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Affinity Labeling of μ Opioid Receptors by Sulfhydryl Alkylating Derivatives of Morphine and Morphinone

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SUMMARY

After reduction of a disulfide bond at or near the μ opioid binding site in rat brain membranes, incubating membranes with 14β-bromoacetamido derivatives of either morphine, dihydromorphine, morphinone, or dihydromorphinone resulted in the irreversible inhibition of μ opioid binding to rat brain membranes. Without the addition of the disulfide bond-reducing reagent dithiothreitol, these affinity ligands bound reversibly to opioid binding sites. Binding to either δ or κ opioid binding sites was not altered by alkylation of the membranes with the affinity ligands. The percentage of irreversible inhibition of μ opioid binding was dependent on the time and temperature of the incubation of membranes with the affinity ligands and on the concentrations of dithiothreitol and the affinity ligands. Incubating membranes with morphine afforded almost complete protection from alkylation of the μ opioid binding site. Naloxone and the *l*-isomer levorphanol also protected the site from alkylation, whereas the *d*-isomer dextrorphan and the κ-selective opioid U50,488H did not protect the site. The μ-selective peptide [D-Ala^2, (Me)Phe^4,Gly(ol)^5]enkephalin was the peptide that afforded the greatest protection. These studies have shown that, after the reduction of a disulfide bond at or near the μ opioid binding site, this sulfhydryl group can be specifically alkylated, resulting in the affinity labeling of the μ opioid binding site.

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 in order to obtain irreversible labeling of the μ opioid receptor. β-FNA has been most extensively used to label μ opioid receptors (1). In the guinea pig ileum, β-FNA irreversibly inhibited the agonist potencies of μ-selective opioids, while not significantly altering the effects of κ opioids (2–5). Irreversible inhibition of opioid binding by β-FNA to homogenates from brain (6–9) and tissues used in bioassays (5, 10, 11) has been more difficult to demonstrate. β-FNA has been shown to interact both reversibly and irreversibly with opioid binding sites in mouse brain homogenates (6) and brain slices (12). While having a preference for μ opioid binding sites, β-FNA also irreversibly decreased binding to δ opioid sites from mouse brain (6). Partial irreversible inhibition of opioid binding to rat (7, 9) and guinea pig (8) brain membranes has been obtained by treating membranes with β-FNA. Other studies using rat brain homogenates (13), guinea pig brain membranes (5), and homogenates from the myenteric plexus-longitudinal muscle from the guinea pig (5, 10, 11) did not find irreversible inhibition of opioid binding to these tissues after incubation of the membrane homogenates with β-FNA. These authors suggested that the effects of β-FNA on agonist potency at the μ opioid receptor were due to an interference with the coupling mechanism between the μ opioid receptors and the effector system. When bovine striatal membranes were incubated with [^{3}H]β-FNA and then separated under denaturing and reducing conditions on polyacrylamide gels, a broad band corresponding to molecular weights of 68,000–97,000 was obtained by autoradiography (14). If the samples were treated with *N*-glycosidase F to digest glycogen moieties, bands corresponding to a molecular weight of 57,000 and 49,000 were labeled (14).

Other affinity ligands that have been used to affinity label μ opioid receptors include both alkaloids and peptides. Naloxonazine (15) and a series of 14-hydroxydihydromorphinone hydrazones (16–18) have been used *in vivo* and *in vitro* to

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ABBREVIATIONS: β-FNA, β-funaltrexamine; BAM, 14β-(bromoacetamido)morphine; BAMO, 14β-(bromoacetamido)morphinone; H_2BAM, 14β-(bromoacetamido)-7,8-dihydromorphine; H_2BAMO, 14β-(bromoacetamido)-7,8-dihydromorphinone; DTT, dithiotreitol; MBTA, [4-(*N*-maleimido)benzyl] trimethylammonium iodide; NEM, *N*-ethylmaleimide; DAGO, [D-Ala^2,(Me)Phe^4,Gly(ol)^5] enkephalin; DPDPE, [D-penicillamine^2,D-penicillamine^5]enkephalin; β_h-endorphin, human β-endorphin; ^{125}I-β_h-endorphin, human β-endorphin with Tyr^{27} radiolabeled; DADLE, [D-Ala^2,D-Leu^5]enkephalin; DSLET, [D-Ser^2,Leu^5,Thr^6]enkephalin; DALECK, Tyr-D-Ala-Gly-Phe-Leu-CH_2Cl; U69,593, (5α,7α,8β)-(−)-*N*-methyl-*N*-(7-(1-pyrrolidinyl)-1-oxaspiro(4,5)dec-8-y)benzeneacetamide; U50,488H, (trans)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)-cyclohexyl]benzeneacetamide methanesulfonate hydrate; BSA, bovine serum albumin; KIU, kallikrein inhibiting unit; TLC, thin layer chromatography.

irreversibly block μ_1 opioid receptors. When rat brain membranes were incubated with the peptide [^{3}H]DALECK at an alkaline pH, a 58,000-Da protein was specifically labeled (19). Based on competition studies, the authors suggested that this labeling was to the μ 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 and then extensive washing of the membranes, greater than 90% of the μ opioid binding to membranes was inhibited, whereas binding to δ and κ opioid sites was not altered by BAM alkylation of membranes (20). Previous studies have shown that opioid binding was inhibited by disulfide bond-reducing reagents (21–25) and sulfhydryl-alkylating compounds (26–30), suggesting the presence of a disulfide bond and sulfhydryl groups near opioid binding sites. Binding to μ opioid sites was more sensitive to disulfide bond-reducing reagents than binding to δ sites, and κ opioid binding was relatively unaltered by reducing reagents (25). By taking advantage of this disulfide bond near the μ opioid binding site, the specific labeling of this site was possible. A similar approach has been used to label nicotinic cholinergic receptors with bromoacetylcholine (31–34) and MBTA (33). After sequencing of this receptor (35), the specific cystinyl residues labeled with [^{3}H]MBTA were identified (36).

The study reported here describes the use of the affinity ligands H_2BAMO, BAMO, H_2BAM, and BAM to irreversibly inhibit μ opioid binding to rat brain membranes. BAMO, H_2BAM, and H_2BAMO irreversibly inhibited μ opioid binding at concentrations 8- to 25-fold lower than were needed with BAM (20). Because of their higher affinity for the μ opioid binding site, H_2BAMO, BAMO, and H_2BAM, at concentrations routinely used in reversible binding experiments, will irreversibly and specifically label the μ opioid binding site.

Materials and Methods

Synthesis of BAM, BAMO, and H_2BAMO. BAM and BAMO were synthesized as previously described (37). H_2BAMO was synthesized from 14β-aminomorphinone by the following procedure. To obtain 14 β-aminomorphinone, a solution of 530 mg (1.48 mM) of 14β-aminocodeinone dimethyl ketal (37) in 65 ml of dry CH_2Cl_2 was cooled to −15° and a solution of 2.65 g (10.6 mM) of BBr_3 in 15 ml of dry CH_2Cl_2 was added dropwise with stirring. After 3 hr, the solution was poured onto ice and treated with excess cold 2% NaOH solution. After 45 min, the layers were separated and the pH of the aqueous phase was adjusted to 7 before being extracted with CH_2Cl_2/CH_3OH (9:1). Evaporation of the solvent gave almost pure product, weight 230 mg (52%). Recrystallization from CH_3OH gave pure material, which decomposed at 210–240°. The IR spectrum showed a strong peak at 1675 cm^{-1}, characteristic of an α,β-unsaturated ketone.

To prepare H_2BAMO, a solution of 10 mg of recrystallized 14β-aminomorphinone[1] in 15 ml of CH_3OH containing 67 μl of 1 N HCl was hydrogenated in the presence of 10 mg of Pd/C for 10 min. The catalyst was removed and the solution was taken to dryness. The IR spectrum showed the presence of a peak at 1720 cm^{-1} and no trace of the 1675 cm^{-1} absorbance.

A solution of 20 mg of the above HCl salt was dissolved in 20 ml of dry $CHCl_3$ containing 32 mg of triethylamine. The stirred solution was cooled to −15° and a solution of 25 mg (0.124 mM) of bromoacetylbromide in 5 ml of dry $CHCl_3$ was added dropwise over a period of 5 min. After stirring for an additional 30 min, the ice bath was removed and stirring was continued another 4 hr. The reaction mixture was washed with saturated $NaHCO_3$ solution and then with H_2O, followed by evaporation to dryness. The residue was dissolved in 5 ml of CH_3OH containing 200 μl of HCl. The mixture was heated with stirring at 50–60° for 45 min. Evaporation of the solvent left 25 mg of a mixture that was chromatographed on silica gel plates using CH_3OH/$CHCl_3$ (1:9) as the developing solvent. There was obtained 11 mg of pure H_2BAMO,[1] which was dissolved in 1 ml of CH_3OH containing 30 μl of 1 N HCl. The solution was evaporated to dryness to leave a residue that, after trituration with ether, left 11.5 mg (47%) of HCl salt, m.p. 230–240° (decomposed). IR: 1720 (ketonic C=O), 1685 cm^{-1} (amide C=O); mass spectrometry (chemical ionization): 421, 423 (M + 1); NMR [$(CD_3)_2SO$] δ: 2.90 (s, 3 H, NCH_3), 4.40 (q, 2 H, J = 16 Hz, CH_2Br), 5.18 (s, 1 H, 5-H), 6.70 (q, 2 H, arom. H), 9.05 (s, 1 H, OH).

Synthesis of H_2BAM. To a solution of 14β-aminomorphine (190 mg) in 25 ml of CH_3OH, a suspension of 10% palladium on charcoal (25 mg) in 1 ml of H_2O was added and the mixture was hydrogenated at room temperature and 15 psi H_2 pressure for 90 min. The mixture was then filtered and washed with CH_3OH. Evaporation of the filtrate *in vacuo* and drying of the residue in a desicator over predried $CaCl_2$ at 12 mm Hg furnished 190 mg of almost pure (by TLC) product, which was used in the bromoacetylation reaction without purification.

To the stirred solution of 14β-amino-7,8-dihydromorphine (190 mg, 0.63 mmol) in 40 ml of dry $CHCl_3$ and $(CH_3CH_2)_3N$ (198 mg, 1.96 mmol), a solution of bromoacetylbromide (388 mg, 1.92 mmol) in 10 ml of dry $CHCl_3$ was dropped during 15 min at approximately −15°. The reaction mixture was then slowly warmed up to room temperature and stirring with exclusion of moisture was continued for 90 min. The reaction solution was then washed with H_2O (3 × 20 = 60 ml) and the $CHCl_3$ phase was evaporated *in vacuo* to leave an oil, which was taken up in 20 ml of CH_3OH, and then 2 ml of 1 N HCl were added and heated under reflux for 2.5 hr. The methanolic solution was concentrated to 2 ml and left at room temperature. Upon cooling and scratching, the desired product crystallized, which was filtered and washed with a little CH_3OH and dried to give 40 mg of H_2BAM·HCl,[1] m.p. > 250°.

The methanolic filtrate was evaporated and the residue was purified by preparative TLC (silica gel, $CHCl_3$/CH_3OH, 9:1) to give 170 mg of TLC pure H_2BAM·HCl, which was dissolved in 5 ml of CH_3OH; 410 μl of 1 N HCl were added and the solution was stirred for 1 hr. Evaporation of the solvent (CH_3OH) and trituration of the residue in diethyl ether furnished 170 mg of H_2BAM·HCl. Total yield was 170 mg + 40 mg = 210 mg (72.5%). XL-200 NMR, IR, and mass spectra were recorded.

Opioid binding to rat brain membranes. Rat brain membranes, excluding cerebellar tissue, were prepared from male Sprague-Dawley rats and washed at 37° for 30 min in 50 mM Tris·HCl, pH 7.5, as previously described (20). Membranes were resuspended in 50 mM Tris·HCl, pH 7.5, at a protein concentration of approximately 20 mg/ml and were stored at −80° until use. Protein concentration was determined by the method of Bradford (38), with BSA as the standard.

To measure the binding of the μ-selective peptide [^{3}H]DAGO, the δ-selective peptide [^{3}H]DPDPE, or the antagonist [^{3}H]naloxone, 0.1 to 0.5 mg of membrane protein was incubated in 50 mM Tris·HCl, pH 7.5, with the radiolabeled ligand in a final volume of 1 ml at 25°. Incubation times of 60 min were used for [^{3}H]DAGO and [^{3}H]naloxone and a 2-hr incubation was used for [^{3}H]DPDPE. Nonspecific binding was measured by the inclusion of 1 μM DAGO, 1 μM naloxone, or 1 μM DADLE for the radiolabeled ligands [^{3}H]DAGO, [^{3}H]naloxone, and [^{3}H]DPDPE, respectively. Binding to κ opioid receptors was measured by two methods. In one, membranes were incubated with 0.2 nM (−)-[^{3}H]bremazocine in the presence of 400 nM DAGO and 400 nM DADLE, acting as μ and δ blockers, for 60 min at 25° in a final volume of 1 ml of 50 mM Tris·HCl, pH 7.5. Nonspecific binding was measured by the inclusion of 1 μM U50,488H. Binding of the κ-selective ligand [^{3}H]U69,593 was also measured by incubating 0.5 mg of membrane protein in 1 ml of 50 mM Tris·HCl, pH 7.5, for 60 min with 1 nM [^{3}H]U69,593.

[1] The elemental analyses of all new compounds were within 0.4% of the calculated values.

Binding assays were terminated by filtering the samples through Schleicher & Schuell No. 32 glass fiber filters using a Brandel 48-well cell harvester. The filters were subsequently washed three times with 4 ml of cold 50 mM Tris·HCl, pH 7.5, and were counted in 2 ml of Liquiscint or Ecoscint A scintillation fluid. For [^{3}H]U69,593 binding, the filters were soaked in 0.25% polyethylenimine for at least 60 min before use.

For ^{125}I-β_h-endorphin binding assays, 30–50 μg of membrane protein were added to polypropylene tubes in a final volume of 0.5 ml of 50 mM Tris·HCl, pH 7.5, containing 0.20% BSA and 0.01% bacitracin. ^{125}I-β_h-Endorphin, obtained lyophilized, was reconstituted according to the manufacturer's recommendations in 0.25% BSA, 5% lactose, 0.2% L-cysteine hydrochloride, 10 mM citric acid, and 800 KIU/ml aprotinin. The reconstituted ^{125}I-β_h-endorphin was aliquoted in 4-ml Nunc polypropylene tubes at 1 μCi in 10 μl/tube and was stored at −20° until use. One tube (1 μCi) was used for each assay, consisting of 8–12 samples assayed in triplicate for a total of 24–36 separate tubes. Just before use, unlabeled β_h-endorphin in 50 mM Tris·HCl, pH 7.5, 0.20% BSA, and 0.01% bacitracin was added to the 1 μCi of ^{125}I-β_h-endorphin to a final concentration of 0.25 nM. Nonspecific binding was measured by the inclusion of 1 μM β_h-endorphin. After a 60-min incubation at 25°, the contents of the tubes were filtered through Schleicher & Schuell No. 32 filters that had been soaked in 0.25% polyethylenimine for at least 60 min. After filtration, the filters were washed three times with 4 ml of ice-cold 50 mM Tris-HCl, pH 7.5, and counted in a γ-counter.

To determine the IC_{50} values for the inhibition of opioid binding to brain membranes by morphine, H_2BAMO, BAMO, H_2BAM, and BAM, 12 different concentrations of each ligand were incubated with the membranes along with the radiolabeled opioids. IC_{50} values were calculated by least squares fit to a logarithm-probit analysis.

Optimization of the alkylation of the μ opioid binding by H_2BAMO, BAMO, H_2BAM, and BAM. To determine the optimal temperature for the irreversible inhibition of opioid binding by H_2BAMO, 20 mg of membrane protein were incubated with 8 mM DTT in 1.8 ml of 50 mM Tris·HCl, pH 7.5, for 30 min at either 37°, 25°, or 4°. Controls consisted of membranes incubated at the various temperatures in the absence of DTT. Buffer or 50 nM H_2BAMO was added to the membranes to bring the final volume to 2.4 ml and the final DTT concentration to 6 mM. After an additional 30-min incubation at the same temperature, the membranes were diluted to 40 ml by the addition of cold 50 mM Tris·HCl, pH 7.5. The membranes were centrifuged at 48,000 × g for 15 min. The pellets were homogenized with a Polytron (Brinkmann) in 40 ml of cold 50 mM Tris·HCl, pH 7.5, and the washing step was repeated three additional times. Membranes were finally resuspended in 4.5 ml of 50 mM Tris·HCl, pH 7.5. The binding of 0.25 nM [^{3}H]DAGO to 0.2 ml of membranes, approximately 0.4 mg of protein, was measured as described above.

To determine the effect of the order of addition of DTT and BAMO on the irreversible inhibition of [^{3}H]DAGO binding, 20 mg of membrane protein were incubated for 30 min at 37° in 1.8 ml of 50 mM Tris·HCl, pH 7.5, with either DTT or BAMO. DTT, BAMO, or buffer was added to bring the volume to 2.4 ml. The final concentrations of DTT and BAMO were 6 mM and 50 nM, respectively. The incubation was continued for an additional 30 min. When DTT and BAMO were added together, membranes were incubated with both compounds for 30 min. The membranes were diluted to 40 ml with cold 50 mM Tris·HCl, pH 7.5 and washed four times, and the binding of 0.25 nM [^{3}H]DAGO to 0.2 ml of membranes was measured as described above.

The concentration of DTT was titrated from 0.375 to 12 mM. The concentration of BAMO was titrated from 1.6 to 400 nM. H_2BAMO was titrated from 0.78 to 50 nM, whereas H_2BAM was titrated from 1 to 2000 nM. BAM was titrated from 24 to 6000 nM. To determine whether, in the absence of DTT, the affinity ligands could be completely washed from the membranes, 20 mg of membrane protein were incubated with varying concentrations of the affinity ligands in 2.4 ml of 50 mM Tris·HCl, pH 7.5. After a 30-min incubation, membranes were washed four times as described above. The binding of 0.25 nM [^{3}H]DAGO to membranes incubated with the affinity ligands was compared with buffer-treated control membranes. The highest concentration of each affinity ligand used in the titration experiments was completely removed from membranes by four washes, when membranes were incubated with the affinity ligand in the absence of DTT.

To determine the time that was necessary for H_2BAMO alkylation of membranes, 20 mg of membrane protein were incubated with 8 mM DTT in 1.8 ml of 50 mM Tris·HCl, pH 7.5, for 30 min. The volume was increased to 2.4 ml by the addition of 50 nM H_2BAMO. The reaction was terminated by diluting the membranes to 40 ml with cold 50 mM Tris·HCl, pH 7.5, at time points ranging from 15 sec to 64 min after the addition of H_2BAMO. After four washes, the membranes were resuspended in 4.5 ml of 50 mM Tris·HCl, pH 7.5, and the binding of 0.25 nM [^{3}H]DAGO to 0.2 ml of membranes was measured as described above.

Multiple opioid binding to membranes alkylated with H_2BAM. Because under reversible binding conditions 2 μM H_2BAM could be completely removed from membranes with four centrifugal washes, H_2BAM was used to alkylate greater than 90% of the μ opioid binding sites. The binding of a number of radiolabeled opioid alkaloids and peptides to membranes that had been alkylated with 2 μM H_2BAM was measured to determine the specificity of the alkylation. Rat brain membranes, 20 mg of protein, were incubated at 37° for 30 min in 1.8 ml of 50 mM Tris·HCl, pH 7.5, with DTT at a final concentration of 6 mM. After an additional 30-min incubation at 37° with 2 μM H_2BAM, the membranes were washed four times by centrifugation as described above. Membranes were resuspended in 4.5 ml of 50 mM Tris·HCl, pH 7.5, and opioid binding to the membranes was measured. The binding of radiolabeled opioids to 0.2 ml of the membranes treated with DTT and H_2BAM was determined as described above and was compared with membranes treated with only DTT. The binding of 0.2 nM [^{3}H] diprenorphine to 0.2 ml of membranes was measured in a final volume of 1 ml of 50 mM Tris·HCl, pH 7.5, at 25° for 60 min. Nonspecific binding was measured by the inclusion of 1 μM naloxone. The binding of 0.7 nM [^{3}H]DADLE to 0.2 ml of membranes was measured in a 1-ml final volume of 50 mM Tris·HCl, pH 7.5, for 60 min at 25°. Nonspecific binding was measured by the inclusion of 1 μM DADLE. Samples were filtered through glass fiber filters as described above.

Titrating [^{3}H]DAGO concentrations. Rat brain membranes, 20 mg of protein, in 1.8 ml of 50 mM Tris·HCl, pH 7.5, were incubated with 8 mM DTT at 37° for 30 min. The volume was increased to 2.4 ml by the addition of 2 μM H_2BAM, and the incubation was continued for an additional 30 min. The membranes were washed four times as described above. They were resuspended in 4.5 ml of 50 mM Tris·HCl, pH 7.5, and [^{3}H]DAGO binding to 0.2 ml of membranes was measured. [^{3}H]DAGO concentrations were varied from 0.03 to 4 nM. After a 60-min incubation at 25°, the samples were filtered onto glass fiber filters as described above.

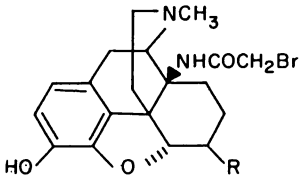
Protection of the μ opioid binding site from alkylation with H_2BAMO and BAMO by incubating membranes with opioids. To determine whether opioids could protect the opioid binding site from alkylation by H_2BAMO or BAMO, the following experiments were performed. Membranes, 20 mg of protein, were incubated in 1.6 ml of 50 mM Tris·HCl, pH 7.5, for 10 min at 37° with a 100 nM final concentration of either morphine, naloxone, levorphanol, dextrorphan, U50,488H, DAGO, DADLE, or DSLET. DTT at a final concentration of 6 mM was added to bring the volume to 1.8 ml. After a 10-min incubation at 37°, the volume was increased to 2.4 ml by the addition of 10 nM H_2BAMO, and the incubation was continued for an additional 10 min. Membranes treated with 20 nM BAMO were incubated at 37° for 30 min after the addition of DTT and after the addition of 20 nM BAMO. After diluting the contents of the tubes to 40 ml by the addition of cold 50 mM Tris·HCl, pH 7.5, and four washes at 48,000 × g, the membranes were resuspended in 4.5 ml of 50 mM Tris·HCl, pH 7.5, and the binding of 0.25 nM [^{3}H]DAGO to 0.2 ml membranes was measured as described above. All protecting ligands were tested for their ability to be removed from the membranes by the four washes.

With the exception of membranes treated with 100 nM DAGO, the binding of 0.25 nM [^{3}H]DAGO to membranes treated with only 100 nM concentrations of the protecting ligands resulted in binding greater than 90% of buffer-treated control membranes. Membranes treated with 100 nM DAGO followed by four washes bound 86 ± 2% of the 0.25 nM [^{3}H]DAGO that was bound by buffer-treated membranes.

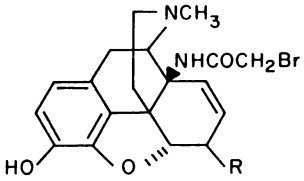
Materials. Male Sprague-Dawley rats (125–150 g) were purchased from Charles River Laboratories. DTT, Ultrol grade, was purchased from Calbiochem. [^{3}H]DAGO (60 Ci/mmol), [^{3}H]DPDPE (27 Ci/mmol), [^{3}H]U69,593 (60 Ci/mmol), [^{3}H]naloxone (56.1 Ci/mmol), [^{3}H]diprenorphine (34 Ci/mmol), [^{3}H]DADLE (44.1 Ci/mmol), and ^{125}I-labeled β_h-endorphin (1957 Ci/mmol), labeled at the Tyr^{27} residue, were purchased from Amersham. (−)-[^{3}H]Bremazocine (22.1 Ci/mmol) was purchased from New England Nuclear. Opioid peptides were obtained from Bachem. U50,488H was a gift from Upjohn (Kalamazoo, MI), and U69,593 was purchased from Amersham. Glass fiber filter sheets No. 32 were purchased from Schleicher & Schuell. Ecoscint A and Liquiscint scintillation fluids were purchased from National Diagnostics.

Results

Determination of the ability of the affinity ligands to inhibit opioid binding to the multiple opioid binding sites. The structures of the four affinity ligands H_2BAMO, H_2BAM, BAMO, and BAM are shown in Fig. 1. In the absence of a disulfide bond-reducing reagent, these affinity ligands bound reversibly to opioid binding sites in rat brain membranes. Table 1 shows the IC_{50} values obtained for morphine and the affinity ligands for the inhibition of opioid binding to the multiple opioid binding sites. Morphine and the affinity ligands were more potent in inhibiting μ opioid binding than the binding to δ or κ opioid sites. The rank order of potency for inhibiting 0.25 nM [^{3}H]DAGO binding to rat brain membranes was H_2BAMO > morphine > BAMO = H_2BAM > BAM. All of the affinity ligands had approximately the same receptor selectivity as morphine. The addition of a 14β-bromoacetamido group to morphine did not greatly diminish its affinity or selectivity for the μ opioid binding site. In addition, the 14β-bromoacetamido derivatives of dihydromorphine, morphinone, and dihydromorphinone had an enhanced affinity for the μ opioid binding site, in comparison with BAM.



R	COMPOUND
=O	H_2BAMO
-OH	H_2BAM



R	COMPOUND
=O	BAMO
-OH	BAM

Fig. 1. Structure of H_2BAMO, H_2BAM, BAMO, and BAM.

Determination of the effect of temperature, the order of addition of DTT and the affinity ligands, and incubation time on the irreversible inhibition of [^{3}H]DAGO binding to membranes. To determine whether the affinity ligands would irreversibly inhibit opioid binding to rat brain membranes and to determine whether the incubation temperature altered the irreversible inhibition of [^{3}H]DAGO binding to membranes, the following experiment was performed. Membranes were incubated with either 50 nM H_2BAMO, 6 mM DTT, or 6 mM DTT followed by the addition of 50 nM H_2BAMO, for 30 min at either 37°, 25°, or 4°. After four centrifugal washes, the binding of 0.25 nM [^{3}H]DAGO to treated membranes was compared with buffer-treated control membranes. As shown in Table 2, [^{3}H]DAGO binding to membranes incubated with 50 nM H_2BAMO followed by washing was the same as buffer-treated controls regardless of the incubation temperature. Incubating membranes at 37° or 25° with 6 mM DTT followed by washing had no effect on opioid binding to membranes. At 4°, there was a slight enhancement in opioid binding to membranes that had been incubated with DTT, in comparison with buffer-treated controls. When membranes were incubated with 6 mM DTT, followed by the addition of 50 nM H_2BAMO and then extensive washing, [^{3}H]DAGO binding to these membranes was irreversibly inhibited. The percentage of inhibition of binding increased with increasing temperature. Similar results were found with the other three affinity ligands (data not shown). Because the greatest alkylation of the opioid binding site occurred at 37°, this incubation temperature was used in all other experiments.

Table 3 shows the effect of the order of addition of DTT and 50 nM BAMO on the irreversible inhibition of 0.25 nM [^{3}H]DAGO binding to membranes. Whether DTT was added to membranes before the addition of BAMO or whether these two compounds were added together did not effect the percentage of irreversible inhibition of opioid binding that was measured. However, [^{3}H]DAGO binding to membranes incubated with 50 nM BAMO before the addition of DTT was the same as to control membranes. No irreversible inhibition of binding was observed if the affinity ligand bound to the μ opioid binding site before the addition of the disulfide bond-reducing reagent. These results suggest that, when the affinity ligand bound

TABLE 1

IC_{50} values for the inhibition of opioid binding to rat brain membranes by morphine, BAM, H_2BAM, BAMO, and H_2BAMO

Rat brain membranes were incubated with 12 different concentrations of either morphine, BAM, H_2BAM, BAMO, or H_2BAMO in the presence of the radiolabeled opioid in 50 mM Tris-HCl, pH 7.5, as described in Materials and Methods. Data are presented as the mean IC_{50} value ± standard error obtained from three experiments, performed in triplicate.

Radiolabeled Opioid	IC_{50}				
	Morphine	BAM	H_2BAM	BAMO	H_2BAMO
			nM		
[^{3}H]DAGO, 0.25 nM	0.76 ± 0.13	3.2 ± 0.69	1.8 ± 0.03	1.5 ± 0.67	0.41 ± 0.02
[^{3}H]DPDPE, 1 nM	83 ± 20	210 ± 13	97 ± 18	42 ± 14	21 ± 3.2
[^{3}H]U69,593, 1 nM	49 ± 8.8	440 ± 12	45 ± 4.1	130 ± 18	61 ± 13
[^{3}H]Bremazocine, 0.2 nM, + μ and δ blockers	170 ± 13	550 ± 94	300 ± 75	470 ± 40	120 ± 21
[^{3}H]Naloxone, 2 nM	6.1 ± 2.7	26 ± 3.4	14 ± 6.0	4.6 ± 0.74	1.5 ± 0.30
^{125}I-β_h-Endorphin, 1 nM	3.1 ± 0.50	14 ± 0.65	3.8 ± 0.19	4.0 ± 1.6	2.3 ± 0.27

TABLE 2

Effect of temperature on the irreversible inhibition of [^{3}H]DAGO binding to membranes by H_2BAMO

Rat brain membranes, 20 mg of protein in 1.8 ml of 50 mM Tris·HCl, pH 7.5, were incubated with either 8 mM DTT or buffer at the temperature stated for 30 min. Buffer or 50 nM H_2BAMO was added to the samples to bring the final volume to 2.4 ml and the final DTT concentrations to 6 mM. After an additional 30-min incubation at the stated temperature, the membranes were diluted to 40 ml by the addition of cold 50 mM Tris·HCl, pH 7.5. The membranes were centrifuged at 48,000 × *g* for 15 min. The pellets were resuspended in 40 ml of buffer, and the washing step was repeated three additional times. Membranes were finally resuspended in 4.5 ml of 50 mM Tris·HCl, pH 7.5. The binding of 0.25 nM [^{3}H]DAGO to 0.2 ml of membranes was measured as described in Materials and Methods. Control binding consisted of membranes incubated with buffer at the different temperatures. Data are presented as the mean percentage bound ± standard error from three experiments, performed in triplicate.

Condition	Binding		
	37°	25°	4°
	% of control		
50 nM H_2BAMO	102 ± 3	87 ± 5	96 ± 9
6 mM DTT	104 ± 6	98 ± 8	123 ± 7
6 mM DTT + 50 nM H_2BAMO	42 ± 6	56 ± 3	82 ± 5

TABLE 3

Effect of the order of addition of DTT and BAMO on the irreversible inhibition of [^{3}H]DAGO binding to membranes

Rat brain membranes, 20 mg of protein, were incubated at 37° in 1.8 ml of 50 mM Tris·HCl, pH 7.5, with either DTT or BAMO for 30 min. DTT, BAMO, or buffer was added to bring the volume to 2.4 ml. After an additional 30-min incubation, the membranes were washed four times and were ultimately resuspended in 4.5 ml of 50 mM Tris·HCl, pH 7.5. The binding of 0.25 nM [^{3}H]DAGO to 0.2 ml of membranes was measured as described in Materials and Methods. Membranes incubated with 6 mM DTT at 37° for 30 min were regarded as controls. Data are presented as the mean percentage bound ± standard error from three experiments performed in triplicate.

Condition	Binding
	% of control
6 mM DTT, followed by 50 nM BAMO	60 ± 3
50 nM BAMO, followed by 6 mM DTT	104 ± 4
6 mM DTT and 50 nM BAMO together	61 ± 2

reversibly to the μ opioid binding site, this bound ligand was able to protect the disulfide bond at or near the binding site from reduction with DTT. By protecting this site, the affinity ligand prevented the reduction and, therefore, the subsequent alkylation of this μ opioid binding site.

Fig. 2 demonstrates the time course of irreversible inhibition of [^{3}H]DAGO binding to membranes that had been treated with DTT followed by the addition of 50 nM H_2BAMO. Membranes were incubated with DTT at 37° for 30 min before the addition of H_2BAMO. A 30-min incubation with DTT was sufficient to reduce the disulfide bond at or near the opioid binding site. Greater than 50% of the total irreversible inhibition that was obtained by treating membranes with DTT and 50 nM H_2BAMO occurred within the first 2 min after the addition of H_2BAMO. After a 15-min incubation, the inhibition of [^{3}H]DAGO binding started to reach a plateau, and a 60-min incubation at 37° did not significantly increase the percentage of irreversible inhibition of binding over the percentage obtained with a 30-min incubation. Similar time courses were obtained with the other affinity ligands.

Determination of the effect of the concentrations of DTT and the affinity ligands on the irreversible inhibition of [^{3}H]DAGO binding. To determine the DTT concentration that was necessary to obtain optimal irreversible inhibition of opioid binding by the affinity ligands, membranes were incubated with DTT at final concentrations ranging from 0.375 to 12 mM for 30 min at 37°. After this incubation, 20 nM concentrations of either H_2BAMO or BAMO were added to the samples, and the incubation was continued 30 min before the membranes were washed to remove noncovalently bound ligand. As shown in Table 4, the percentage of control binding obtained with both H_2BAMO and BAMO was dependent on the concentration of DTT. With DTT concentrations of less than 1 mM, little irreversible inhibition of opioid binding was obtained. With increasing concentrations of DTT, the irreversible inhibition of [^{3}H]DAGO binding to membranes treated with an affinity ligand increased. Maximal inhibition of opioid binding was obtained at 6 mM DTT. Consequently, a final concentration of 6 mM DTT was used in all other experiments. As shown in Table 2, incubating membranes with 6 mM DTT followed by washing did not alter [^{3}H]DAGO binding to these membranes.

Fig. 3 demonstrates that the degree of inhibition of [^{3}H]DAGO binding to membranes treated with 6 mM DTT was dependent on the concentration of the affinity ligands. With a 30-min incubation at 37°, a concentration of 13 ± 1.6 nM H_2BAMO resulted in 50% of the 0.25 nM [^{3}H]DAGO binding sites being irreversibly inhibited. Under identical conditions, 33 ± 8.1 nM BAMO and 41 ± 1.8 nM H_2BAM resulted in half of the [^{3}H]DAGO binding sites being inhibited. In contrast, a concentration of 330 ± 15 nM BAM was necessary to achieve a 50% reduction in the number of [^{3}H]DAGO binding sites. H_2BAMO, BAMO, and H_2BAM were 8- to 25-fold more potent in irreversibly inhibiting [^{3}H]DAGO binding to membranes than was BAM.

The highest concentration of each affinity ligand used in the irreversible inhibition studies was the highest concentration that, when incubated with membranes in the absence of DTT followed by four washes, resulted in the attainment of at least 95% of 0.25 nM [^{3}H]DAGO binding obtained with buffer-treated control membranes. The morphinone derivatives BAMO and H_2BAMO were more difficult to remove from the membranes than the morphine derivatives. With four washes, 50 nM H_2BAMO and 400 nM BAMO were the highest concentrations that could be completely removed from the membranes under reversible binding conditions. Thus, H_2BAMO and BAMO were the best affinity ligands to use when less than 70% of the [^{3}H]DAGO binding sites needed to be inhibited. When an irreversible inhibition of [^{3}H]DAGO binding of greater than 70% was desired, H_2BAM was the ligand of choice because, at 2 μM H_2BAM, 90% of the binding was inhibited and yet under reversible binding conditions 2 μM H_2BAM could be easily removed from the membranes.

Determination of the selectivity of the opioid binding site that was alkylated with the affinity ligands. The binding of radiolabeled opioids specific for the different types of opioid receptors to membranes treated with 6 mM DTT and 2 μM H_2BAM was investigated to determine whether alkylation of the opioid binding site with the affinity ligands was specific for a certain type of opioid binding site. Table 5 demonstrates that almost 90% of the μ opioid binding, as measured with 0.25 nM [^{3}H]DAGO, was irreversibly inhibited by alkylation of membranes with H_2BAM. The binding of the δ-selective peptide [^{3}H]DPDPE or κ opioid binding, as measured by (−)-[^{3}H]bremazocine binding in the presence of μ and δ blockers, was not inhibited in membranes that had been treated with DTT

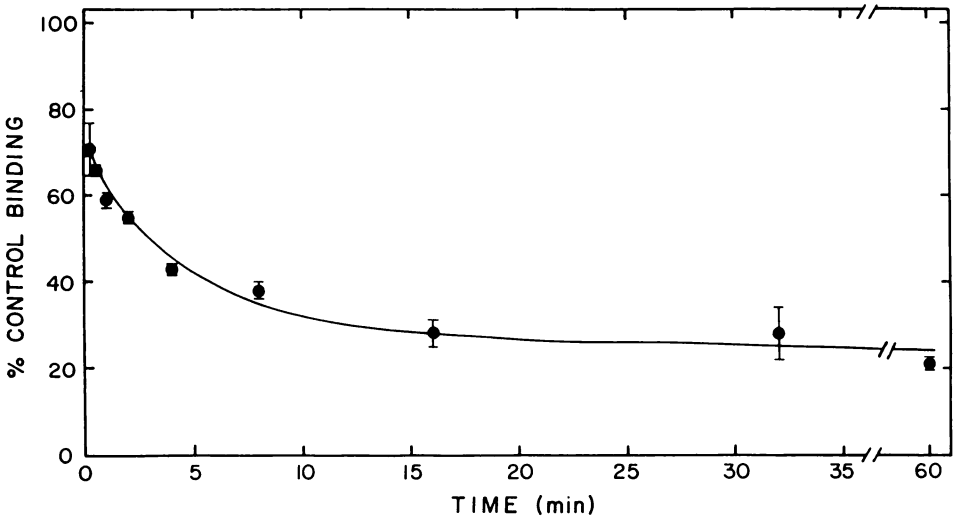


Fig. 2. Time course for H_2BAMO to irreversibly inhibit [^{3}H]DAGO binding to membranes. Rat brain membranes, 20 mg of protein, were incubated in 1.8 ml of 50 mM Tris·HCl, pH 7.5, with 8 mM DTT for 30 min at 37°. The volume was increased to 2.4 ml by the addition of 50 nM H_2BAMO. The incubation at 37° was terminated by diluting the contents of the tubes to 40 ml with cold 50 mM Tris·HCl, pH 7.5, at times ranging from 15 sec to 64 min after the addition of H_2BAMO. Samples were centrifuged at 48,000 × *g* for 15 min. The membrane pellet was resuspended in 40 ml of 50 mM Tris·HCl, pH 7.5, and the washing step was repeated three additional times before the membrane pellet was ultimately resuspended in 4.5 ml of 50 mM Tris·HCl, pH 7.5. The binding of 0.25 nM [^{3}H]DAGO was measured to 0.2 ml of resuspended membranes. Controls consisted of membranes incubated with only DTT. *Points* represent the mean percentage bound ± standard error from four experiments performed in triplicate.

TABLE 4

Effect of the DTT concentration on the irreversible inhibition of [^{3}H]-DAGO binding by H_2BAMO and BAMO

Rat brain membranes, 20 mg of protein, were incubated at 37° in 1.8 ml of 50 mM Tris·HCl, pH 7.5, for 30 min with varying concentrations of DTT. The sample volume was increased to 2.4 ml by the addition of either 20 nM H_2BAMO or BAMO, followed by an additional 30-min incubation. After four washes, the membranes were resuspended in 4.5 ml of 50 mM Tris·HCl, pH 7.5, and the binding of 0.25 nM [^{3}H]DAGO to 0.2 ml of membranes was measured as described in Materials and Methods. DTT concentrations refer to the final DTT concentration. Data are presented as the mean percentage of DTT control binding ± standard error from four experiments.

DTT concentration	Binding	
	H_2BAMO	BAMO
mM	*% of control*	
0.375	89 ± 3	97 ± 2
0.75	83 ± 5	82 ± 5
1.5	68 ± 5	67 ± 4
3.0	61 ± 3	61 ± 6
6.0	46 ± 2	51 ± 5
12.0	46 ± 2	59 ± 5

and H_2BAM. Similar results were obtained with the other affinity ligands.

β-Endorphin binds equally well to μ and δ opioid binding sites in brain (39). The binding of 0.25 nM ^{125}I-β_h-endorphin to alkylated membranes was reduced by 58%, suggesting that the remaining ^{125}I-β_h-endorphin binding was to δ opioid binding sites. Similarly, the binding of the antagonists [^{3}H]naloxone and [^{3}H]diprenorphine was also partially inhibited by treating membranes with DTT and H_2BAM. [^{3}H]Naloxone has a slight preference for μ opioid binding sites (39), whereas [^{3}H]diprenorphine binds equally well to all opioid binding sites. In alkylated membranes, [^{3}H]naloxone binding was inhibited to a greater degree than [^{3}H]diprenorphine binding, implying that the alkylation with the affinity ligands was specific for the μ opioid binding site and that this alkylation altered both agonist and antagonist binding.

Titrating [^{3}H]DAGO binding to membranes treated with DTT and H_2BAM. To determine whether the percentage of inhibition of [^{3}H]DAGO binding to alkylated membranes was dependent on the [^{3}H]DAGO concentration used in the binding assay, the binding of 0.03–4 nM [^{3}H]DAGO to membranes that had been treated with 6 mM DTT and 2 μM H_2BAM, followed by four washes was measured. The results are depicted in Fig. 4. At [^{3}H]DAGO concentrations of 0.5 nM and below, the percentage of DTT-treated control membrane binding for membranes treated with DTT and H_2BAM ranged from 8 to 16%. At [^{3}H]DAGO concentrations of greater than 0.5 nM, there was a gradual increase in the percentage of control binding obtained by treating membranes with DTT and H_2BAM. A K_d value of 0.4 nM [^{3}H]DAGO was obtained for the binding of this μ-selective opioid peptide to control membranes that had been treated with DTT and washed extensively (20). At [^{3}H]DAGO concentrations above the K_d value, [^{3}H]DAGO bound to additional sites that were not altered by the affinity labeling of membranes. At [^{3}H]DAGO concentrations below the K_d value, all of the [^{3}H]DAGO binding appeared to be to the μ opioid binding site(s) that were specifically alkylated with H_2BAM. Similar results were obtained with the other three affinity ligands.

Determination of the ability of opioids to protect the μ opioid binding site from alkylation with H_2BAM. Because adding BAMO before the addition of DTT completely protected the opioid binding site from alkylation with the affinity ligand, as is shown in Table 3, a number of different opioid alkaloids and peptides were tested for their ability to prevent alkylation of the opioid binding site and subsequent irreversible inhibition of [^{3}H]DAGO binding to membranes. Opioids were incubated with membranes, followed by the addition of DTT and then either H_2BAMO or BAMO. After washing, [^{3}H]DAGO binding to membranes was determined and compared with DTT-treated control membranes and membranes treated with DTT and the affinity ligand. As shown in Table 6, similar results were obtained with both H_2BAMO and BAMO. Morphine afforded the greatest protection. Naloxone and levorphanol also protected the site from alkylation with the affinity ligands. The *d*-isomer dextrorphan and the κ-selective compound U50,488H were ineffective in protecting the site from alkylation. Of the peptides tested, the μ-selective peptide DAGO afforded the greatest protection, approximately 40%, and this estimate may

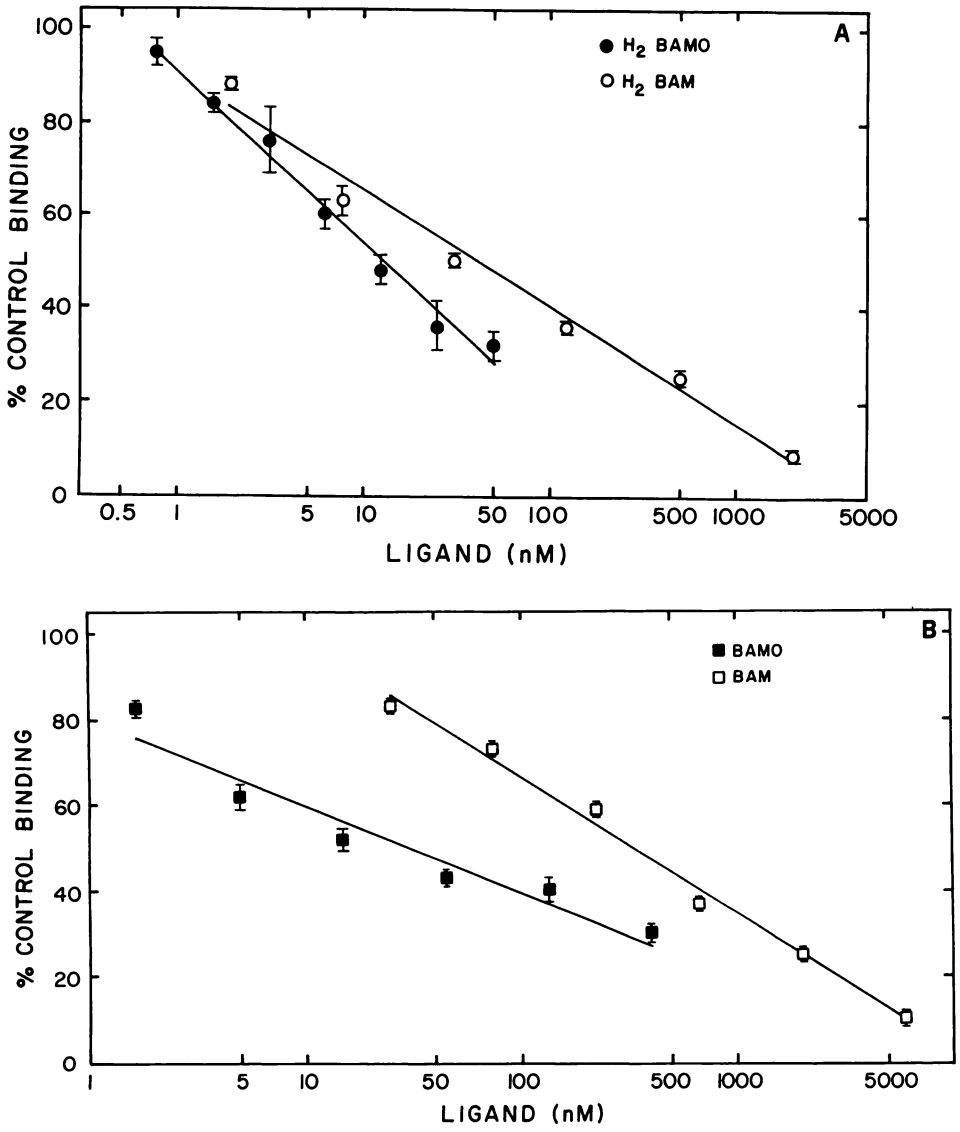


Fig. 3. Determination of the concentration of H_2BAMO, H_2BAM, BAMO, and BAM necessary for irreversible inhibition of [^{3}H] DAGO binding. In 1.8 ml of 50 mM Tris·HCl, pH 7.5, 20 mg of membrane protein were incubated with 8 mM DTT at 37° for 30 min. The affinity ligands were added at varying concentrations, and the incubation was continued for an additional 30 min. H_2BAMO was titrated from 0.78 to 50 nM, and BAMO was titrated from 1.6 to 400 nM (A). The concentration of H_2BAM was varied from 1 to 2000 nM and BAM was titrated from 24 to 6000 nM (B). After four washes, the binding of 0.25 nM [^{3}H]DAGO to the resuspended membranes was measured as described in Fig. 2. Binding to membranes treated only with DTT was regarded as control binding. The highest concentration of each affinity ligand used in the titrations was the highest concentration that could be completely washed from the membranes in the absence of DTT. *Points* are presented as the mean percentage bound ± standard error from three experiments performed in triplicate.

TABLE 5

Opioid binding to membranes treated with DTT and H_2BAM

Rat brain membranes, 20 mg of protein, in 1.8 ml of 50 mM Tris·HCl, pH 7.5, were incubated with 8 mM DTT at 37° for 30 min. The volume was increased to 2.4 ml by the addition of 2 μM H_2BAM, and the incubation was continued for 30 min at 37°. After four washes, the membranes were resuspended in 4.5 ml of 50 mM Tris·HCl, pH 7.5. The binding of the radiolabeled opioids to these membranes was measured as described in Materials and Methods. Controls consisted of membranes incubated with only DTT. Data are presented as the mean percentage bound ± standard error from three or more experiments.

Radiolabeled ligand	Binding
	% of control
[^{3}H]DAGO, 0.25 nM	12 ± 2
[^{3}H]DPDPE, 1 nM	106 ± 7
[^{3}H]Bremazocine, 0.2 nM, + μ and δ blockers	99 ± 6
[^{3}H]DADLE, 0.7 nM	42 ± 5
^{125}I-β_h-Endorphin, 0.25 nM	42 ± 3
[^{3}H]Naloxone, 0.8 nM	31 ± 4
[^{3}H]Diprenorphine, 0.2 nM	51 ± 5

be low. With the exception of DAGO, concentrations of 100 nM of all the ligands listed in Table 6 were removed from the membranes by four washes. Binding to membranes treated with only 100 nM DAGO was 86 ± 2% of control binding. In other words, DAGO may have afforded 15–20% more protection of the μ binding site from alkylation than is reported in Table 6. The peptides DADLE and DSLET bind to both δ and μ opioid binding sites but have a slight preference for the δ opioid site. These peptides slightly protected the site from alkylation. The δ-selective peptide DPDPE could not be tested for its ability to protect the site due to the fact that DPDPE contains a disulfide bond, which is broken upon the addition of DTT rendering the peptide inactive. In agreement with the data presented in Table 5, demonstrating the binding of many radiolabeled opioids to affinity-labeled membranes, μ-selective opioids protected the site from alkylation, whereas ligands that do not interact with the μ opioid binding site were ineffective at protecting the site from affinity labeling.

Discussion

The study reported here has shown that 14β-bromoacetamido derivatives of morphine, dihydromorphine, morphinone, and dihydromorphinone bind to opioid binding sites in rat brain membranes with high affinity and selectivity for the μ opioid binding site. By adding the reactive group to the 14 position,

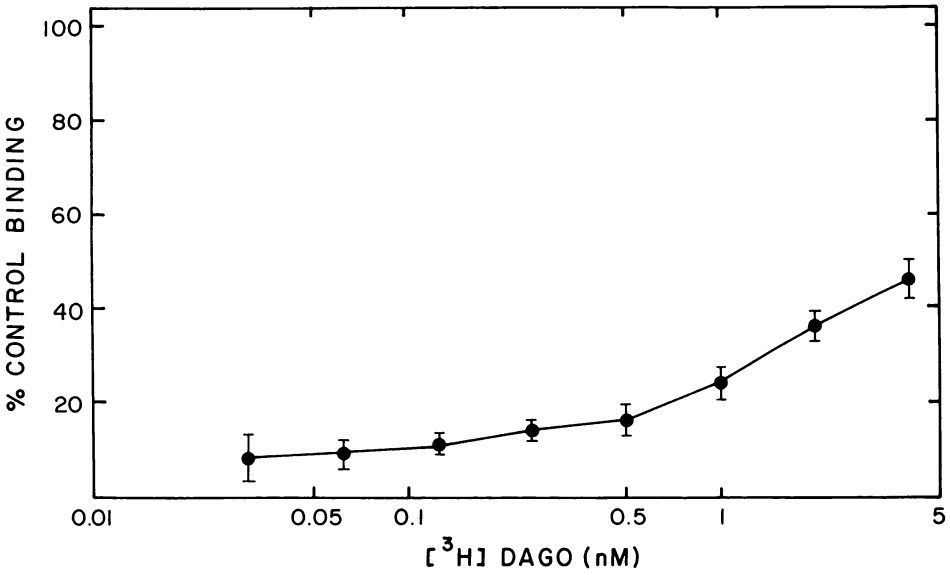


Fig. 4. The effect of the concentration of [^{3}H]DAGO on the irreversible inhibition of opioid binding obtained with membranes treated with DTT and H_2BAM. In 1.8 ml of 50 mM Tris·HCl, pH 7.5, 20 mg of membrane protein were incubated with 8 mM DTT for 30 min at 37°. The volume was increased to 2.4 ml by the addition of 2 μM H_2BAM. After an additional 30-min incubation, the contents of the tubes were washed as described in Fig. 2. The binding of [^{3}H]DAGO to 0.2 ml of resuspended membranes was measured, using [^{3}H]DAGO concentrations ranging from 0.03 to 4 nM. Control binding consisted of membranes incubated with only DTT. *Points* are presented as the mean percentage bound ± standard error from four experiments performed in triplicate.

TABLE 6

Ability of opioids to protect the μ opioid binding site from alkylation with H_2BAMO and BAMO

Protection experiments were performed with the ligands listed below at a final concentration of 100 nM, as described in Materials and Methods. Incubation times of 10 min after each addition were used with 10 nM H_2BAMO-treated membranes and 30-min incubation times were used with BAMO-treated membranes. After four centrifugal washes, the binding of 0.25 nM [^{3}H]DAGO to 0.2 ml of membranes was measured. Membranes treated with DTT and either 10 nM H_2BAMO or 20 nM BAMO without protecting ligands bound 53 ± 2% and 50 ± 5%, respectively, of DTT-treated control membranes. Data are presented as the mean percentage of protection ± standard error afforded by each ligand, where 100% protection was set equal to the binding obtained with DTT-treated membranes and 0% protection was set equal to the binding obtained with DTT and either H_2BAMO- or BAMO-treated membranes.

Ligand	Protection	
	H_2BAMO	BAMO
	%	
Morphine	90 ± 11	96 ± 8
Naloxone	82 ± 16	82 ± 4
Levorphanol	63 ± 15	80 ± 1
Dextrorphan	(−2) ± 7	(−8) ± 2
U50,488H	1 ± 10	1 ± 8
DAGO	43 ± 6	37 ± 8
DADLE	29 ± 4	24 ± 9
DSLET	20 ± 12	17 ± 8

the affinity or selectivity of these compounds was not significantly compromised. The affinity for the μ opioid binding site was increased either by reduction of the double bond in the 7,8 position or by the addition of a ketone instead of an alcohol in position 6 of morphine. The combination of both of these modifications resulted in H_2BAMO, the ligand with the highest affinity for μ opioid binding sites, as shown in Table 1.

Incubation of rat brain with any of the affinity ligands followed by extensive washing of the membranes did not result in any irreversible inhibition of opioid binding to the membranes. This result implies that the affinity ligands did not bind covalently to the binding site of the opioid receptor or at any other position on the receptor that could allosterically influence opioid binding. Only by the use of radiolabeled derivatives of these affinity ligands will it be possible to definitively determine whether these affinity ligands bind covalently to any protein in the rat brain membranes. Opioid binding to membranes has been shown to be inhibited by DTT (21, 23–25), with the μ opioid binding site being the most sensitive (25). In the presence of DTT, the high affinity μ opioid binding site is probably reduced to a low affinity site (24). This finding is consistent with the fact that higher concentrations of the affinity ligands were needed to obtain irreversible inhibition of μ opioid binding to membranes than were necessary to reversibly inhibit μ opioid binding. As shown in Table 2, once DTT was removed from membranes, opioid binding to these membranes returned to levels obtained with buffer-treated control membranes. During the four washes that were used to remove noncovalently bound affinity ligands and DTT, the reduced disulfide was reoxidized, returning the μ opioid binding site to a high affinity state. In the presence of DTT, the affinity ligands covalently bound to the newly formed sulfhydryl group at the μ opioid binding site. The time course for the irreversible inhibition of [^{3}H]DAGO binding was quite rapid, occurring within the first 2 min after the addition of the affinity ligand. The rapid binding of the affinity ligands to the binding site probably accounts for the specific alkylation of the receptor despite the presence of DTT concentrations that greatly exceeded the concentrations of the affinity ligands. The maximal amount of inhibition obtained was dependent on the time and temperature of the incubation and on the concentrations of DTT and the affinity ligands. The only difference among the four affinity ligands was the concentration of each ligand that was necessary to obtain irreversible inhibition of opioid binding. The rank order of potency of the affinity ligands for irreversibly inhibiting μ opioid binding was the same as their order of potency in reversibly inhibiting μ opioid binding to membranes.

Additional support for the idea of a critical disulfide bond at the μ opioid binding site comes from the fact that BAMO could protect the opioid binding site from alkylation. Table 3 shows that the same percentage of irreversible inhibition of binding was obtained regardless of whether DTT was incubated with membranes before the addition of BAMO, or whether DTT and BAMO were added to the membranes together. However, if membranes were incubated with BAMO first and then DTT was added, no irreversible inhibition of binding was detected.

These data suggest that BAMO bound noncovalently to the μ opioid binding site and that this binding protected the disulfide bond at the μ opioid binding site from reduction with DTT and, consequently, subsequent alkylation with BAMO.

Only μ opioid binding to membranes was irreversibly inhibited by alkylation of membranes with the affinity ligands, as shown in Table 5. Opioid binding to δ and κ sites was not altered by the affinity ligands. Alkylation of membranes with the affinity ligands partially inhibited the binding of opioids that bind to other types of opioid binding sites in addition to μ opioid binding sites. Both μ agonist and antagonist binding to affinity-labeled membranes was irreversibly inhibited, demonstrating that the alkylation of the μ opioid binding site did not result in discrimination between agonist and antagonist binding.

The selectivity of the affinity labeling for the μ opioid binding site was further demonstrated by protection studies. As shown in Table 3, the affinity ligand itself was able to afford virtually complete protection from the alkylation of the binding site. With regard to other opioids, only opioids that are known to bind to the μ opioid binding site were able to protect the site from alkylation (Table 6). The alkaloids were slightly more potent than the μ-selective peptide DAGO, suggesting that the alkaloids may bind with closer proximity to the critical disulfide bond than the peptide. Also, the amount of protection was dependent on the concentration and affinity of the opioid for the μ opioid binding site. High concentrations of opioids could not be tested for their ability to protect the site from alkylation because many of the opioids, particularly the peptides, were difficult to wash completely from the membranes.

All of the data presented here suggest that there is an important disulfide bond at or near the μ opioid binding site. This observation raises the question of whether there are free sulfhydryl groups at the μ opioid binding site. Most of the evidence for the presence of free sulfhydryl groups comes from studies that have used NEM and other sulfhydryl-alkylating compounds (21, 26–30). NEM is known to react most rapidly with sulfhydryl groups, but NEM has also been shown to react with amino and imidazole groups (40–42).[2] Only partial inhibition of opioid binding to membranes has been observed with NEM (21, 26–30), suggesting that NEM may be allosterically altering the receptor instead of binding at the opioid binding site. NEM has also been shown to inhibit GTP regulation of opioid binding to brain membranes (29). This uncoupling of the μ opioid receptor from the G_i protein complex may also account for the inhibition of opioid binding to NEM-treated membranes.

The group that the μ-selective irreversible affinity ligand β-FNA alkylates has not been definitively reported, although it has been suggested that β-FNA probably binds to a sulfhydryl group (1, 2, 8, 43). NEM decreased [^{3}H]β-FNA binding to guinea pig brain membranes (8). Some studies have raised the possibility that β-FNA may not bind covalently to the opioid binding site but instead may bind to a site on the receptor distant from the binding site and that the alkylation of this site interferes with the coupling of the μ opioid receptor with effector systems (5, 10, 11, 13). Other studies support β-FNA alkylation of the μ opioid binding site (6–9, 12, 14). The specific location, at the μ opioid binding site or adjacent to the binding site, of the sulfhydryl group that β-FNA alkylates remains to be determined.

Most extracellular domains of proteins have been shown not to contain free sulfhydryl groups (44). The α-subunit of the nicotinic acetylcholine receptor is the site of acetylcholine binding, and a critical disulfide bond is located at this binding site. After the receptor was treated with DTT, Cys-192 was specifically labeled with the affinity label [^{3}H]MBTA, demonstrating that Cys-192 is half of the disulfide bridge, which is known to exist near the acetylcholine binding site (36). Both a site-specific mutation at Cys-192 and deletions in this region abolished ligand binding (45).

A disulfide bond located at the extracellular domain of the μ opioid receptor is probably critical for opioid binding. With the use of affinity ligands described in this study, the selective affinity labeling of the μ opioid receptor will be possible, as well as the identification of the critical cysteine residues at the μ opioid binding site.

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