protein)	
	$+h\nu$	$+h\nu$ + 1 μM naloxone
1	2 262	1 826
3	6 148	5 236

[a] Rat brain membranes (0.45 mg of protein/mL) were preincubated with 1 or 3 nM of [^{3}H]NUPA for 30 min at 37 °C in a Tris–EDTA buffer containing 1 mM GSH and 100 mM NaCl in the absence or presence of 1 μM naloxone. After irradiation at 255 nm for 20 min, samples were incubated for 30 min with 1 μM naloxone and rinsed 10 times with 3 mL buffer supplemented with 1 μM naloxone. Data are from two experiments in triplicate.

et al., 1988), NG 108-15 cells (δ), and guinea pig cerebellum μ+δ/κ 1.5/8.5, Kosterlitz et al., 1981).

Rat Brain Photolabeling: Filter Assay. Rat brain membranes preincubated with [^{3}H]NUPA were irradiated in the presence of 1 mM GSH as a nitrene scavenger, adsorbed to filters (0.45 mg of protein/filter), and subjected to repetitive rinsing steps with a washing buffer supplemented with 1 μM naloxone. Table II shows the [^{3}H]NUPA radioactivity remaining on filters after photolabeling of rat brain membranes with varying concentrations (1 and 3 nM) of [^{3}H]NUPA in the presence or absence of naloxone (1 μM). The incorporation of radioactivity appears to be naloxone-sensitive and dependent on the probe concentration. These data clearly showed a protectable radioactive photoincorporation into rat brain membranes, a result which confirms the observation made with the nonradioactive compound (**4**).

Rat Brain Photolabeling: SDS–PAGE. In order to identify the labeled polypeptide(s), analysis of the photolabeled membranes by SDS electrophoresis was carried out. Figure 5 shows the results obtained when opioid receptors of rat brain membranes were photolabeled in the presence of [^{3}H]NUPA (3 nM) and then subjected to SDS–PAGE. An optimal ratio between specific and total photolabeling was obtained upon removal by centrifugation of excess free probe after the incubation prior to UV irradiation. Under these conditions,

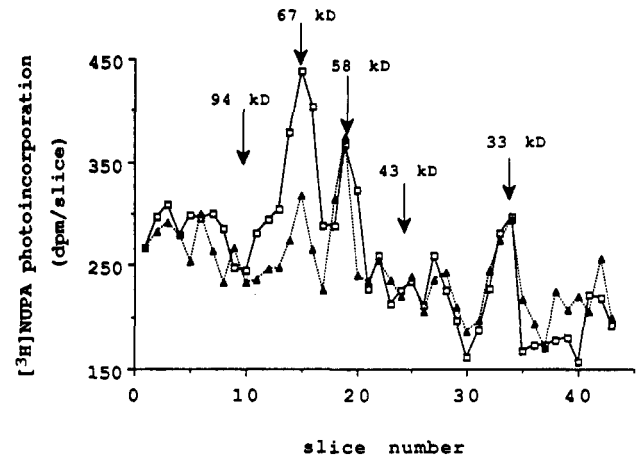

FIGURE 5: Photoaffinity labeling pattern of rat brain membranes by [^{3}H]NUPA. Aliquots of membranes (1 mg of protein/mL) were incubated with [^{3}H]NUPA (3 nM) alone or with naloxone 10^{-6} M, washed, and photolyzed as described under Materials and Methods. Samples were subjected to SDS–PAGE, and after Coomassie blue staining, gels were cut in 2-mm slices. Radioactivity recovered from the SDS–polyacrylamide gel slices was measured as described: (□) membrane preparation labeled by [^{3}H]NUPA and (▲) same experiment in the presence of naloxone 10^{-6} M. The results shown are representative of four experiments. Arrows indicate the relative molecular mass of known proteins used as standards.

Table III: [^{3}H]NUPA Incorporation into Molecular Mass = 67 kDa Band in Presence of Selected Protecting Agents[a]

protecting agent	radioactivity incorporated into 67-kDa band (% of control)
none	100
DPDPE, 50 nM	97
U 50, 488H, 100 nM	93
fentanyl, 100 nM	22
(−)naloxone, 1 μM	18
(+)naloxone, 1 μM	78

[a] Photolabeling of rat brain membranes was performed in the absence or presence of different protecting agents, at the indicated concentration, as described under Materials and Methods. Radioactivity incorporated into the molecular mass = 67 kDa band was counted, and the data are expressed as a percentage of the maximum incorporation measured in the absence of any protecting agent where 100% represented 1760 dpm. Results are from three independent experiments in duplicate.

[^{3}H]NUPA incorporates mainly into three bands at 67, 58, and 33 kDa. When the labeling was performed in the presence of an excess (1 μM) of naloxone, the radioactivity incorporation was inhibited in the 67-kDa band only. Both bands at molecular mass = 58 and 33 kDa did not appear to be receptor-specific since they were not protected by naloxone.

In order to establish the pharmacological specificity of the covalent labeling of the molecular mass = 67 kDa peptide, labeling of rat brain membranes was performed in the presence of various opioid drugs. As shown in Table III, the μ-selective agonist fentanyl (100 nM) provided significant protection of the labeling of the 67-kDa polypeptide (78% inhibition). In contrast, when the incubation was carried out in the presence of 50 nM DPDPE (δ-selective) or 100 nM U 50,488H (κ-selective), at concentrations which would completely inhibit reversible binding to their respective receptors, the incorporation of [^{3}H]NUPA into the molecular mass = 67 kDa band was inhibited by less than 10%. Moreover, a 1 μM (−)naloxone completely blocked [^{3}H]NUPA photoincorporation whereas the (+) isomer was nearly inert. These results indicate that the photoincorporation follows the pharmacological specificity of the μ-opioid receptor and suggest that the molecular mass

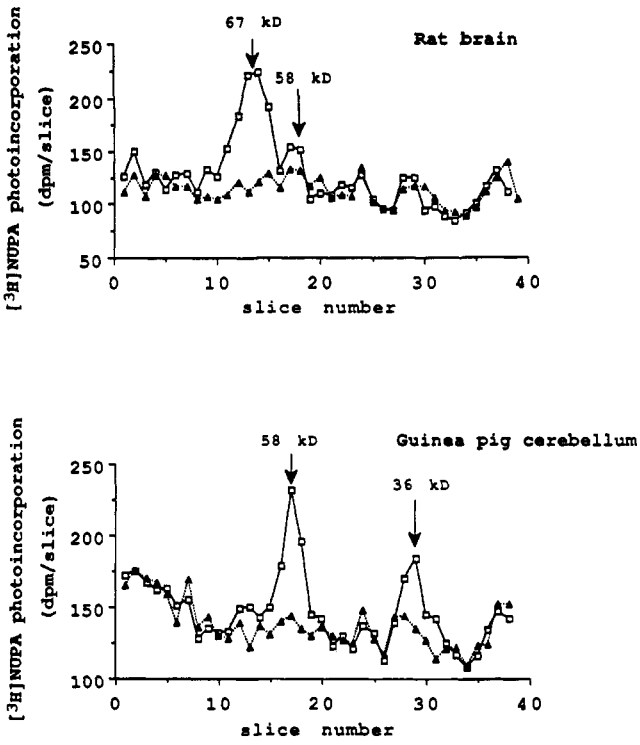

FIGURE 6: Comparison of [^{3}H]NUPA photolabeling in rat brain (A) and guinea pig cerebellum (B) membranes in the presence of GSH. Membranes (1 mg of protein/mL) were incubated with [^{3}H]-NUPA (3 nM) alone (□) or with 1 μM naloxone (▲), washed, and irradiated in the presence of 1 mM GSH. Samples (300 μg of protein) were subjected to SDS–PAGE, and radioactivity was quantified as described under Materials and Methods. The results shown are representative of three experiments.

= 67 kDa peptide contains the ligand binding site of the μ-opioid receptor.

A further increase in the ratio between specific and total labeling could be obtained with the inclusion of GSH (1 mM) as a nitrene scavenger during photolysis (Bayley & Knowles, 1978; Ruoho et al., 1984). Figure 6A shows the pattern obtained when rat brain membranes were photoaffinity labeled with [^{3}H]NUPA in the presence of 1 mM GSH. The photoligand still incorporated into a polypeptide with molecular mass = 67 kDa on SDS–PAGE while the bands at molecular mass = 33 and 58 kDa were markedly reduced. Surprisingly, the labeling of the 58-kDa band appeared partially sensitive to naloxone (1 μM), suggesting the possibility of an opioid binding component in this molecular weight range.

Using these optimized conditions, the specific irreversible incorporation of [^{3}H]NUPA into the molecular mass = 67 kDa band ranged from 7 to 10% of the ligand that was specifically and reversibly bound. Further evidence for the specificity of labeling is provided by electrophoretic analysis of experiments conducted in the presence of varying concentrations of [^{3}H]NUPA (data not shown). The amount of specific radioactivity incorporated into the 67-kDa band increased with the [^{3}H]NUPA concentration and reached a maximum at a radioligand concentration of 3 nM.

Photolabeling of Guinea Pig Cerebellum Membranes. To further clarify the assignment of the labeled peptides to receptor types (i.e., μ vs κ), photoaffinity labeling experiments were carried out in guinea pig cerebellum and in NG 108-15 cells. Figure 6B shows the electrophoretic pattern obtained after photolabeling of guinea pig cerebellum membranes in the presence of 3 nM of [^{3}H]NUPA and 1 mM GSH. A major labeled band at molecular mass = 58 kDa and a minor one at 36 kDa are detected. Incorporation into the molecular mass = 58-kDa peptide was blocked by an excess of the antagonist naloxone (1 μM) (data not shown) or the agonist U 50,488H (1 μM). The effectiveness of U 50,488H in blocking incorporation into the 58-kDa polypeptide demonstrates the κ-opioid specificity of this labeled band and suggests that the specifically labeled [^{3}H]NUPA molecular mass = 58 kDa band in rat brain membranes (Figure 6A) may represent labeling of the κ-receptor fraction of opioid binding sites present in this membrane preparation. As calculated from the radioactivity incorporated into the molecular mass = 58-kDa band and the enrichment of guinea pig cerebellum membranes in κ-receptor (B_{max} = 135 fmol/mg of protein), the efficiency of incorporation of [^{3}H]NUPA into the 58-kDa band was estimated to be about 7%. The labeling of the lower molecular weight polypeptide (molecular mass = 36 kDa) was partially inhibited by either naloxone (1 μM) (data not shown) or U 50,488H (1 μM). Further work will be required to determine the specificity of this 36-kDa labeled band.

NG 108-15 Cells Labeling. Photolabeling of NG 108-15 cell membranes was performed in the presence of [^{3}H]NUPA using the same experimental procedures as described for rat brain photolabeling experiments, including protein concentration, time, and energy of irradiation. However, for [^{3}H]-NUPA concentrations ranging from 30 to 300 nM, no specific radioactivity incorporation was observed.

DISCUSSION

We report the synthesis of a new photoactivatable naltrexyl urea phenylazido derivative (NUPA) as a potent stereospecific photoaffinity label of μ- and κ-opioid receptors.

Reversible binding studies with NUPA (**4**) showed that the addition of an aryl moiety at position 6 of naltrexone enhanced the affinity of the compound for the μ-opioid receptor 5-fold (K_i NUPA = 0.25 nM) (Jiang et al., 1977; Sayre et al., 1983; Tam & Liu-Chen, 1986) whereas it exhibits about 100-fold lower affinity for δ sites (K_i NUPA = 21 nM), compared to naltrexone itself. This binding is totally reversible in the dark, whereas after irradiation the azido derivative behaves as an irreversible ligand of [^{3}H]sufentanil binding sites (57% of μ sites alkylated at 10 nM NUPA). This irreversible inactivation was partially prevented by the opioid antagonist naloxone. These data suggested the usefulness of the probe in radioactive form for visualization and identification of the μ-opioid binding site and perhaps the δ and κ sites.

The synthesis of tritiated NUPA ([^{3}H]NUPA) took advantage of the availability of a heterobifunctional radioactive precursor of arylazido compounds, *N*-[^{3}H]methyl-*N*-(chloroformyl)-*N'*-(*tert*-butoxycarbonyl)-*p*-phenylenediamine ([^{3}H]-**2**) (Klotz et al., 1991), which can be conveniently coupled to a nucleophilic group of any ligand. In our case, this reaction was performed on 6β-naltrexamine (**1**) (Sayre & Portoghese, 1980) as starting material and was transformed to the azido derivative, yielding a new photoactivatable opioid ligand of sufficiently high specific radioactivity (70 Ci/mmol) to detect opioid binding sites.

Saturation experiments on rat brain and guinea pig cerebellum show that [^{3}H]NUPA exhibits high affinity binding to both μ- and κ-opioid receptors (K_D μ = 0.11 nM; K_D κ = 0.24 nM).

Irreversible experiments using the filter method revealed a protectable radioactive incorporation into the filter-trapped membranes. The specific incorporation of radioactivity retained on filters represents a significant fraction of the total amount of radioactivity 19% and 15%, respectively, at 1 and

3 nM of [^{3}H]NUPA. Analysis by SDS electrophoresis of the photolabeled rat brain membranes revealed a major labeled band of molecular mass = 67 kDa and showed that the amount of radioactivity incorporated into this band increased linearly with increasing [^{3}H]NUPA concentration up to 3 nM. Comparison of the filter assay with SDS–PAGE indicates that the protectable radioactivity remaining on filters occurs in a manner closely paralleling the [^{3}H]NUPA incorporation into the 67-kDa band until a 3 nM probe concentration is reached.

As calculated from the amount of radioactivity specifically incorporated into the 67-kDa band and the number of μ binding sites present in the membrane preparation (250 fmol/mg of protein), the efficiency of incorporation of [^{3}H]NUPA ranges from 7 to 10% of the total μ binding sites. This efficiency agrees well with the values for the efficiency of incorporation of many arylazide derivatives into various enzymes and other hormone receptors (Kerlavage & Taylor, 1980). However under the same photolabeling conditions with the nonradioactive compound, about 36% of μ binding sites are irreversibly blocked. Variation in dissociation procedure efficacy (filter assay versus electrophoresis) could account for this apparent discrepancy.

Photolabeling of rat forebrain membranes, which contain approximately 40% μ, 50% δ, and 10% κ sites, revealed a major labeled band of molecular mass = 67 kDa. The inhibition of the labeling by an excess of naloxone or fentanyl and the slight attenuation of photoincorporation observed with DPDPE or U 50,488H are consistent with a μ specificity for the labeling of the molecular mass = 67 kDa polypeptide. The molecular mass of 67 kDa closely correlates with apparent sizes estimated by affinity labeling experiments with [^{3}H]β-FNA (68–97 kDa) (Liu-Chen & Phillips, 1987) and cross-linking studies (65 kDa) (Howard et al., 1985, 1986).

Analysis of photolabeled guinea pig cerebellum membranes by SDS electrophoresis revealed a major band at molecular mass = 58 kDa and a minor band at molecular mass = 36 kDa. The complete inhibition of [^{3}H]NUPA incorporation into the molecular mass = 58 kDa band by naloxone or U 50,488H, and the high proportion of κ sites in the guinea pig cerebellum preparations is consistent with the photolabeling of κ sites.

A 58-kDa band, naloxone sensitive, was also found labeled, to a low extent, in the rat brain membranes. Also, as limited proteolysis cannot definitely rule out, it is interesting to note that the ratio of radioactivity incorporated into the 67- and 58-kDa peptides (10:1) from rat brain photolabeling patterns is in good agreement with the proportion of μ/κ binding sites detected in this tissue (Mansour et al., 1988; Kosterlitz et al., 1981).

Previous molecular mass estimate by affinity chromatography of solubilized frog brain membranes (65 kDa) (Simon et al., 1987, 1990) or by purification of the κ-opioid receptor from human placenta (63 kDa) (Ahmed et al., 1989) are in reasonable agreement with our proposed molecular size for the κ binding site (58 kDa). The efficiency of irreversible incorporation (7–10%) of [^{3}H]NUPA into the molecular mass = 67 kDa band from rat brain membranes was apparently identical to that found on guinea pig cerebellum membranes for the molecular mass = 58 kDa labeled band, suggesting that the coupling ability of [^{3}H[NUPA was the same for each receptor type. Guinea pig cerebellum photolabeling patterns also showed a minor labeled peptide at molecular mass = 36 kDa. Howard et al. (1985) identified a minor band at 38 kDa in rat brain membranes by covalent cross-linking, which was attributed to proteolytic fragments or distinct binding peptides cross-linked to [^{125}I]β-endorphin at a lower efficiency. This low molecular weight band has also been described in cross-linking experiments on rat brain membranes using a κ-specific agonist, [^{3}H]dynorphin 1-17 (Ueda et al., 1987). The relationship of this 36-kDa labeled band to opioid receptor structure remains unclear.

Despite many attempts, we did not succeed in labeling the δ-opioid receptor. Although NG 108-15 membranes are considerably enriched in δ-opioid receptor, no specific incorporation of radioactivity could be detected using [^{3}H]NUPA at concentrations up to 300 nM. These results could be explained by the lower affinity of [^{3}H]NUPA for the δ sites (K_i = 25 nM) and/or insufficient specific radioactivity of the probe. The complete absence of photoincorporation of [^{3}H]-NUPA into NG 108-15 cell membranes gives strong support to the μ-specificity of the molecular mass = 67 kDa band observed in rat brain membranes.

Based on these results, we propose that μ binding sites reside on a peptide of 67 kDa apparent molecular mass, whereas κ sites reside on a peptide of 58 kDa. The reproducibility of the observed labeling patterns and the marked difference in the molecular weights determined for μ and κ binding proteins lend support to the validity of the existence of distinct glycoproteic species. As proposed by Gioannini et al. (1982), the difference in molecular size for μ and κ binding proteins may reflect differences in their primary structure or different degree or type of glycosylation of the same protein. The identification of the amino acid residues labeled in those two proteins may allow a more direct insight of these structural differences.

In summary, as observed by Howard et al. (1985) for the μ- and δ-opioid receptor types, our labeling data support the notion that μ and κ types consist of different glycoproteic species. Furthermore, the sodium chloride insensitivity of [^{3}H]NUPA binding makes it a potent photoaffinity label of solubilized and purified species.

The coexistence of multiple receptor types seems to be a common feature of the members of the large family of G-protein-coupled receptors. Interestingly, gene cloning studies have shown that for some neurotransmitter receptors, such as the muscarinic and adrenergic receptors, the molecular heterogeneity observed using photolabeling techniques (Leeb-Lundberg et al., 1984; Lavin et al., 1983) or purification processes (Shorr et al., 1982) can be correlated to the existence of distinct gene products.

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