Affinity Labeling of the μ Opioid Receptor in Bovine Striatal Membranes with [^{3}H]-14β-(Bromoacetamido)-7,8-dihydromorphine†

Jean M. Bidlack,*,‡ Richard A. Kaplan,‡ Ramu A. Subbramanian,‡ Ahmad Seyed-Mozaffari,§ and Sydney Archer§

Department of Pharmacology, The University of Rochester School of Medicine and Dentistry, Rochester, New York 14642, and Department of Chemistry, Cogswell Laboratory, Rensselaer Polytechnic Institute, Troy, New York 12181

Received January 22, 1993; Revised Manuscript Received April 21, 1993

ABSTRACT: [^{3}H]-14β-(Bromoacetamido)-7,8-dihydromorphine ([^{3}H]H_2BAM) was synthesized and tested for its ability to selectively label μ opioid receptors in bovine striatal membranes. Incubating membranes with *N*-tosyl-L-phenylalanine chloromethyl ketone and dithiothreitol before the addition of [^{3}H]H_2BAM reduced nonspecific [^{3}H]H_2BAM binding so that [^{3}H]H_2BAM binding to opioid receptors was up to 70% of the total [^{3}H]H_2BAM binding and was dependent on [^{3}H]H_2BAM concentration, incubation time, and pH of the reaction. At pH 7.5, [^{3}H]H_2BAM bound selectively to the μ opioid receptor, but mainly noncovalently. After the initial binding of [^{3}H]H_2BAM to the receptor, membranes were washed and then incubated at 37 °C in 50 mM Tris-HCl, pH 8.5, for 3 h, a time that resulted in greater than 80% of the [^{3}H]H_2BAM associated with the receptor becoming covalently bound to the opioid receptor. The μ-selective peptide [D-Ala^2,(Me)Phe^4,Gly(ol)^5]enkephalin inhibited [^{3}H]H_2BAM labeling of membranes, while δ- or κ-selective compounds were ineffective. Both NaCl and the nonhydrolyzable guanine nucleotide analog guanylyl 5′-imidodiphosphate reduced the incorporation of [^{3}H]H_2BAM into membranes. When [^{3}H]H_2BAM-labeled striatal membranes were separated under reducing conditions on a sodium dodecyl sulfate–polyacrylamide gel, two proteins with molecular weights of 54 000 and 44 000 were specifically labeled. The 54-kDa protein was present in a greater amount than the 44-kDa protein. Both proteins bound to wheat germ agglutinin–Sepharose and concanavalin A–Sepharose, suggesting that both proteins contain multiple carbohydrate moieties. Despite the inclusion of protease inhibitors, the 44-kDa protein may be a proteolytic fragment of the 54-kDa protein. Alternatively, these two proteins may be either different types of μ opioid receptors, or different degrees of posttranslational modification may account for the difference in the apparent molecular weight of the two proteins.

Affinity labeling of the different types of opioid receptors allows the further elucidation of the pharmacological and molecular properties of the multiple opioid receptors. Affinity labeling of the μ opioid receptor has been difficult due to the lack of specific affinity ligands and the fact that high concentrations of most affinity ligands have been necessary to obtain irreversible labeling of the μ opioid receptor. β-FNA[1] has been extensively used to label μ opioid receptors (Portoghese et al., 1980). When bovine striatal membranes were incubated with [^{3}H]-β-FNA and then separated under denaturing and reducing conditions on SDS–polyacrylamide gels, a broad band corresponding to molecular weights of 68 000–97 000 was detected by autoradiography (Liu-Chen & Phillips, 1987). Bands corresponding to molecular weights of 57 000 and 49 000 were obtained when the [^{3}H]-β-FNA-labeled proteins were treated with *N*-glycosidase F to digest carbohydrate moieties, before the sample was separated under denaturing and reducing conditions. Other affinity ligands that have been used to affinity label μ opioid receptors include both alkaloids and peptides. Naloxonazine (Hahn et al., 1982) and a series of 14-hydroxydihydromorphinone hydrazones (Pasternak & Wood, 1986; Heyman et al., 1988; Williams et al., 1988) have been used *in vivo* and *in vitro* to irreversibly block μ_1 opioid receptors. [^{3}H]Naloxone benzoylhydrazone has been used as a photoaffinity ligand to label μ and κ_3 opioid receptor subtypes in bovine striatal membranes. SDS–polyacrylamide gel electrophoresis of the photoaffinity-labeled membranes revealed a number of ^{3}H-labeled proteins (Standifer et al., 1991). When rat brain membranes were incubated at an alkaline pH with the peptide [^{3}H]DALECK (Newman & Barnard, 1984) or a ^{3}H-labeled chloromethyl ketone derivative of the μ-selective peptide DAMGO (Varga et al., 1988), a 58-kDa protein was specifically labeled. On the basis of competition studies, the authors suggested that these chloromethyl ketones labeled the μ opioid receptor. Wolleman (1987) reported that [^{3}H]DALECK labeled additional proteins of higher and lower molecular weight. After UV irradiation for 20 min at 10 °C at a wavelength of 255 nm, the photoaffinity probe [^{3}H]NUPA labeled a 67-kDa, a 58-kDa, and a 33-kDa protein in rat brain membranes, but only the labeling of the 67-kDa protein was partially blocked by naloxone (Mejean et al., 1992). In guinea pig cerebellum membranes, [^{3}H]NUPA labeled a 58-kDa and a 36-kDa protein. The labeling of both proteins was partially reduced

† This work was supported by U.S. Public Health Service Grants DA03742 and DA01674 from the National Institute on Drug Abuse and in part by the Aaron Diamond Foundation.

* Author to whom correspondence should be addressed.

‡ The University of Rochester School of Medicine and Dentistry.

§ Rensselaer Polytechnic Institute.

[1] Abbreviations: H_2BAM, 14β-(bromoacetamido)-7,8-dihydromorphine; BAM, 14β-(bromoacetamido)morphine; BAMO, 14β-(bromoacetamido)morphinone; H_2BAMO, 14β-(bromoacetamido)-7,8-dihydromorphinone; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; DTT, dithiothreitol; β-FNA, β-funaltrexamine; DALECK, Tyr-D-Ala-Gly-Phe-Leu-CH_2Cl; DAMGO, [D-Ala^2,(Me)Phe^4,Gly(ol)^5]enkephalin; ICI174 864, *N*,*N*-diallyl-Tyr-Aib-Aib-Phe-Leu-OH, where Aib is α-aminoisobutyric acid; U50 488, *trans*-(±)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide; FIT, fentanyl isothiocyanate; SUPERFIT, (+)-*cis*-3-methylfentanyl isothiocyanate; azido-DTLET, Tyr-D-Thr-Gly-pN_3Phe-Leu-Thr; Gpp(NH)p, guanylyl 5′-imidodiphosphate; NUPA, naltrexyl urea phenylazido; PMSF, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid dipotassium salt; SDS, sodium dodecyl sulfate; WGA, wheat germ agglutinin; Con A, concanavalin A.

0006-2960/93/0432-6703$04.00/0

by the κ-selective ligand U50 488.

A number of irreversible affinity ligands have been synthesized to label the δ opioid receptor. These include the δ-selective acylating agonists FIT (Rice et al., 1983) and SUPERFIT (Burke et al., 1986). In the neuroblastoma × glioma NG108-15 cell line, which contains only δ-type opioid receptors, both FIT and SUPERFIT irreversibly inhibited opioid binding. When NG108-15 cell membranes were incubated with [^{3}H]FIT and then separated under denaturing and reducing conditions on SDS–polyacrylamide gels, a 58-kDa protein was specifically labeled (Klee et al., 1982). While these ligands have yielded very specific labeling of the δ receptor in NG108-15 membranes, they have not been used successfully to label δ opioid receptors in brain. A 33-kDa protein was labeled when NG108-15 cell membranes were incubated with the photoaffinity peptide, [^{125}I]azido-DTLET, while 44- and 34-kDa proteins were labeled when the photoaffinity probe was incubated with rat brain membranes (Bochet et al., 1988). A derivative of [D-Ala^2,D-Leu^5]enkephalin, containing a cysteine residue in the sixth position, has been shown to inhibit δ opioid binding to rat brain membranes in a wash-resistant manner, suggesting that this peptide bound covalently to the δ opioid receptor (Bowen et al., 1987). Yeung (1987) prepared a photoreactive ^{125}I-labeled enkephalin derivative that labeled a 46-kDa protein in rat brain. The labeling of this protein was blocked by μ, δ, and κ opioids, suggesting a lack of specificity. While numerous affinity ligands have been synthesized for the specific labeling of μ and δ opioid receptors, they have suffered from the lack of high affinity and selectivity for a certain type of opioid receptor. The design of specific, selective, and irreversible affinity ligands may be facilitated by taking advantage of a molecular characteristic unique to a certain type of opioid receptor.

Recently, we have shown that when rat brain membranes were incubated with the disulfide bond reducing agent DTT, followed by the addition of BAM, BAMO, H_2BAM, or H_2-BAMO and then extensive washing of the membranes, greater than 90% of μ opioid binding to membranes was inhibited, while δ and κ opioid binding was not altered by alkylation of membranes with the affinity ligands (Bidlack et al., 1989, 1990). In the absence of DTT, these affinity ligands did not inhibit opioid binding to brain membranes in a wash-resistant manner. These studies initially suggested that there was a disulfide bond at the μ opioid binding site. However, as demonstrated in this paper, DTT enhanced the alkylation of the opioid receptor by reducing the binding of the affinity ligands to nonspecific sulfhydryl groups, not by breaking a disulfide bond at the μ opioid binding site. We have also recently shown that the endogenous thiol-containing compound glutathione is associated with bovine striatal membranes at a concentration of approximately 1 μM and DTT may be reacting with glutathione, thereby allowing a higher concentration of the affinity ligands to reach the receptor (J. M. Bidlack, unpublished data). Previous studies have shown that opioid binding was inhibited by disulfide bond reducing reagents (Pasternak et al., 1975; Marzullo & Hine, 1980; Nozaki & Cho, 1985; Kamikubo et al., 1988; Gioannini et al., 1989) and sulfhydryl alkylating compounds (Simon & Groth, 1975; Smith & Simon, 1980; Larsen et al., 1981; Childers, 1984; Ofri & Simon, 1992), suggesting the presence of at least one and possibly two (Ofri & Simon, 1992) thiol groups at or near opioid binding sites. By taking advantage of this sulfhydryl group near the μ opioid binding site, the specific radioactive labeling of the μ opioid receptor is possible.

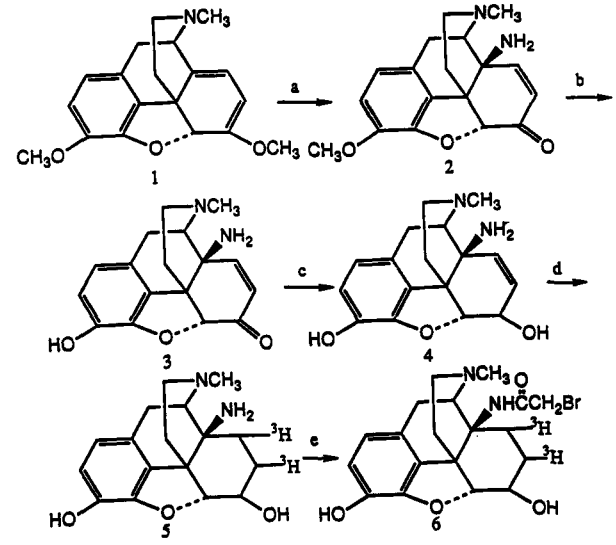

FIGURE 1: Synthesis of [^{3}H]H_2BAM. The synthesis of [^{3}H]H_2-BAM was performed as described under Experimental Procedures.

EXPERIMENTAL PROCEDURES

Materials. [^{3}H]H_2BAM (38.4 Ci/mmol) was synthesized by Amersham Corp., and the [^{14}C]methylated proteins, used as molecular weight markers on the SDS–polyacrylamide gels, were obtained from Amersham (Arlington Heights, IL). The tissue solubilizer, Soluene 350, and Triton X-100, obtained as a 10% solution, were purchased from Packard Instruments (Meridan, CT). Cytoscint ES scintillation fluid was obtained from ICN Radiochemicals (Covina, CA). Entensify was purchased from New England Nuclear (Wilmington, DE). X-Omat XAR film was purchased from Eastman Kodak Co. (Rochester, NY). *N*-Acetyl-D-glucosamine, methyl α-D-glucopyranoside, TPCK, and Con A–Sepharose were obtained from Sigma Chemical Co. (St. Louis, MO). DTT, ultrol grade, was purchased from Calbiochem (La Jolla, CA). The protease inhibitors were obtained from Boehringer Mannheim (Indianapolis, IN). U50 488 was purchased from Research Biochemicals Inc. (Natick, MA). DAMGO and ICI174 864 were purchsaed Bachem (Torrance, CA) and Cambridge Research Biochemicals (Wilmington, DE), respectively. Dextrorphan and levorphanol were gifts from Hoffmann-La Roche Inc. (Nutley, NJ), and naloxone was a gift from ENDO Laboratories (Garden City, NY). Etorphine was obtained from the National Institute on Drug Abuse (Rockville, MD). The WGA–Sepharose was purchased from EY Laboratories Inc. (San Mateo, CA).

Synthesis of [^{3}H]H_2BAM. Starting with thebaine, [^{3}H]H_2-BAM was synthesized to a specific activity of 38.4 Ci/mmol, as shown in Figure 1. Thebaine (**1**) was converted to 14β-aminocodeinone (**2**) according to the method of Kirby and McLean (1985). Demethylation with boron bromide gave the corresponding morphinone **3**. The latter was reduced with sodium borohydride to 14β-aminomorphine (**4**). Reduction with tritium gas gave **5**, which was treated with bromoacetyl bromide to furnish the required [^{3}H]H_2BAM (**6**). The synthesis was performed by Amersham, and by three different chromatography procedures the purity was determined to be ≥96%.

Covalent Labeling of the μ Opioid Receptor in Bovine Striatal Membranes with [^{3}H]H_2BAM. The affinity labeling experiments used bovine calf striatal membranes due to the high concentration of μ opioid receptors in this tissue. Calf striata were homogenized in 10 times the wet weight of tissue in 50 mM Tris-HCl, pH 7.5, followed by centrifugation at

39000g for 20 min at 4 °C. The membranes were resuspended in the original volume of buffer and incubated at 37 °C for 30 min, followed by centrifugation at 39000g for 20 min at 4 °C. The membranes were resuspended at a protein concentration of 8–12 mg/mL in 50 mM Tris-HCl, pH 7.5, and stored at −80 °C until use. The membrane protein concentration was determined according to the method of Bradford (1976), using bovine serum albumin as the standard.

Bovine striatal membranes, 20 mg of protein, were incubated in 4.8 mL of 50 mM Tris-HCl, pH 7.5, with a final concentration of 100 μM TPCK. After a 30-min incubation at 25 °C, 100 μM DTT was added for an additional 30-min incubation. The volume was brought to 5 mL by the addition of 8 nM [^{3}H]H_2BAM, which was incubated with the membranes for 10 min, followed by the dilution of the contents of the tubes to 40 mL by the addition of cold 50 mM Tris-HCl, pH 8.5. After centrifugation at 39000g for 15 min at 4 °C, the membranes were resuspended in 40 mL of 50 mM Tris-HCl, pH 8.5, followed by an additional centrifugation at 39000g for 15 min. The supernatant was removed, and the membrane pellets were incubated in a 37 °C shaking water bath for 3 h, followed by resuspension in 2 mL of 50 mM Tris-HCl, pH 6.8. To determine total counts associated with the membranes, an equal volume of membranes was added to 10% SDS, resulting in a final SDS concentration of 5%. The samples were incubated at 50 °C for 2 h, followed by centrifugation in a microfuge at 16000g for 10 min at 25 °C. Virtually all of the membrane protein was solubilized. An 80-μL aliquot incubated with 0.5 mL of the tissue solubilizer, Soluene 350, at 50 °C for at least 60 min before the addition of 6 mL of Cytoscint ES scintillation fluid.

To determine the [^{3}H]H_2BAM that was bound covalently to membrane proteins, an aliquot of SDS-solubilized membranes was precipitated with methanol and chloroform as described by Wessel and Flügge (1984). Briefly, 400 μL of methanol was added to 100 μL of the SDS-solubilized membranes, followed by centrifugation at 9000g for 10 s in a microfuge. Then 100 μL of chloroform was added, and the samples were mixed and centrifuged at 9000g for 10 s. After the addition of 300 μL of H_2O, the samples were centrifuged at 9000g for 1 min. The upper phase was removed by aspiration, and an additional 300 μL of methanol was added to the lower phase. The samples were mixed and centrifuged at 9000g for 2 min. The supernatant was aspirated, and the pellets were allowed to air-dry. The protein was solubilized by the addition of 100 μL of 5% SDS. An aliquot of 80 μL was counted in Soluene 350 and Cytoscint ES, as described above.

Optimal conditions for [^{3}H]H_2BAM labeling of the membranes were determined by varying the addition of TPCK and the concentrations of DTT and [^{3}H]H_2BAM. The time of incubation of membranes with [^{3}H]H_2BAM and the time of incubation of [^{3}H]H_2BAM-treated membranes at 37 °C were also varied, as well as the pH of the reaction. Protection experiments were performed to determine if selective opioids could block [^{3}H]H_2BAM alkylation of membranes. In these experiments, membranes were incubated with 100 nM of the opioids and 100 μM TPCK for 30 min at 25 °C, followed by the addition of 100 μM DTT and 8 nM [^{3}H]H_2BAM as described above. In experiments directed at determining the effect of NaCl and the nonhydrolyzable guanine nucleotide Gpp(NH)p on [^{3}H]H_2BAM labeling of the μ opioid receptor, membranes were incubated with 150 mM NaCl and/or 100 μM Gpp(NH)p and 100 μM TPCK for 30 min at 25 °C, followed by the addition of 100 μM DTT and 8 nM [^{3}H]H_2BAM as described above. Protease inhibitors, consisting of 1 mM EDTA, 0.2 mM PMSF, 0.7 μg/mL pepstatin, and 0.5 μg/mL leupeptin, were included throughout the experiment to determine if the [^{3}H]H_2BAM-labeling pattern changed as a result of the inclusion of protease inhibitors.

Determining if the [^{3}H]H_2BAM-Labeled Proteins Bound to Wheat Germ Agglutinin–Sepharose or Con A–Sepharose. To determine whether the [^{3}H]H_2BAM-labeled proteins were glycoproteins, the [^{3}H]H_2BAM-labeled, solubilized membrane proteins were applied to WGA–Sepharose and Con A–Sepharose columns. Bovine striatal membranes were incubated with 8 nM [^{3}H]H_2BAM as described above and resuspended in 50 mM Tris-HCl, pH 7.5. To 18 mL of [^{3}H]H_2BAM-labeled membranes was added 18 mL of a solution containing 0.6% SDS and 20 mM DTT in 50 mM Tris-HCl, pH 7.5. The sample was boiled for 3 min to inactivate proteases, followed by centrifugation at 100000g for 60 min at 18 °C, resulting in the solubilization of greater than 90% of the membrane protein. To remove SDS from the sample, this anionic detergent was exchanged for Triton X-100. The SDS-containing supernatant was diluted 6-fold by the addition of Triton X-100 at a final concentration of 0.5%, with a resulting SDS concentration of 0.05%, below the critical micelle concentration for SDS of 0.24% (Furth, 1980). The sample was concentrated 10-fold on an Amicon PM-10 membrane. Most of the Triton X-100 detergent, having a critical micelle concentration of 0.02% (Furth, 1980), remained associated with the solubilized protein. The supernatant was diluted 2-fold by the addition of NaCl to a final NaCl concentration of 0.8 M and a final Triton X-100 concentration of approximately 2%. The sample was centrifuged at 100000g for 60 min. Greater than 90% of the protein remained in solution. The protein concentration was determined according to the modified Lowry procedure, using SDS (Peterson, 1983). The solubilized [^{3}H]H_2BAM-labeled proteins were mixed overnight at room temperature with 5 mL of WGA–Sepharose, which had been equilibrated with 50 mM Tris-HCl, pH 7.5, containing 0.5 M NaCl. The slurry was poured into a 1 × 20 cm column and was washed with column buffer containing 0.5 M NaCl and 0 1% Triton X-100 in 50 mM Tris-HCl, pH 7.5. Fractions containing 3.5 mL were collected, and when the absorbance at 280 nm reached the baseline, the column buffer was switched to the eluting buffer, which contained 0.5 M *N*-acetyl-D-glucosamine in column buffer. Protein elution was monitored by absorbance and counting 200 μL of each fraction in 1 mL of Soluene 350 and 10 mL of Cytoscint ES scintillation fluid.

To determine if any of the [^{3}H]H_2BAM-labeled protein that did not bind to the WGA–Sepharose would bind to a different lectin, [^{3}H]H_2BAM-solubilized proteins that did not bind to WGA–Sepharose were incubated with Con A–Sepharose. The void sample from a WGA–Sepharose column was reapplied to the WGA column, and only approximately 15% of the original amount of binding to the WGA column was obtained. The void fraction from the second WGA column was then applied to the Con A–Sepharose column. To enhance glycoprotein binding to Con A, $MnCl_2$ and $CaCl_2$ were added to the void sample from the WGA column to a final concentration of 1 mM. The [^{3}H]H_2BAM-labeled protein was mixed overnight at room temperature with the Con A–Sepharose, which had been equilibrated with column buffer containing 0.5 M NaCl, 0.1% Triton X-100, 1 mM $CaCl_2$, and 1 mM $MnCl_2$ in 50 mM Tris-HCl, pH 7.5. The slurry was poured into a column and washed with column buffer. Protein was eluted from the Con A–Sepharose by the addition

of 0.5 M methyl α-D-glucopyranoside in column buffer. Void and eluted samples from both lectin columns were concentrated either on an Amicon PM-10 or on an Amicon Centriprep 10 membrane. The concentrated samples were then separated by SDS–polyacrylamide electrophoresis.

SDS–Polyacrylamide Gel Electrophoresis of [^{3}H]H_2BAM-Labeled Proteins. [^{3}H]H_2BAM-labeled membranes were solubilized in sample buffer containing 0.125 M Tris-HCl, pH 6.8, 5% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.005% bromophenol blue (Laemmli, 1970). After incubating at 50 °C for at least 2 h, samples were centrifuged at 16000*g* for 10 min in a microfuge to remove any unsolubilized material. The samples, containing up to 0.8 mg of protein/gel lane, were separated on a 12 × 18 × 0.3 cm running slab gel containing 8.5% acrylamide and a 3 × 18 × 0.3 cm 4% acrylamide stacking gel. The samples were electrophoresed overnight at a constant current of 11 mA/gel. [^{14}C]-Methylated proteins consisting of [^{14}C]methylated-myosin (200 000), phosphorylase *b* (97 400), bovine serum albumin (69 000), ovalbumin (46 000), carbonic anhydrase (30 000), and lysozyme (14 300) were used as the molecular weight standards. Either the gel lanes were sliced in 2-mm slices or the gel was stained with Coomassie blue, followed by destaining. To elute [^{3}H]H_2BAM-labeled proteins from the gel, the 2-mm gel slices were incubated in 1 mL of Soluene 350 at 50 °C for at least 18 h before the addition of 10 mL of Cytoscint ES scintillation fluid. For fluorography, the gels were soaked in Entensify solutions as recommended by the manufacturer. The gel was then dried and exposed to Kodak X-Omat XAR film that had been hypersensitized according to the method of Smith et al. (1985), using the hypersensitizing kit from Lumicon (Livermore, CA). The films were incubated Du Pont intensifying screens at −80 °C for 3–4 months before developing.

RESULTS

Determining the Effect of TPCK and DTT on [^{3}H]H_2BAM Alkylation of Bovine Striatal Membrane Proteins. When membranes were incubated with 8 nM [^{3}H]H_2BAM at 25 °C for 10 min, followed by two centrifugal washes in 50 mM Tris-HCl, pH 8.5, and incubation at 37 °C for 3 h, [^{3}H]H_2BAM bound covalently to membrane proteins, as shown in Table I. In the absence of either TPCK or DTT, specific binding, as measured by the inclusion of 10 μM naloxone, was obtained but was only 29% of the total binding. Inclusion of either 100 μM TPCK or 100 μM DTT increased the amount of [^{3}H]H_2BAM bound covalently to opioid receptors. The specificity of [^{3}H]H_2BAM binding increased to approximately 43% in the presence of either TPCK or DTT. Incubating membranes with both TPCK and DTT before the addition of [^{3}H]H_2BAM resulted in a 55% increase in the amount of [^{3}H]H_2BAM specifically bound to opioid receptors and a doubling of the specificity of binding in comparison to untreated membranes. These results suggest that a sulfhydryl group is present at the μ opioid binding site but that other sulfhydryl groups associated with the membranes compete with the receptor for reactivity with [^{3}H]H_2BAM. Both TPCK and DTT occupy some of the nonspecific thiol groups, resulting in a greater amount of [^{3}H]H_2BAM reaching the opioid binding site.

TPCK at concentrations of up to 100 μM did not alter the binding of the μ-selective peptide [^{3}H]DAMGO to membranes (data not shown). DTT at concentrations ranging from 50 to 200 μM enhanced, equally well, the specificity of [^{3}H]H_2BAM binding in comparison to membranes only incubated with TPCK. In all other experiments, membranes were first incubated at 25 °C for 30 min with 100 μM TPCK, followed by the addition of 100 μM DTT for an additional 30-min incubation before the addition of [^{3}H]H_2BAM.

Table I: Effect of TPCK and DTT on [^{3}H]H_2BAM Alkylation of Bovine Striatal Membranes[a]

condition	covalent [^{3}H]H_2BAM bound (cpm) total	nonspecific	specific	% specificity
control	1300 ± 119	1040 ± 123	420 ± 156	29 ± 8
TPCK	1330 ± 115	750 ± 69	590 ± 56	44 ± 2
DTT	1130 ± 126	810 ± 85	470 ± 62	42 ± 9
TPCK + DTT	1110 ± 116	470 ± 50	650 ± 83	58 ± 3

[a] Bovine striatal membranes, 20 mg of membrane protein, were incubated in 4.8 mL of 50 mM Tris-HCl, pH 7.5, with 100 μM TPCK, where stated. Nonspecific binding was determined by the inclusion of 10 μM naloxone. After a 30-min incubation at 25 °C, 100 μM DTT was added for an additional 30-min incubation. The volume was brought to 5 mL by the addition of 8 nM [^{3}H]H_2BAM, which was incubated with the membranes for 10 min, followed by the dilution of the contents of the tubes to 40 mL by the addition of cold 50 mM Tris-HCl, pH 8.5. After centrifugation at 39000*g* for 15 min at 4 °C, the membranes were resuspended in 40 mL of 50 mM Tris-HCl, pH 8.5, followed by an additional centrifugation at 39000*g*. The supernatant was removed and the membrane pellets were incubated in a 37 °C shaking water bath for 3 h, followed by resuspension in 2 mL of 50 mM Tris-HCl, pH 6.8. To determine the [^{3}H]H_2BAM that was bound covalently, an aliquot of membranes was solubilized with 5% SDS and was subsequently precipitated with methanol–chloroform, as described under Experimental Procedures. The results are expressed as the mean counts per minute/80 μL of precipitated and then resolubilized protein ± SEM, corresponding to approximately 75 μg of protein, or the mean percent specificity ± SEM from at least three experiments performed in triplicate.

Optimizing the [^{3}H]H_2BAM Alkylation of the μ Opioid Receptor. To determine an optimal pH for the covalent binding of [^{3}H]H_2BAM to proteins, membranes in 50 mM Tris-HCl, pH 7.5, were first incubated for 10 min with 8 nM [^{3}H]H_2BAM, with and without 10 μM naloxone in order to measure binding to opioid receptors. Membranes were then diluted with 50 mM Tris-HCl, at pH values ranging from 7.0 to 8.5, followed by two centrifugal washes. After membranes were incubated with [^{3}H]H_2BAM, varying the pH of the wash buffer from 7.0 to 8.5 did not significantly alter the [^{3}H]H_2BAM associated with the membranes. However, raising the pH increased the covalent binding of [^{3}H]H_2BAM to the opioid receptor. At pH 8.5, [^{3}H]H_2BAM alkylation of opioid receptors increased by 62% in comparison to samples that had been incubated at pH 7.0. Nonspecific [^{3}H]H_2BAM binding did not vary as a function of pH. It was not possible to test more alkaline pH values than 8.5 due to the solubilization of membrane proteins by alkali.

Since incubating membranes at pH 8.5 provided the greatest specific [^{3}H]H_2BAM alkylation of the opioid binding site, the optimal time for the incubation of [^{3}H]H_2BAM-bound membrane pellets at 37 °C was determined to obtain maximal specific alkylation of the opioid binding site. Figure 2A shows that without any incubation at 37 °C the specific covalent [^{3}H]H_2BAM bound to opioid receptors was less than 25% of the [^{3}H]H_2BAM bound to the receptor after a 4-h incubation at 37 °C. Nonspecific [^{3}H]H_2BAM binding did not change from 0 to 4 h, but the specific binding increased linearly. Figure 2B shows the percent of [^{3}H]H_2BAM associated with the membranes that became covalently bound to the membranes. Greater than 80% of the nonspecific [^{3}H]H_2BAM associated with the membranes was covalently bound regardless of whether a 37 °C incubation step was included. In contrast, only 25% of the [^{3}H]H_2BAM associated with opioid receptors was covalently bound when no 37 °C incubation

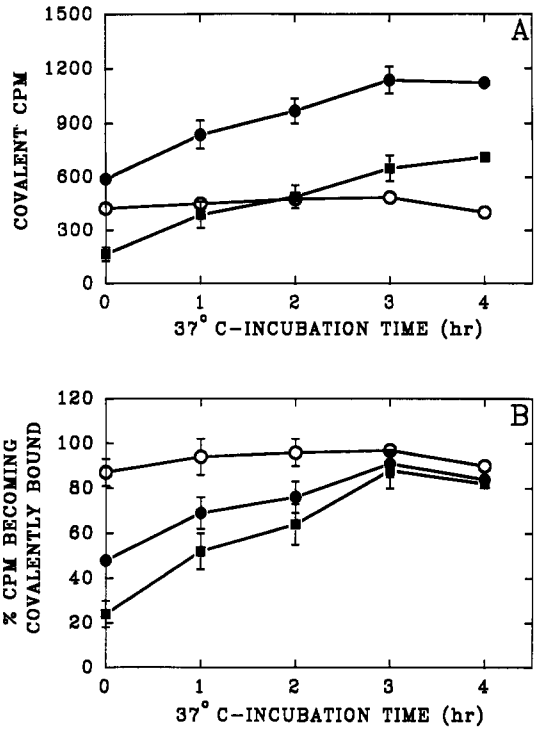

FIGURE 2: 37 °C time course for [^{3}H]H_2BAM covalent binding to membranes. Bovine striatal membranes, 20 mg of protein, were incubated in 4.8 mL of 50 mM Tris-HCl, pH 7.5, with a final concentration of 100 μM TPCK, in the absence (●) and presence of 10 μM naloxone (○). After a 30-min incubation at 25 °C, 100 μM DTT was added for an additional 30-min incubation. The volume was brought to 5 mL by the addition of 8 nM [^{3}H]H_2BAM, which was incubated with the membranes for 10 min, followed by the dilution of the contents of the tubes to 40 mL by the addition of cold 50 mM Tris-HCl, pH 8.5. After an additional centrifugal wash, the membrane pellets were incubated in a 37 °C shaking water bath for 0–4 h, followed by resuspension in 2 mL of 50 mM Tris-HCl, pH 6.8. Covalent [^{3}H]H_2BAM binding to proteins was determined as described under Experimental Procedures. Specific covalent binding (■) to 75 μg of membrane protein was calculated as the total covalent counts per minute minus the nonspecific covalent counts per minute. Points represent the mean covalent counts per minute ± SEM from three experiments performed in triplicate (A). The mean percent ± SEM of the counts per minute associated with the membranes that became covalently bound to protein (B) was determined by counting an aliquot of solubilized membranes before and after methanol–chloroform precipitation.

step was included. After a 3-h incubation, greater than 80% of the [^{3}H]H_2BAM associated with opioid receptors was covalently bound. These results indicate that [^{3}H]H_2BAM first bound to the opioid binding site noncovalently, and then at 37 °C under alkaline conditions, the [^{3}H]H_2BAM, associated with the opioid binding site became covalently bound.

To determine the optimal time for the incubation of membranes with [^{3}H]H_2BAM, membranes were incubated with 8 nM [^{3}H]H_2BAM at 25 °C for 5–80 min in the absence or presence of 10 μM naloxone to measure specificity. As shown in Figure 3, covalent nonspecific [^{3}H]H_2BAM binding increased linearly with time. Covalent [^{3}H]H_2BAM binding to the opioid receptor reached a maximum after a 20-min incubation of [^{3}H]H_2BAM with membranes. From 20 to 80 min, the amount of specific [^{3}H]H_2BAM binding remained constant. The percent of specific [^{3}H]H_2BAM binding in comparison to total [^{3}H]H_2BAM binding decreased from 66 ± 6% with a 5-min incubation to 44 ± 5% with an 80-min incubation. With 8 nM [^{3}H]H_2BAM, incubation times of 20 min and less provided greater than 60% specific binding of [^{3}H]H_2BAM to an opioid binding site.

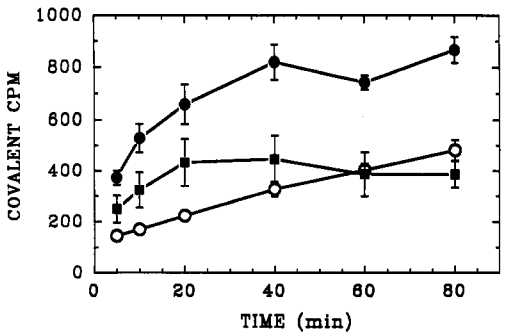

FIGURE 3: Time course for [^{3}H]H_2BAM binding to membranes. Bovine striatal membranes were incubated with 8 nM [^{3}H]H_2BAM for varying times at 25 °C as described under Experimental Procedures. After a 3-h incubation at 37 °C at pH 8.5, the membranes were solubilized with 5% SDS, and protein was precipitated with methanol–chloroform. Specific covalent binding (■) was calculated as the total binding (●) minus the nonspecific binding (○), determined by the inclusion of 10 μM naloxone. Points represent the mean covalent counts per minute ± SEM from three experiments performed in triplicate.

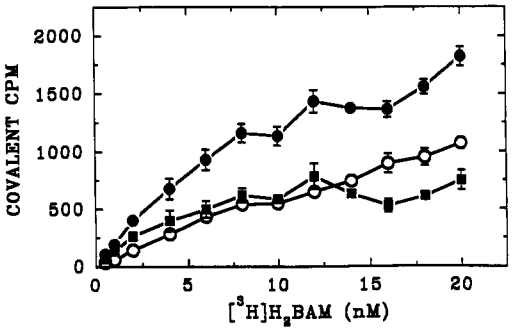

FIGURE 4: Titration of [^{3}H]H_2BAM binding to membranes. Membranes were incubated with varying concentrations of [^{3}H]H_2-BAM at 25 °C for 10 min, as described under Experimental Procedures. Specific covalent binding (■) was calculated as the total binding (●) minus the nonspecific binding (○), determined by the inclusion of 10 μM naloxone. Points represent the mean covalent counts per minute ± SEM from three experiments performed in triplicate.

Figure 4 shows the saturation profile of [^{3}H]H_2BAM binding to membranes. With a 10-min incubation at 25 °C, specific covalent binding was saturated at a [^{3}H]H_2BAM concentration of 8 nM. The percent of [^{3}H]H_2BAM associated with the receptor that became covalently bound after a 3-h incubation did not vary as a function of the [^{3}H]H_2-BAM concentration, remaining at 82 ± 5% of the [^{3}H]H_2-BAM associated with the receptor becoming covalently bound.

Determining the Type of Opioid Binding Site That Was Alkylated with [^{3}H]H_2BAM and whether NaCl and Gpp(NH)p Altered the Alkylation. Table II compares the effects of 100 nM of the μ-selective peptide DAMGO, the δ-selective peptide ICI174 864, and the κ-selective compound U50 488 on [^{3}H]H_2BAM alkylation of membranes in the absence and presence of protease inhibitors. The μ-selective peptide DAMGO inhibited [^{3}H]H_2BAM alkylation of membranes by 53%, while the δ- and κ-selective ligands were ineffective. The inhibition of [^{3}H]H_2BAM alkylation of membranes by DAMGO was concentration dependent, as shown in Figure 5. Half-maximal inhibition of [^{3}H]H_2BAM alkylation of membranes was obtained with approximately 1 nM DAMGO, and DAMGO concentrations of greater than 10 nM did not further reduce the amount of [^{3}H]H_2BAM incorporated into membranes. The labeling of the μ opioid binding site with [^{3}H]H_2BAM was also stereoselective. Levorphanol was as potent as DAMGO, but the (+)-isomer dextrorphan was

Table II: Effect of Opioids, NaCl, and Gpp(NH)p on [^{3}H]H_2BAM Alkylation of Membranes in the Absence and Presence of Protease Inhibitors[a]

compound	covalent [^{3}H]H_2BAM bound (cpm)
−protease inhibitors	
control	1110 ± 101
DAMGO	510 ± 43
ICI174 864	1090 ± 94
U50 488	1020 ± 78
dextrorphan	1020 ± 66
levorphanol	480 ± 20
etorphine	490 ± 57
NaCl	660 ± 62
Gpp(NH)p	900 ± 135
NaCl + Gpp(NH)p	410 ± 102
+protease inhibitors	
control	820 ± 71
DAMGO	470 ± 19
ICI174 864	880 ± 26
U50 488	870 ± 51

[a] Bovine striatal membranes, 20 mg of protein, were incubated for 30 min at 25 °C in 4.8 mL of 50 mM Tris-HCl, pH 7.5, with either 100 nM opioids, 150 mM NaCl, or 100 μM Gpp(NH)p in the presence of 100 μM TPCK. The samples were then incubated with 100 μM DTT and 8 nM [^{3}H]H_2BAM, followed by solubilization, and precipitated with methanol–chloroform, as described in Table I. The results are expressed as the mean counts per minute/80 μL of precipitated and then resolubilized protein ± SEM, corresponding to approximately 75 μg of protein, from at least three experiments performed in triplicate.

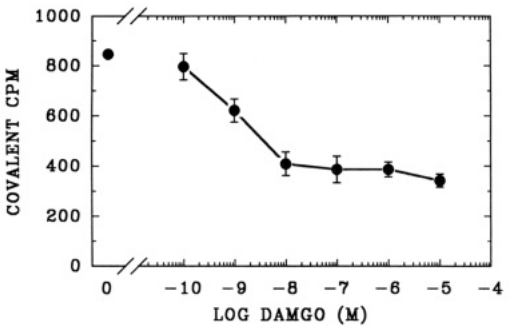

FIGURE 5: Titration of DAMGO inhibition of [^{3}H]H_2BAM binding to membranes. Striatal membranes were incubated with varying concentrations of DAMGO in the presence of 8 nM [^{3}H]H_2BAM for 10 min at 25 °C, as described under Experimental Procedures. After a 3-h incubation at 37 °C at pH 8.5, the membranes were solubilized with 5% SDS and proteins were precipitated with methanol–chloroform. Points represent the mean covalent counts per minute ± SEM from three experiments performed in triplicate.

ineffective at blocking [^{3}H]H_2BAM alkylation (Table II). The nonselective high-affinity opioid etorphine also reduced [^{3}H]H_2BAM binding by an amount equivalent to that obtained with DAMGO and levorphanol. Inclusion of protease inhibitors did not alter the specificity of the [^{3}H]H_2BAM alkylation of the μ opioid binding site. As shown in Table II, protease inhibitors reduced the total [^{3}H]H_2BAM alkylation, probably by leupeptin and pepstatin competing with the receptor for [^{3}H]H_2BAM alkylation.

Inclusion of 150 mM NaCl reduced [^{3}H]H_2BAM alkylation of membranes by 40%, and 100 μM Gpp(NH)p reduced the labeling by 18%. The inhibition by NaCl and Gpp(NH)p appeared to be additive in that the combination of these compounds resulted in 63% inhibition in [^{3}H]H_2BAM alkylation. The addition of 10 μM naloxone to membranes incubated with NaCl and Gpp(NH)p did not further reduce the labeling, suggesting that all [^{3}H]H_2BAM labeling of the opioid receptor was inhibited by the combination of NaCl and Gpp(NH)p.

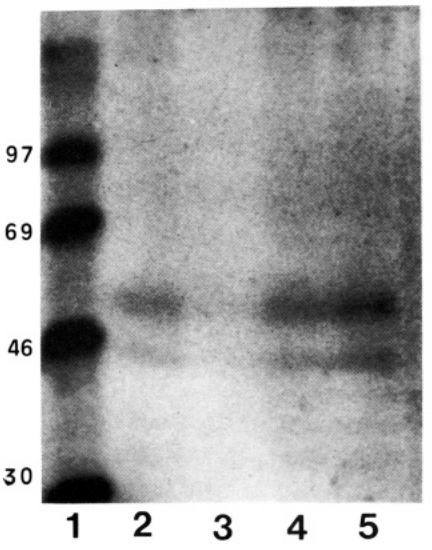

FIGURE 6: Autoradiograph of [^{3}H]H_2BAM-labeled membranes separated on a SDS–polyacrylamide gel, followed by autoradiography. Bovine striatal membranes, in the presence of protease inhibitors, were incubated with 8 nM [^{3}H]H_2BAM for 10 min at 25 °C in the absence (lane 2) or presence of 100 nM of either DAMGO (lane 3), ICI174 864 (lane 4), or U50 488 (lane 5), followed by washing and a 3-h incubation at 37 °C. The membranes, 0.8 mg of protein, were separated on an 8.5% acrylamide–SDS gel. Lanes 2–5 were loaded with 6230, 2910, 6290, and 6530 cpm, respectively. The gel was treated with Extensify, followed by drying and exposure in the presence of intensifying screens to Kodak XAR film that had been hypersensitized. Lane 1 contained 4100 cpm of ^{14}C-methylated proteins, used as molecular weight markers. The gel was exposed for 4 months before the film was developed.

Determining the Molecular Weight of Membrane Proteins Alkylated with [^{3}H]H_2BAM. Bovine striatal membranes, in the presence of protease inhibitors, were alkylated with 8 nM [^{3}H]H_2BAM in the absence and presence of DAMGO, ICI174 864, and U50 488 to determine the receptor specificity of the labeling. [^{3}H]H_2BAM-labeled membranes were separated on an 8.5% acrylamide–SDS gel under reducing conditions, followed by fluorography of the gel. The autoradiograph, shown in Figure 6, demonstrates that [^{3}H]H_2BAM alkylated two proteins with molecular weights of 54 000 and 44 000, on the basis of the [^{14}C]methylated protein standards. Inclusion of neither the δ-selective peptide ICI174 864 nor the κ-selective alkaloid U50 488 altered the labeling pattern. However, the μ-selective peptide DAMGO blocked most of the labeling of both the 54- and 44-kDa proteins.

Due to the long time needed for ^{3}H fluorography, most experiments sliced the SDS–polyacrylamide gel into 2-mm sections and then extracted the proteins from the gel. Figure 7 shows that, like the results obtained with fluorography, [^{3}H]H_2BAM, in the absence of protease inhibitors, labeled primarily two major proteins with molecular weights of 54 000 and 44 000. DAMGO blocked most of the labeling of both proteins, while ICI174 864 and U50 488 were ineffective. Of the two [^{3}H]H_2BAM-labeled proteins, 70% of [^{3}H]H_2BAM was associated with the 54-kDa protein and 30% with the 44-kDa protein. The ratio of labeling did not change regardless of whether protease inhibitors were included in the incubation. Figure 8 shows that the labeling of both proteins was reduced with the inclusion of either NaCl or Gpp(NH)p and that the alkylation of neither protein was preferentially reduced.

As an additional control to address the possibility of nonspecific labeling, an equal number of counts per minute was added to each gel lane instead of an equal amount of

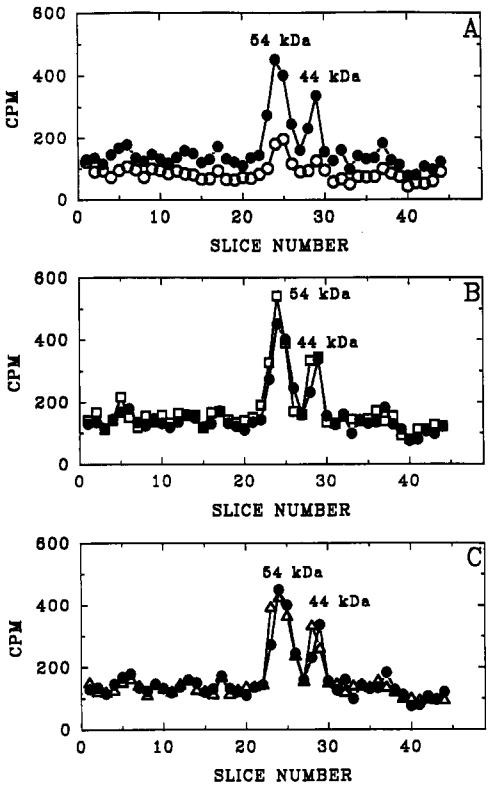

FIGURE 7: Determining the ability of μ-, δ-, and κ-selective opioids to inhibit [^{3}H]H_2BAM alkylation of membranes. Bovine striatal membranes, in the absence of protease inhibitors, were incubated with 8 nM [^{3}H]H_2BAM for 10 min at 25 °C in the absence (●) (A–C) or presence 100 nM of either DAMGO (○) (A), ICI174 864 (□) (B), or U50 488 (△) (C), followed by washing and a 3-h incubation at 37 °C. The membranes, 0.8 mg of protein, were separated on an 8.5% acrylamide–SDS gel. The control, DAMGO, ICI174 864, and U50 488 lanes contained 4460, 2270, 4720, and 5300 cpm, respectively. The gel was sliced into 2-mm sections, and each section was incubated with 1 mL of Soluene 350 at 50 °C for greater than 18 h before the addition of 10 mL of Cytoscint ES scintillation fluid.

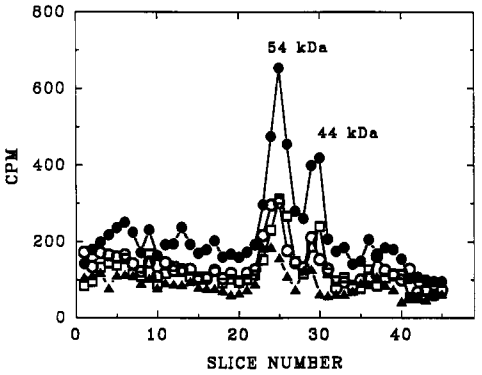

FIGURE 8: Determining whether NaCl or Gpp(NH)p selectively reduced the [^{3}H]H_2BAM alkylation of the 54- or 44-kDa proteins. Membranes were incubated with 8 nM [^{3}H]H_2BAM for 10 min at 25 °C in the absence (●) or presence of either 150 mM NaCl (○), 100 μM Gpp(NH)p (□), or both NaCl and Gpp(NH)p (▲), followed by washing and a 3-h incubation at 37 °C. The membranes, 0.8 mg of protein, were separated on an 8.5% acrylamide–SDS gel. The control, NaCl, Gpp(NH)p, and NaCl + Gpp(NH)p lanes contained 9390, 5440, 6160, and 4180 cpm, respectively. The gel was sliced into 2-mm sections, and each section was incubated with 1 mL of Soluene 350 at 50 °C for greater than 18 h before the addition of 10 mL of Cytoscint ES scintillation fluid.

membrane protein. Under these conditions, the labeling pattern remained unchanged, but the number of counts per minute associated with the 54- and 44-kDa proteins was reduced by approximately half in the control lanes in comparison to the counts per minute obtained in control lanes

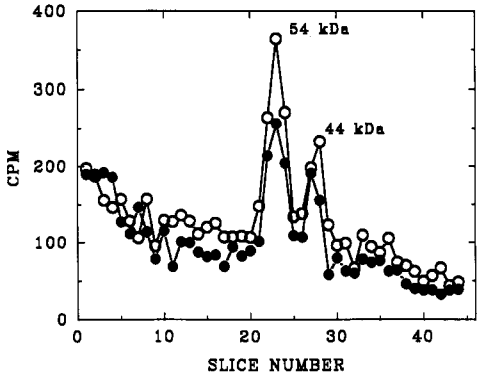

FIGURE 9: Determining whether the [^{3}H]H_2BAM-labeled proteins bound to WGA–Sepharose and Con A–Sepharose. Membranes were alkylated with 8 nM [^{3}H]H_2BAM, and the protein was solubilized as described under Experimental Procedures. The solubilized [^{3}H]H_2-BAM-labeled protein was incubated with the WGA–Sepharose gel overnight before the gel was poured, washed, and then eluted with 0.5 mM *N*-acetyl-D-glucosamine. The void volume that did not bind to the WGA–Sepharose was reapplied to the WGA–Sepharose. The second void volume was then incubated overnight with Con A–Sepharose before the gel was poured, washed, and eluted with 0.5 M methyl α-D-glucopyranoside. The eluted protein from each lectin column was concentrated and then separated on an 8.5% acrylamide–SDS gel, followed by extraction of the protein from the gel, as described in Figure 7. The lane containing the sample eluted from the WGA–Sepharose (●) had 3890 cpm applied, and the lane containing the sample eluted from the Con A–Sepharose (○) had 4760 cpm applied.

containing 0.8 mg of protein (Figure 7). Since in control samples more [^{3}H]H_2BAM was associated with the membranes during the 37 °C incubation than in samples containing [^{3}H]H_2BAM and DAMGO, it could be argued that during the 3-h incubation at 37 °C [^{3}H]H_2BAM dissociates from the μ opioid receptor and randomly labels proteins. To control for nonspecific labeling, membranes were incubated with [^{3}H]H_2BAM in the absence or presence of DAMGO. After two centrifugal washes, DAMGO and [^{3}H]H_2BAM, an amount approximately equal to counts per minute associated with control membranes, were added to the DAMGO-treated membranes, resulting in the control and DAMGO-treated membranes containing an equal amount of [^{3}H]H_2BAM during the 3-h 37 °C incubation. While the overall background labeling was increased slightly, the addition of [^{3}H]H_2BAM just before the 37 °C incubation step did not result in an increased labeling of either the 54- or 44-kDa protein, suggesting that the labeling of these two proteins represents the specific labeling of μ opioid binding proteins.

Determining if either the 54- or 44-kDa Proteins Were Glycoproteins. Receptors are known to be glycosylated, while many membrane-associated proteins, such as G proteins, do not contain a carbohydrate moiety. Therefore, experiments were performed to determine if both the 54- and 44-kDa proteins were glycosylated. [^{3}H]H_2BAM-labeled membranes were solubilized with 0.6% SDS in the presence of DTT, to completely dissociate the receptor from the G-protein complex. The anionic detergent was then replaced with the nonionic detergent Triton X-100 before the sample was applied to the lectin column. After application of the sample, the WGA–Sepharose column washed, and glycoproteins were eluted with *N*-acetyl-D-glucosamine. Of the total [^{3}H]H_2BAM-labeled protein applied to the column, approximately 20% was retained. The eluted fraction was separated under reducing conditions on a SDS–polyacrylamide gel, followed by gel slicing and counting. As shown in Figure 9, both the 54- and 44-kDa proteins bound to the WGA–Sepharose column and were eluted with the *N*-acetyl-D-glucosamine.

To determine whether the two proteins possess multiple carbohydrate moieties, with different specificities for various lectins, experiments were performed to determine whether [^{3}H]H_2BAM-labeled proteins that did not bind to the WGA–Sepharose would bind to Con A–Sepharose. The void volume obtained after two passes through the WGA column was applied to Con A–Sepharose. Protein specifically bound to the Con A was eluted with methyl α-D-glucopyranoside and analyzed under the same conditions as the fractions from the WGA–Sepharose column. Some of the [^{3}H]H_2BAM-labeled protein that did not bind to the WGA did bind to Con A, suggesting multiple carbohydrate moieties on both proteins. Figure 9 shows that both the 54- and 44-kDa proteins bound to Con A and that the ratio of the two proteins remained the same. These results suggest that both proteins are part of the opioid receptor and not part of the G-protein complex that is coupled to μ opioid receptors.

DICUSSION

In this study, we demonstrated that [^{3}H]H_2BAM stereoselectively labeled the μ opioid receptor in bovine striatal membranes. The excellent specificity of the labeling was in part derived from the fact that at neutral pH thiol groups are very poor nucleophiles and thus do not displace the bromine atom in the 14β-bromoacetamido group. At pH 7.5, [^{3}H]H_2BAM first bound, primarily noncovalently, to the μ opioid receptor (Figure 2) and exhibited binding kinetics (Figure 3) similar to the time courses obtained with most opioid ligands. At 25 °C, equilibrium was reached within 20 min. The reactivity of [^{3}H]H_2BAM with a sulfhydryl group at the μ opioid binding site was dependent on the temperature and pH of the reaction. The anion of the thiol group, S^-, was the reactive species, and the more alkaline the pH, the larger the concentration of anion that was present. Bednar (1990) has determined that the difference in nucleophilicity between RSH and RS is about 10 orders of magnitude. Hence, when striatal membranes were first incubated with [^{3}H]H_2BAM at physiological pH, [^{3}H]H_2BAM bound primarily noncovalently to the μ opioid receptor. Then by changing the pH from 7.5 to 8.5 and increasing the temperature of the reaction from 25 to 37 °C, greater than 80% of the [^{3}H]H_2BAM bound to the receptor became covalently bound.

The addition of TPCK and DTT enhanced the magnitude and the specificity of labeling the μ opioid binding site (Table I). On the basis of our previous studies, which showed that H_2BAM and related compounds inhibited [^{3}H]DAMGO binding in a wash-resistant manner only when DTT was present (Bidlack et al., 1990), we hypothesized that the μ opioid binding site contained a disulfide bond that needed to be reduced before the affinity ligands would react with the receptor (Bidlack et al., 1991). However, as shown in Table I, [^{3}H]H_2BAM labeled the binding site in the absence of DTT. Therefore, the binding site probably does contain a thiol group, as has been proposed by other investigators (Childers, 1984; Ofri & Simon, 1992). By reducing nonspecific [^{3}H]H_2BAM binding, the addition of TPCK and DTT enhanced the labeling of the opioid binding site, by allowing a larger amount of unreacted [^{3}H]H_2BAM to reach the binding site. Recently, we have also demonstrated that the endogenous thiol-containing compound, glutathione, is associated with bovine striatal membranes at a concentration of approximately 1 μM (Bidlack, unpublished data). TPCK and DTT may also react with glutathione, thereby reducing glutathione's reactivity with [^{3}H]H_2BAM. Both thiol-alkylating compounds, such as *N*-ethylmaleimide (Childers, 1984; Ofri & Simon, 1992), and thiol-reducing compounds, such as DTT (Pasternak et al., 1975; Gioannini et al., 1989), have been shown to inhibit opioid binding to membranes. The alkylating compounds act directly by reacting with sulfhydryl groups. Reducing compounds, such as DTT, have always been proposed to break disulfide bonds as their mechanism of action. DTT concentrations ranging from 1 to 150 mM (Gioannini et al., 1989) have been shown to inhibit opioid binding to membranes. While DTT does break disulfide bonds, it may have another mechanism of action in producing its inhibition of opioid binding. DTT can interact with sulfhydryl groups and establish an equilibrium between itself and the sulfhydryl group. Thus, high DTT concentrations may be required to observe inhibition of opioid binding due to the large number of thiol groups that are associated with membranes. DTT would first react with easily accessible thiol groups. Finally, when sufficiently high DTT concentrations are present, DTT may react with the sulfhydryl group at the opioid binding site and thereby inhibit opioid binding. Alternatively, an equilibrium may exist between thiol(s) on the receptor and other thiol-containing compounds, such as glutathione, that are associated with membranes. In other words, some receptor thiols may be reduced while other receptor thiols are oxidized either intramolecularly or as a mixed disulfide between the receptor and endogenous thiol-containing compounds. The addition of DTT would disrupt this thiol–disulfide equilibrium. This hypothesis explains why the addition of either sulfhydryl-alkylating compounds or disulfide-reducing compounds results in an inhibition of binding.

Both NaCl and the nonhydrolyzable guanine nucleotide analog Gpp(NH)p inhibited [^{3}H]H_2BAM alkylation of the opioid receptor. The effects of both NaCl and Gpp(NH)p are consistent with [^{3}H]H_2BAM acting as an agonist. Antagonist binding, such as was seen with [^{3}H]-β-FNA (Liu-Chen & Phillips, 1987), would not be expected to be inhibited by sodium or guanine nucleotides. We have recently shown that H_2BAMO, which is identical to H_2BAM, except for a ketone in the C-6 position instead of the alcohol, acted as a short-term, μ-selective opioid agonist in the mouse tail-flick assay (Jiang et al., 1992). Thus, in both *in vivo* and *in vitro* experiments, the 14β-bromoacetamido derivatives of dihydromorphine acted as agonists. Under *in vivo* physiological conditions, H_2BAMO did not bind covalently to the μ opioid receptor, a result consistent with the pH dependence of the reaction that we have shown here.

Two proteins with molecular weights of 54 000 and 44 000 were specifically labeled with [^{3}H]H_2BAM. Inclusion of protease inhibitors did not change the labeling of these proteins but did reduce the amount of labeling, probably due to the protease inhibitors leupeptin and pepstatin reacting with [^{3}H]H_2BAM. Approximately 70% of the [^{3}H]H_2BAM was associated with the 54-kDa protein and 30% with the 44-kDa protein. A similar [^{3}H]H_2BAM-labeling pattern has been observed with rat brain membranes (Bidlack, unpublished data). Both the 54- and 44-kDa proteinis are glycoproteins and appear to possess multiple carbohydrate moieties in that these proteins bound to both WGA and Con A. The difference in molecular weight of the two proteins may be due to different degrees of posttranslational modification, similar to that found in the β_2-adrenergic receptor (Stiles et al., 1984).

Other approaches, besides affinity labeling, that have been used to determine the molecular weight of the μ opioid receptor have included cross-linking and purification studies. Cross-linking studies using [^{125}I]-β-endorphin have shown many proteins, ranging in molecular weight from 23 000 to 92 000,

being labeled (Howard et al., 1985, 1986; Helmeste et al., 1986). Similarly purification studies have suggested molecular weights ranging from 23 000 to 94 000 for the μ receptor under reduced conditions (Bidlack et al., 1981; Cho et al., 1983, 1986; Gioannini et al., 1985; Maneckjee et al., 1985). Since the μ opioid receptor is negatively coupled to adenylyl cyclase by a G_i protein, this receptor is thought to belong to the superfamily of G-linked receptors and, consequently, probably to have a molecular weight between 35 000 and 70 000, as has been reported for other G-protein-linked receptors. The molecular weight of the two proteins labeled with [^{3}H]H_2BAM is consistent with the μ opioid receptor belonging to this superfamily.

Since the δ opioid receptor from the NG108-15 cells has been recently cloned (Evans et al., 1992; Kieffer et al., 1992), this opioid receptor may serve as a model for the μ opioid receptor. More than a decade ago, affinity labeling studies using [^{3}H]FIT reported that the δ opioid receptor in NG108-15 cells had a molecular weight of 58 000 (Klee et al., 1982). The cloned δ opioid receptor from NG108-15 cells has a calculated molecular weight of 40 810 (Kieffer et al., 1992) or less (Evans et al., 1992). The difference between this molecular weight and the molecular weight observed in affinity labeling studies using [^{3}H]FIT is probably due to posttranslational modification of the protein. The cloned δ opioid receptor has two sites where N-linked glycosylation could occur (Evans et al., 1992). Similar results would be expected for the μ opioid receptor. The 54- and 44-kDa proteins are both glycosylated, as demonstrated by their ability to bind to lectins (Figure 9). Currently, we cannot rule out the possibility that the 44-kDa protein is a proteolytic product of the 54-kDa protein. However, alternatively, the two proteins may represent subtypes of μ opioid receptors, as has been previously postulated (Wolozin & Psternak, 1981), or the two proteins may represent different degrees of posttranslational modifications of the same protein.

REFERENCES

Bednar, R. A. (1990) *Biochemistry 29*, 3684–3690.

Bidlack, J. M., Abood, L. G., Osei-Gyimah, P., & Archer, S. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 636–639.

Bidlack, J. M., Frey, D. K., Seyed-Mozaffari, A., & Archer, S. (1989) *Biochemistry 28*, 4333–4339.

Bidlack, J. M., Frey, D. K., Kaplan, R. A., Seyed-Mozaffari, A., & Archer, S. (1990) *Mol. Pharmacol. 37*, 50–59.

Bidlack, J. M., Seyed-Mozaffari, A., & Archer, S. (1991) *Med. Chem. Res. 1*, 43–46.

Bochet, P., Icard-Liepkalns, C., Pasquini, F., Garbay-Jaureguiberry, C., Beaudet, A., Roques, B., & Rossier, J. (1988) *Mol. Pharmacol. 34*, 436–443.

Bowen, W. D., Hellewell, S. B., Kelemen, M., Huey, R., & Stewart, D. (1987) *J. Biol. Chem. 262*, 13434–13439.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Burke, T. R., Jr., Jacobson, A. E., Rice, K. C., Silverton, J. V., Simonds, W. F., Streaty, R. A., & Klee, W. A. (1986) *J. Med. Chem. 29*, 1087–1093.

Childers, S. R. (1984) *J. Pharmacol. Exp. Ther. 230*, 684–691.

Cho, T. M., Ge, B. L., Yamato, C., Smith, A. P., & Loh, H. H. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 3176–3180.

Cho, T. M., Hasegawa, J.-I., Ge, B. L., & Loh, H. H. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 4138–4142.

Evans, C. J., Keith, D. E., Jr., Morrison, H., Magendzo, K., & Edwards, R. H. (1992) *Science 258*, 1952–1955.

Furth, A. J. (1980) *Methods Enzymol. 109*, 207–215.

Gioannini, T. L., Howard, A. D., Hiller, J. M., & Simon, E. J. (1985) *J. Biol. Chem. 260*, 15117–15121.

Gioannini, T. L., Liu, Y. F., Park, Y. H., Hiller, J. M., & Simon, E. J. (1989) *J. Mol. Recognit. 2*, 44–48.

Hahn, E. F., Carroll-Buatti, M., & Pasternak, G. W. (1982) *J. Neurosci. 2*, 572–576.

Helmeste, D. M., Hammonds, R. G., Jr., & Li, C. H. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 4622–4625.

Heyman, J. S., Williams, C. L., Burks, T. F., Mosberg, H. I., & Porreca, F. (1988) *J. Pharmacol. Exp. Ther. 245*, 238–243.

Howard, A. D., de La Baume, S., Gioannini, T. L., Hiller, J. M., & Simon, E. J. (1985) *J. Biol. Chem. 260*, 10833–10839.

Howard, A. D., Sarne, Y., Gioannini, T. L., Hiller, J. M., & Simon, E. J. (1986) *Biochemistry 25*, 357–360.

Jiang, Q., Seyed-Mozaffari, A., Archer, S., & Bidlack, J. M. (1992) *J. Pharmacol. Exp. Ther. 262*, 526–531.

Kamikubo, K., Murase, H., Murayama, M., Matsuda, M., & Miura, K. (1988) *J. Neurochem. 50*, 503–509.

Kieffer, B. L., Befort, K., Gaveriaux-Ruff, C., & Hirth, C. G. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 12048–12052.

Kirby, G. W., & McLean, D. (1985) *J. Chem. Soc., Perkin Trans. 1*, 1443–1445.

Klee, W. A., Simonds, W. F., Sweat, F. W., Burke, T. R., Jr., Jacobsen, A. E., & Rice, K. C. (1982) *FEBS Lett. 150*, 125–128.

Laemmli, U. K. (1970) *Nature 227*, 680–685.

Larsen, N. E., Mullikin-Kilpatrick, K., & Blume, A. J. (1981) *Mol. Pharmacol. 20*, 255–262.

Liu-Chen, L.-Y., & Phillips, C. A. (1987) *Mol. Pharmacol. 32*, 321–329.

Maneckjee, R., Zukin, R. S., & Archer, S. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 594–598.

Marzullo, G., & Hine, B. (1980) *Science 208*, 1171–1173.

Mejean, A., Hawkinson, J., Guthapfel, R., Goeldner, M., & Hirth, C. (1992) *Biochemistry 31*, 9685–9693.

Newman, E. L., & Barnard, E. A. (1984) *Biochemistry 23*, 5383–5389.

Nozaki, M., & Cho, T. M. (1985) *J. Neurochem. 45*, 461–464.

Ofri, D., & Simon, E. J. (1992) *Receptors 2*, 109–119.

Pasternak, G. W., & Wood, P. L. (1986) *Life Sci. 38*, 1889–1898.

Pasternak, G. W., Wilson, H. A., & Snyder, S. H. (1975) *Mol. Pharmacol. 11*, 340–351.

Peterson, G. L. (1983) *Methods Enzymol. 91*, 95–119.

Portoghese, P. S., Larson, D. L., Sayre, L. M., Fries, D. S., & Takemori, A. E. (1980) *J. Med. Chem. 23*, 233–234.

Rice, K. C., Jacobsen, A. E., Burke, T. R., Jr., Bajwa, B. S., Streatly, R. A., & Klee, W. A. (1983) *Science 220*, 314–316.

Simon, E. J., & Groth, H. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 2404–2407.

Smith, A. G., Phillips, C. A., & Hahn, E. J. (1985) *J. Imaging Technol. 11*, 27–32.

Smith, J. R., & Simon, E. J. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 281–284.

Standifer, K. M., Murthy, L. R., Kinouchi, K., Steele, L., & Pasternak, G. W. (1991) *Mol. Pharmacol. 39*, 290–298.

Stiles, G. L., Benovic, J. L., Caron, M. G., & Lefkowitz, R. J. (1984) *J. Biol. Chem. 259*, 10117–10123.

Varga, E., Toth, G., Hepp, J., Benyhe, S., Simon, J., Medzihradszky, K., & Borsodi, A. (1988) *Neuropeptides 12*, 135–139.

Wessel, D., & Flügge, U. I. (1984) *Anal. Biochem. 138*, 141–143.

Williams, C. L., Bodnar, R. J., Clark, J. E., Hahn, E. F., Burks, T. F., & Pasternak, G. W. (1988) *J. Pharmacol. Exp. Ther. 245*, 8–12.

Wolleman, M. (1987) *Neurochem. Res. 12*, 129–133.

Wolozin, B. L., & Pasternak, G. W. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 6181–6185.

Yeung, C. W. (1987) *Arch. Biochem. Biophys. 254*, 81–91.