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Synthesis and Characterization of 4,4-Difluoro-4-bora-3a,4a-Diaza-*s*-Indacene (BODIPY)-Labeled Fluorescent Ligands for the Mu Opioid Receptor

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ABSTRACT. A series of opioid ligands utilizing the 4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene (BODIPY) fluorophores 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-propionic acid or 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-*s*-indacene-3-propionic acid were synthesized and characterized for their ability to act as a suitable fluorescent label for the mu opioid receptor. All compounds displaced the mu opioid receptor binding of [^{3}H]Tyr-D-Ala-Gly-(Me)Phe-Gly-ol in monkey brain membranes with high affinity. The binding of fluorescent ligands to delta and kappa receptors was highly variable. 5,7-Dimethyl-BODIPY naltrexamine, "6-BNX," displayed subnanomolar affinities for the mu and kappa opioid receptors (K_i 0.07 and 0.43 nM, respectively) and nanomolar affinity at the delta (K_i 1.4 nM) receptor. Using fluorescence spectroscopy, the binding of 6-BNX in membranes from C_6 glioma cells transfected with the cloned mu opioid receptor was investigated. In these membranes containing a high receptor density (10–80 pmol/mg protein), 6-BNX labeling was saturable, mu opioid specific, stereoselective (as determined with the isomers dextrorphan and levorphanol), and more than 90% specific. The results describe a series of newly developed fluorescent ligands for the mu opioid receptor and the use of one of these ligands as a label for the cloned mu receptor. These ligands provide a new approach for studying the structural and biophysical nature of opioid receptors. BIOCHEM PHARMACOL **54**;12:1315–1322, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. mu opioid receptor; fluorescence; BODIPY; receptor binding

The availability of fluorescently labeled ligands for a variety of receptors has permitted the examination of these systems in ways not provided by approaches based on radiolabeled ligands. Utilizing fluorescent ligands, the kinetics of ligand-receptor association and dissociation were examined [1], as well as the interaction of ligand, receptor, and G protein** [2, 3]. Lateral mobility of receptors [4–6] and G proteins [7] within the plasma membrane of intact cells was investigated by fluorescence recovery after photobleaching. Determination of the distance between the epidermal growth factor receptor binding site and the cell membrane utilizing resonance energy transfer allowed for the localization of the receptor binding domain [8]. Additionally, resonance energy transfer has also been utilized to determine the distribution of phospholipids surrounding membrane proteins [9, 10].

Rhodamine-labeled enkephalins [11] were found to specifically bind to cells expressing delta opioid receptors and resulted in clustering of the receptor on the cell surface, suggesting an agonist-induced sequestration or internalization of the receptor [12]. Although these ligands maintained high affinity for the delta receptor, they were found to label mu receptors poorly [13]. Opioid peptides and alkaloids containing different fluorophores including dansyl [14], fluorescein [15], nitrobenzoxadiazole [16], and pyrene [17] have also been synthesized previously. Labeling of receptors with dansyl-enkephalin was hampered by tissue autofluorescence and the destruction of opioid receptors and ligands by light in the ultraviolet spectrum [18]. A derivative of naltrexone with fluorescein (6-FNX) retained high affinity for the mu opioid receptor [15]; however, no fluorescent labeling of opioid receptors with this ligand has been reported. Fluorescent labeling of kappa opioid receptors with a fluorescein-labeled arylacetamide has been demonstrated recently [19, 20]. Secondary binding of *R*-phycoerythrin to the receptor–ligand complex has also

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** *Abbreviations:* G protein, GTP-binding protein; pCl-DPDPE, [2,5-D-Pen, 4-*p*-Cl-Phe]enkephalin; DAMGO, Tyr-D-Ala-Gly-(Me)Phe-Gly-ol; BODIPY, 4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene; 5,7-dimethyl-BODIPY, 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-propionic acid; 5-(4-phenyl-1,3-butadienyl)-BODIPY, 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-*s*-indacene-3-propionic acid; 6-FNX, 1-*N*-fluoresceinyl naltrexone thiosemicarbazone; 6-BNX, WA-III-25; and U69593, 5α,7α,8β-*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro(4,5)dec-8-yl]benzeneacetamide.

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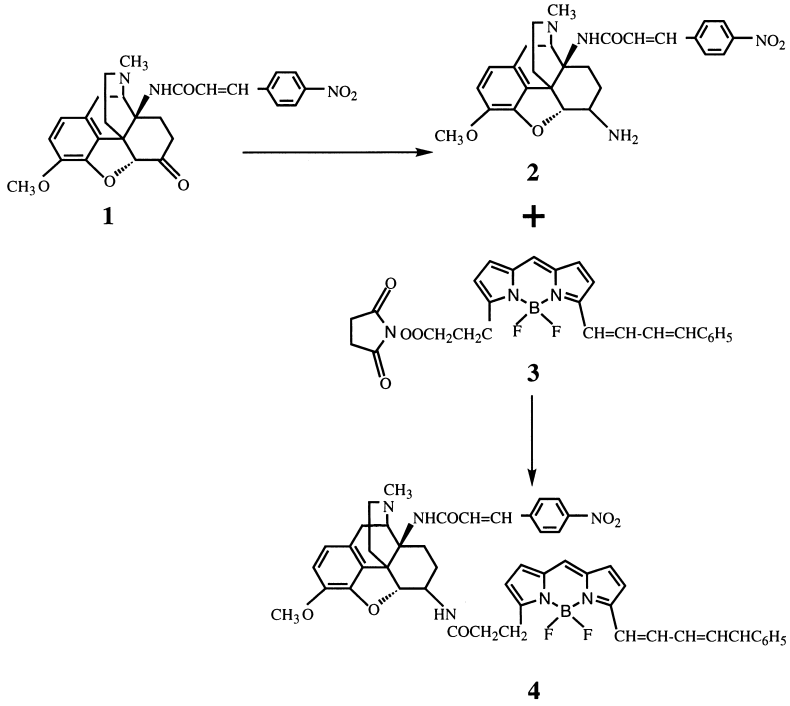


FIG. 1. Synthetic scheme for the synthesis of BODIPY-labeled opioids. Details of the synthesis are described under "Materials and Methods." Compound 1: 7,8-dihydro-4,5-epoxy-14β-*p*-nitrocinnamoylaminocodeinone; compound 2: 6β-amino-7,8-dihydro-4,5-epoxy-14β-*p*-nitrocinnamoylaminocodeinone; compound 3: 5-(4-phenyl-1,3-butadienyl)-BODIPY; and compound 4: the fluorescent opioid WA-III-62.

proven useful for the detection of kappa receptors at very low density [19].

The purpose of the present study was to develop a fluorescent-opioid ligand for examination of the biophysical and biochemical properties of the mu opioid receptor. The high fluorescence quantum yield, photostability, and low pH dependence of BODIPY [4] suggested that this fluorophore might provide a useful label for opioid ligands. The high intensity of BODIPY fluorescence emission has been used to visualize the specific labeling of β-adrenergic receptors on individual cells [21], and to examine the lateral mobility of the glycine receptor on spinal cord neurons [4]. The small size of this fluorophore also provided a significant advantage over larger fluorophores (e.g. fluorescein and rhodamine) in the retention of high affinity after conjugation to ligands for dopamine [22] and β-adrenergic [21] receptors. Additionally, we demonstrate the specific binding of a fluorescent opioid ligand in membranes from cells stably expressing the cloned rat mu opioid receptor [23] at high levels [24]. A preliminary report on these findings has been presented [25].

MATERIALS AND METHODS

Materials

[^{3}H]DAMGO (sp. act. 60 Ci/mmol), [^{3}H]diprenorphine (sp. act. 30 Ci/mmol), and [^{3}H]U69593 (sp. act. 64 Ci/mmol) were purchased from the Amersham Co. (Arlington Heights, IL). [^{3}H][D-Pen^2-D-Pen^5-pCl-Phe^4]Enkephalin (sp. act. 46 Ci/mmol) was purchased from the New England Nuclear Corp. (Boston, MA). The unlabeled drugs were obtained through the Narcotic Drug and Opiate Peptide Basic Research Center at the University of Michigan (Ann Arbor, MI). Geneticin was from the GIBCO Life Sciences Co. (Gaithersburg, MD). Succinimidyl esters of 5,7-dimethyl-BODIPY and 5-(4-phenyl-1,3-butadienyl)-BODIPY, and 6-FNX were from Molecular Probes Inc. (Eugene, OR). Sigma-Cote and all other biochemicals were from the Sigma Chemical Co. (St. Louis, MO).

Preparation of BODIPY-Labeled Opioids

The following is an example of the general method used to synthesize the ligands described in this paper (Fig. 1).

6β-AMINO-7,8-DIHYDRO-4,5-EPOXY-14β-*P*-NITROCINNAMOYLAMINOCODEINONE (2). Nitrocinnamoylaminocodeinone (**1**) [26] (50 mg) and 120 mg of ammonium acetate were dissolved in 20 mL of methanol and 3 mL of tetrahydrofuran and the solution was adjusted to pH 6.0 by the addition of a few drops of HCl. Then 20 mg of $NaCNBH_3$ was added, and the suspension was stirred for 24 hr at which time a clear solution resulted. The reaction mixture was evaporated to dryness, and the residue was taken up in $CHCl_3$. The solution was washed with dilute $NaHCO_3$, water, and then was dried. The $CHCl_3$ solution was taken to dryness. The residue melted at 243–245° after crystallization; wt, 40 mg. The NMR spectrum was run in $CDCl_3$ and showed a doublet at δ 4.95 (J = 8 Hz), which indicated that the newly introduced NH_2 group was in the β configuration. Calculated analysis (in percent) for $C_{27}H_{30}N_4O_5 \cdot 0.5H_2O$: C, 64.91; H, 6.25; N, 11.13. Found: C, 64.99; H, 6.24; N, 11.20.

SYNTHESIS OF BODIPY-LABELED OPIOIDS. A solution of 5 mg of the amino compound **2** or β-naltrexamine and 4 mg

of the BODIPY succinimidyl ester [5-(4-phenyl-1,3-butadienyl)-BODIPY (compound **3**) or 5,7-dimethyl-BODIPY] in 5 mL of tetrahydrofuran was stirred for 72 hr in an argon atmosphere. The solution was evaporated to dryness, and the residue was chromatographed on a silica gel plate using $CHCl_3$:methanol (19:1) as the developing solvent. Approximately 5 mg of pure BODIPY-labeled opioid was obtained. Structures of the BODIPY-labeled opioids were confirmed by electrospray mass spectrometry (Protein and Carbohydrate Structure Facility, University of Michigan). Calculated and experimentally determined molecular weights were: WA-III-25, 616.5 and 616.8; WA-III-62, 863.9 and 864.8; WA-III-86, 716.2 and 716.8.

Cell Culture

C_6 glioma cells were stably transfected with the cloned rat mu opioid receptor [23] as described earlier [24]. Cells were grown under 5% CO_2 in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum with Geneticin (GIBCO) (1 mg/mL). Cells used for preparation of membranes were grown in the absence of Geneticin for 6 days without loss in receptor number.

Membrane Preparation

Membranes were prepared essentially as described previously [27]. Cells were homogenized repeatedly in 0.32 M sucrose (pH 7.4 at 4°) and centrifuged at 1,000 × *g* for 10 min. Then the combined supernatants were centrifuged at 15,000 × *g* for 20 min, and the upper white layer of the pellet was removed. The upper pellet was washed once with ice-cold 50 mM Tris (pH 7.4) and resuspended at a protein concentration of 1 mg/mL. Aliquots were frozen at −80°. Monkey brain membranes were prepared as described earlier [28].

Radioligand Binding

Ligand binding was carried out as described previously [28]. Monkey brain cortical (MBC) membranes or $C_6(\mu)$ cell membranes were suspended in 50 mM Tris–HCl (pH 7.4) and added to polypropylene tubes. Sodium chloride was added to a final concentration of 150 mM followed by a solution of Tris buffer or increasing concentrations of the tested ligand. Dilutions of fluorescent ligands were prepared in tubes precoated with Sigma-Cote to reduce binding of the fluorescent ligand to the tube surface. Radioligand was added at a final concentration of: 0.5 nM [^{3}H]DAMGO (mu-selective), 1.0 nM [^{3}H]pCl-DPDPE (delta-selective), 1.5 nM [^{3}H]U69593 (kappa-selective), or 20 nM [^{3}H]diprenorphine (non-selective antagonist). Specific binding was determined by the addition of excess unlabeled ligand (1 μM). After incubation to equilibrium (25°), the samples were quickly filtered and subjected to liquid scintillation counting. The binding affinity of the investigated opioids was expressed as their K_i values in the displacement of the radiolabeled indicator ligands. The initial IC_{50} was determined by linear regression from plots that related the inhibition of specific binding in probit units to the log of 7–9 different ligand concentrations, each run in duplicate. The correlation coefficient of the log-probit plot (r^2) was higher than 0.97 in all cases. The corresponding K_i values were calculated as $IC_{50}/(1 + [L]/K_d)$, where [L] is the radioligand concentration [29]. The affinities, K_d, of the radiolabeled ligands in rhesus monkey brain membranes were: [^{3}H]DAMGO, 0.70 nM; [^{3}H]pCl-DPDPE, 0.36 nM; and [^{3}H]U69593, 1.10 nM [28].

Fluorescence Measurements

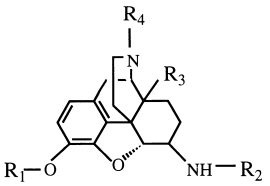
Fluorescence spectra were determined on a Spex Industries FluoroMax spectrofluorometer using a Xenon lamp. Excitation and emission maxima were determined as described in the legends to each figure and using slit widths fixed to 5 nm for all experiments. Polarized emission was detected using a set of computer-driven Glan-Thomsen polarizers. The polarized emission was expressed as the anisotropy (r), where $r = (I_o - I_{90})/(I_o - 2I_{90})$, and I_o and I_{90} represent the intensities of emission when polarizers were in a parallel and perpendicular orientation.

Labeling of Membranes with 6-BNX

$C_6(\mu)$ cell membranes suspended in 50 mM Tris buffer, pH 7.4 (80–120 μg protein), were added to tubes. Where indicated, sodium chloride was added to tubes to yield a final concentration of 150 mM. Specific binding was determined by the addition of 10 μM naltrexone. Fluorescent opioid was added at the concentrations indicated in the legend to each figure, and the tubes were incubated for 90 min at 25°. Free ligand was removed by repetitively centrifuging the membranes (15,000 *g* for 7 min) and washing with Tris buffer (0.75 mL) in the cold. Membranes were suspended in 0.75 mL Tris buffer, then transferred to a quartz cuvette, and the fluorescence emission was detected as described in the figure legends. For fluorescence anisotropy measurements, 1 mg of membrane was suspended in a quartz cuvette in Tris buffer (0.75 mL) containing 150 mM NaCl and prewarmed to 25° or 37°. Then a blank fluorescence scan was collected, after which 6-BNX (15 nM) was added and anisotropy data were collected until a steady-state value was attained (5–10 min). Subsequently, naltrexone (10 μM) was added, and the change in anisotropy was examined (40–60 min). The change in anisotropy was fit to a single phase exponential decay with a variable plateau where $Y = \text{span} \cdot e^{(-kx)} + \text{plateau}$ (Graph Pad Prism, San Diego, CA).

RESULTS

BODIPY-conjugated opioids were prepared as described in Materials and Methods (Fig. 1). The morphinan nucleus was conjugated to either the green 5,7-dimethyl-BODIPY



Fluorescent Ligand	R_1	R_2	R_3	R_4
WA-III-25 (6-BNX)	H	A	OH	CPM
WA-III-62	CH_3	B	PNC	CH_3
WA-III-86	H	B	OH	CPM

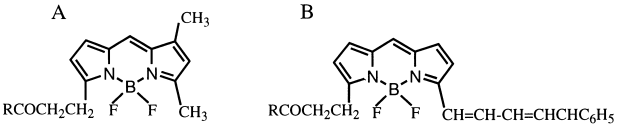


FIG. 2. Structures of BODIPY-labeled opioids. Abbreviations: CPM, cyclopropylmethyl; and PNC, 14β-*p*-nitrocinnamoylamino.

(WA-III-25) or the red 5-(4-phenyl-1,3-butadienyl)-BODIPY (WA-III-62, -86) derivatives (Fig. 2). The excitation maximum of WA-III-25 (6-BNX) was 502 nm with emission at 509 nm (Fig. 3). The excitation and emission maxima for WA-III-86 were 581 and 589 nm (Fig. 3). Fluorescence emission of all the BODIPY opioids in methanol was detectable at 0.1 nM (data not shown).

The affinity and opioid selectivity of these ligands were examined in a preparation of rhesus monkey brain membrane, which contains mu, delta, and kappa receptors. Conjugation of the BODIPY fluorophores to the morphinan nucleus was found to have little effect on their affinity for the mu receptor. 6-BNX exhibited the highest affinity for the mu receptor (K_i 0.07 nM) and retained high affinity for both the delta (1.41 nM) and kappa (0.43 nM) receptors (Table 1). 5-(4-phenyl-1,3-butadienyl)-BODIPY conjugates also maintained low nanomolar affinity for the mu receptor. WA-III-86 was weakly selective for the mu opioid receptor versus the delta (15-fold) and kappa (11-fold) receptors, while the irreversible opioid WA-III-62 was found to bind only to the mu receptor, EC_{50} 24.4 nM (Table 1).

The $C_6(\mu)$ cell line expressing the cloned mu receptor [23] was shown earlier to be a useful model system for the examination of mu opioid binding and receptor signal transduction mechanisms [24]. As determined by [^{3}H]diprenorphine binding, the level of mu receptor in membranes from these cells was 10–80 pmol/mg membrane protein (data not shown). In membranes from these cells, the labeling of the mu receptor by the high-affinity fluorescent opioid 6-BNX was examined. Fluorescence labeling of membranes incubated with ligand was >10-fold that of membrane alone (Fig. 4). Repetitive washing of the membranes in the cold eliminated nonspecific labeling without

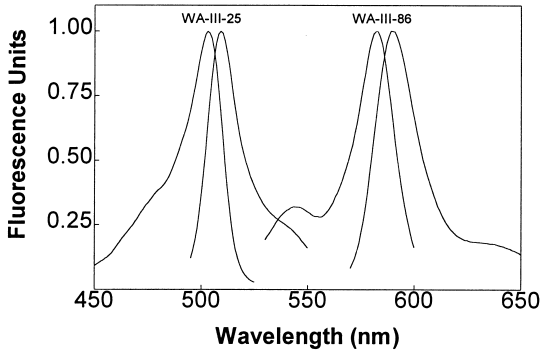


FIG. 3. Excitation and emission spectra of fluorescently labeled opioids. Ligands (WA-III-25 and WA-III-86) were diluted in methanol to 10 nM. Excitation [emission at 525 nm (WA-III-25) and 620 nm (WA-III-86)] and emission [excitation at 475 nm (WA-III-25) and 560 nm (WA-III-86)] spectra were normalized with the Xenon lamp spectrum.

substantial loss in the specific binding. After two washes of the membrane suspension, specific binding was greater than 95%. 6-BNX labeling of these membranes was examined further to illustrate the mu opioid receptor specific nature of this binding. Binding of 6-BNX was shown to be saturable (Fig. 5). Maximum labeling, 31.7 ± 2.2 (N = 3) pmol/mg membrane protein, was found to be quantitatively similar to the content of mu opioid receptor binding sites in these membranes (28 pmol [^{3}H]diprenorphine/mg membrane protein). At the lowest concentrations used in the saturation experiment, the free ligand was calculated to be less than 10% of the total ligand added and, therefore, prevented transformation of the data to a Scatchard plot (bound/free vs bound). Labeling of membranes by 6-BNX was stereoselective: labeling was reduced by the active mu opioid agonist, levorphanol, and not by the inactive isomer, dextrorphan (Fig. 6A). Additionally, the mu opioid ligands etonitazene, naltrexone, and morphine reduced 6-BNX labeling of the membranes in a concentration-dependent manner consistent with their relative affinities at the mu opioid receptor [28], whereas the delta specific agonist [D-Pen^2-D-Pen^5]-enkephalin was inactive (Fig. 6B). The emission intensity of receptor specific labeling of 6-BNX was also compared to that for 6-FNX [15]. Under saturating conditions of both ligands, the specific intensity of fluorescence emission of membranes incubated with 6-BNX was found to be 3-fold higher than the level for 6-FNX (Fig. 4, inset). Binding of both ligands to the membranes was greater than 90% specific (data not shown).

Binding of 6-BNX to the mu receptor could also be detected by alterations in fluorophore mobility as determined by polarized fluorescence emission anisotropy (r) (Fig. 7). Whereas the free ligand in the presence of membranes was freely mobile as indicated by a low anisotropy, restriction of fluorophore motion upon binding of the ligand to the receptor increased the anisotropy. In $C_6(\mu)$ membranes pre-equilibrated with 6-BNX, the rate of ligand dissociation could be assessed by a time-dependent decrease in the anisotropy (increase of 6-BNX mobility)

TABLE 1. Binding affinities of fluorescent opioids

Ligand	K_i (nM) [^{3}H]DAMGO (μ)	[^{3}H]pCl-DPDPE (δ)	[^{3}H]U69593 (κ)	Selectivity ratio δ/μ	κ/μ
WA-III-25 (6-BNX)	0.07 (0.003)	1.4 (0.04)	0.4 (0.04)	20	6.1
WA-III-62	24.4 (3.9)*	>1000*	>1000*	>40	>40
WA-III-86	1.27 (0.31)	18.8 (2.3)	13.9 (1.9)	15	11

Inhibition of the specific binding of the three receptor-selective opioids by the listed compounds was determined in the presence of sodium chloride (150 mM) as described under "Materials and Methods." The parameters shown were obtained from linear regression plots relating the percent inhibition of binding as probit units versus 6–9 concentrations of the ligand. K_i values were calculated from EC_{50} values by the method of Cheng and Prusoff [29]. Shown are the means (SEM values in parentheses) of three to four experiments, each carried out in duplicate. The selectivity ratio represents the ratio of K_i values to inhibit the binding of two receptor-selective radiolabeled opioids.

* EC_{50} values are shown for the irreversible ligand WA-III-62.

after the addition of competing unlabeled naltrexone. The decrease in anisotropy value over time was shown to be temperature dependent. At 25°, dissociation half-time of 6-BNX was 16.4 min. Increasing the temperature of the sample to 37° decreased the dissociation half-time to 5.4 min.

DISCUSSION

The favorable characteristics of BODIPY fluorescence have previously led to its successful use as a probe for the β-adrenergic receptor [30]. Conjugation of the BODIPY fluorophore to a morphinan nucleus resulted in the formation of a series of high-affinity fluorescent opioid ligands. As shown in other studies [21, 22], as well as in our work (Table 1), a common feature of these small fluorescent probes appears to be the preservation of high-affinity receptor binding. The addition of 5,7-dimethyl-BODIPY to naltrexone, "6-BNX" (K_i 0.07 nM, Table 1), may have even resulted in an enhancement of ligand affinity relative to naltrexone (K_i 0.28 nM) [29]. Addition of 5-(4-phenyl-1,3-butadienyl)-BODIPY, WA-III-86, to naltrexone resulted in only modest reduction in the affinity for the mu receptor, 1.27 nM. Derivatives of naltrexone (6-BNX and WA-III-86) were moderately selective for mu receptors in comparison to kappa or delta. WA-III-62, a fluorescent opioid containing the alkylating *p*-nitrocinnamoyl substi-

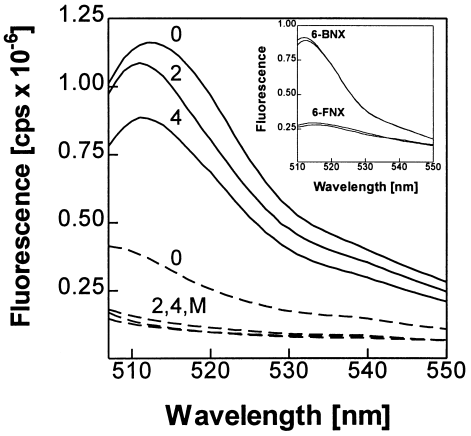


FIG. 4. Fluorescent labeling of C_6(μ) cell membranes. Membranes (4 nM receptor) were incubated (60 min, 25°) in the presence of NaCl (150 mM) and 6-BNX (10 nM) and in the absence (solid lines) and presence of naltrexone (10 μM, broken lines). Blank membranes (M) were incubated in the absence of 6-BNX. The suspension was centrifuged, resuspended in buffer, and kept on ice until all samples were washed (0 washes). Identical samples were subjected to repeated cycles of the washout procedure (2, 4). Sample fluorescence emission was determined after excitation at 492 nm. Data are presented as emission spectra from samples washed 0, 2, or 4 times. Data are from a representative experiment repeated with similar results. *Inset*: Comparison of receptor specific labeling of 6-BNX and 6-FNX. Membranes were incubated with fluorescent ligand (15 nM) for 90 min in the absence and presence of naltrexone (10 μM) and washed 2 times as described above. Fluorescence emission was detected after excitation at 492 nm (6-FNX) or 500 nm (6-BNX). Shown is the difference between emission spectra in the absence and presence of naltrexone. Data are duplicate samples from a single representative experiment repeated with similar results.

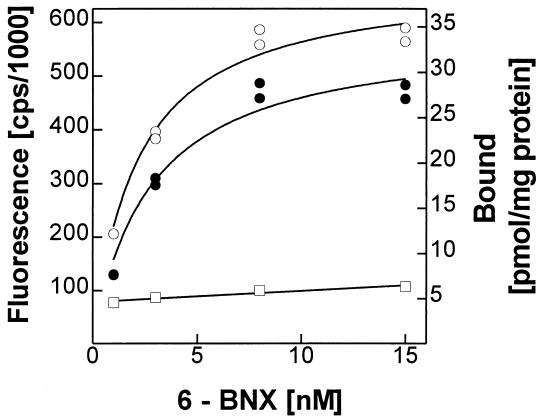


FIG. 5. Equilibrium binding of 6-BNX to C_6(μ) cell membranes. A membrane suspension (2.6 nM receptor) was incubated with increasing concentrations of 6-BNX in the presence of NaCl (150 mM) for 90 min at 25°. Samples were then washed two times as described in the legend to Fig. 4 and "Materials and Methods." Fluorescence emission of samples was detected at 512 nm after excitation at 493 nm. Specific binding (●) was determined by the subtraction of binding in the absence (○) and presence (□) of excess naltrexone (10 μM). Right Y-axis: 6-BNX fluorescence versus concentration curves were determined by addition of free ligand to tubes containing membrane suspension; extrapolation of bound was then based upon the linear regression: Y = 143,000 (cps/nM) · X + 16,500 (cps) r^2 = 0.99. Shown are the data from a representative experiment repeated two times with similar results.

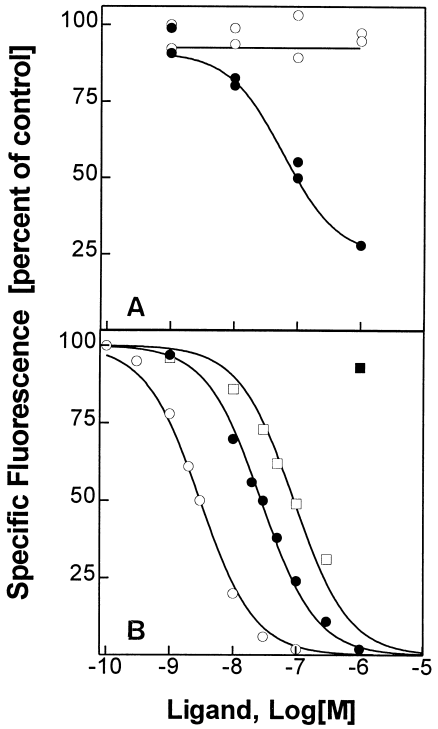


FIG. 6. Inhibition of 6-BNX specific binding by opioid ligands. Membranes were incubated with 6-BNX (10 nM) in the absence and presence of increasing concentrations of (A) dextrorphan (○) or levorphanol (●) in the presence of NaCl (150 mM), or (B) etonitazene (○), naltrexone (●), morphine (□), or DPDPE (10 μM, ■) in the absence of NaCl for 90 min at 25°. Membranes were washed two times, and fluorescence emission was examined at 512 nm after excitation at 493 nm. Data are plotted as the percent reduction of specific fluorescence as defined by incubation with naltrexone (10 μM). Shown are the data from representative experiments, each repeated with similar results.

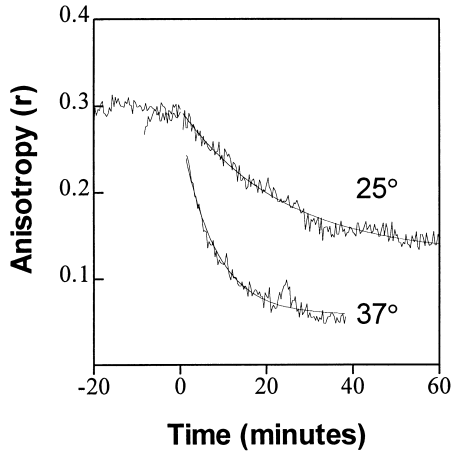


FIG. 7. Temperature dependence of 6-BNX dissociation. Membranes suspended in Tris–HCl buffer (30 nM receptor) were preincubated with NaCl (150 mM) at 25° or 37° for 10 min. 6-BNX (15 nM) was added and anisotropy was determined at 20-sec intervals for 10 (37°) or 20 (25°) min to reach equilibrium. Dissociation of bound 6-BNX was initiated at time zero by the addition of naltrexone (10 μM), and the anisotropy was determined until a plateau was reached, an additional 40 (37°) or 60 (25°) min. Data shown are from representative experiments, each repeated with similar results.

tution [26], favored the mu receptor in comparison to the delta and kappa receptor. This substitution has been suggested to confer covalent binding activity to the ligand [31]. The irreversible binding of this ligand may allow for receptor internalization studies as well as providing a wash-resistant ligand–receptor interaction that would be stable to the extensive washing required to remove nonspecific labeling.

Although many fluorescent opioids have been synthesized [see, for example, Refs. 14, 16, and 17], no demonstration of the utility of these ligands as fluorescent probes for the mu opioid receptor has been reported. Recently, fluorescent labeling of kappa receptors has been demonstrated, utilizing fluorescein-labeled arylacetamide agonists [19, 20]. However, the concentration of fluorescent antagonist required for labeling of the mu receptor in the present study (1–15 nM) illustrates the advantage of these ligands over other labeled agonists (30–50 μM) [19, 20]. This low concentration of fluorescent antagonist affords a low background that is easily removed by washing. Additionally, antagonist binding is insensitive to sodium and the presence of guanine nucleotides, thus allowing low concentrations of ligand to be used to obtain a greater percentage of labeled receptor with lower background.

The use of fluorescent ligands to study opioid receptor mechanisms has been hampered by a combination of the low number of receptors expressed by transformed cell lines (e.g., SH-SY5Y and NG108-15) and the low fluorescence emission of many opioid-fluorophore conjugates [14, 16, 17]. In contrast to the low detection limit for radioligand binding (fmol/mg protein), spectrofluorometric detection required significantly higher levels of opioid receptor (pmol/mg protein). Low receptor number was not easily overcome by increasing the cuvette concentration of receptor due to the high levels of excitation light scattering of the membrane suspension [32] and sample autofluorescence [33]. Specific binding of 6-BNX was detected in membranes from SH-SY5Y cells (700–1000 fmol/mg membrane protein); however, high levels of membrane in the sample cuvette required the use of band pass filters [32] to reduce the substantial amounts of scattered light (data not shown). Cloning of the delta [34, 35], kappa [36, 37], and mu [23, 38] opioid receptors and their homogeneous expression at high levels will provide suitable systems to study these receptors by fluorescence techniques. Isolation of plasma membranes from various passages of C_6 glioma cells stably expressing the mu receptor [24] yields 50–400 times the level of mu receptor binding found in membranes of rat brain prepared by the same method [39]. Addition of 6-BNX to $C_6(\mu)$ cell membranes was found to label the receptor with greater than 90% specific binding. Residual nonspecific binding was easily removable by washing of the membranes. In comparison to 6-fluorescein naltrexone [15], 6-BNX had an affinity nearly 40 times higher for the mu

opioid receptor and specifically labeled the receptor in membranes from $C_6(\mu)$ cells with 3-fold higher emission intensity.

In conclusion, BODIPY-labeled opioids were found to exhibit high affinity for the mu opioid receptor and variable affinity for the delta and kappa. Labeling of the receptor by 6-BNX was found to be saturable and quantitatively similar to that found with the opioid antagonist [^{3}H]diprenorphine. Labeling was also stereoselective and reduced only by ligands that bound with high affinity to the mu receptor. Receptor-bound ligand and free ligand were discriminated by changes in fluorescence anisotropy and indicated a temperature-dependent dissociation of this ligand from the mu receptor. The characteristics of fluorescence shown here confirm that 6-BNX specifically labels the mu opioid receptor in membranes of $C_6(\mu)$ cells.

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