14β-(Bromoacetamido)morphine Irreversibly Labels μ Opioid Receptors in Rat Brain Membranes†

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ABSTRACT: The binding properties of 14β-(bromoacetamido)morphine (BAM) and the ability of BAM to irreversibly inhibit opioid binding to rat brain membranes were examined to characterize the affinity and selectivity of BAM as an irreversible affinity ligand for opioid receptors. BAM had the same receptor selectivity as morphine, with a 3–5-fold decrease in affinity for the different types of opioid receptors. When brain membranes were incubated with BAM, followed by extensive washing, opioid binding was restored to control levels. However, when membranes were incubated with dithiothreitol (DTT), followed by BAM, and subsequently washed, 90% of the 0.25 nM [^{3}H][D-Ala^2,(Me)Phe^4,Gly(ol)^5]enkephalin (DAGO) binding was irreversibly inhibited as a result of the specific alkylation of a sulfhydryl group at the μ binding site. This inhibition was dependent on the concentrations of both DTT and BAM. The μ receptor specificity of BAM alkylation was demonstrated by the ability of BAM alkylated membranes to still bind the δ-selective peptide [^{3}H][D-penicillamine^2,D-penicillamine^5]enkephalin (DPDPE) and (−)-[^{3}H]bremazocine in the presence of μ and δ blockers, selective for κ binding sites. Under conditions where 90% of the 0.25 nM [^{3}H]DAGO binding sites were blocked, 80% of the 0.8 nM [^{3}H]naloxone binding and 50% of the 0.25 nM ^{125}I-labeled β_h-endorphin binding were inhibited by BAM alkylation. Morphine and naloxone partially protected the binding site from alkylation with BAM, while ligands that did not bind to the μ site did not afford protection. These studies have demonstrated that when a disulfide bond at or near μ opioid binding sites was reduced, BAM could then alkylate this site, resulting in the specific irreversible labeling of μ opioid receptors.

Determining the molecular weight of the multiple opioid receptors has been attempted by covalent cross-linking, radiation inactivation, molecular sieve chromatography, and partial purification of the opioid receptor complex. Covalent cross-linking of neural membranes with human [^{125}I-Tyr^{27}]-β-endorphin resulted in multiple proteins, ranging in molecular weight from 23 000 to 68 000, being specifically labeled (Howard et al., 1985, 1986; Helmeste et al., 1986). Radiation inactivation studies have suggested that μ and δ opioid receptor complexes have molecular weights of approximately 100 000 (Lai et al., 1984; Ott et al., 1988). Binding studies that have bound a radiolabeled opioid to detergent-solubilized opioid receptors followed by molecular sieve chromatography have estimated that the opioid receptor complex has a molecular weight ranging from 100 000 to 500 000 (Simonds et al., 1980; Dornay & Simantov, 1982; Howells et al., 1982; Chow & Zukin, 1983; Helmeste & Li, 1986; Simon et al., 1986). Partially purified opioid receptors, separated on sodium dodecyl sulfate–polyacrylamide gels, have revealed proteins with molecular weights ranging from 23 000 to 94 000 (Bidlack et al., 1981; Gioannini et al., 1985; Manekjee et al., 1985; Simonds et al., 1985; Cho et al., 1986; Nakayama et al., 1986; Ueda et al., 1987). A number of purification studies suggest a molecular weight for both μ and δ opioid receptors ranging from 58 000 to 65 000 (Simonds et al., 1985; Cho et al., 1986; Nakayama et al., 1986; Ueda et al., 1987). Another approach to determining the molecular weight of the opioid receptor is to affinity label the receptor.

Affinity labeling of the different types of opioid receptors allows the further elucidation of the pharmacological properties and the molecular characteristics of the multiple opioid receptors. A number of irreversible ligands have been synthesized for the specific labeling of opioid receptors. The peptide DALECK[1] inhibited electrically stimulated contractions in mouse vas deferens and partially inhibited the binding of [^{3}H]DADLE (Venn & Barnard, 1981) and [^{3}H]naloxone (Szücs et al., 1983) to rat brain membranes. When rat brain membranes were incubated with [^{3}H]DALECK at an alkaline pH, a 58 000-dalton protein was specifically labeled (Newman & Barnard, 1984). On the basis of competition studies, the authors suggested that this irreversible peptide was labeling μ opioid receptors, though the specificity of this labeling has not been thoroughly examined (Newman & Barnard, 1984). A photoaffinity derivative of morphiceptin was synthesized as a μ-selective irreversible affinity ligand (Herblin et al., 1987). Naloxonazine (Hahn et al., 1982) and a series of 14-hydroxydihydromorphinone hydrazones have been used in binding studies and in vivo studies to label μ_1 opioid receptors (Hahn et al., 1982; Pasternak & Wood, 1986; Williams et al., 1988; Heyman et al., 1988). The most extensively used affinity

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[1] Abbreviations: BAM, 14β-(bromoacetamido)morphine; 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; DTT, dithiothreitol; DALECK, Tyr-D-Ala-Gly-Phe-Leu-CH_2Cl; DADLE, [D-Ala^2,D-Leu^5]enkephalin; β-FNA, β-funaltrexamine; β-CNA, β-chlornaltrexamine; FIT, fentanyl isothiocyanate; SUPERFIT, (+)-*cis*-3-methylfentanyl isothiocyanate; azido-DTLET, Tyr-D-Thr-Gly-pN_3Phe-Leu-Thr; BSA, bovine serum albumin; KIU, kallikrein inhibiting unit.

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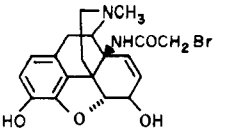
label for μ opioid receptors has been β-FNA (Portoghese et al., 1980). In bioassays, β-FNA irreversibly antagonized morphine's actions in the guinea pig ileum, shifting morphine's dose–response curve to the right (Takemori et al., 1981; Ward et al., 1982). A partial irreversible inhibition of binding to μ binding sites and to a lesser degree δ binding sites in brain has been obtained with β-FNA (Tam & Liu-Chen, 1986; Recht & Pasternak, 1987). 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 a molecular weight of 68 000–97 000 was obtained by autoradiography. If the samples were treated with *N*-glycosidase F, to digest glycogen moieties, a band corresponding to a molecular weight of 57 000 was specifically labeled along with a 49 000-dalton band (Liu-Chen & Phillips, 1987). By the inclusion of protecting opioids, site-directed alkylation of the different types of opioid receptors, particularly κ opioid receptors, has been achieved by the use of the nonspecific alkylating ligand β-CNA (Portoghese et al., 1979; Goldstein & James, 1984; James & Goldstein, 1984; Sheehan et al., 1986).

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 x glioma NG108-15 cell line, which contains only the δ opioid receptor, both FIT and SUPERFIT irreversibly inhibited opioid binding (Rice et al., 1983; Burke et al., 1986). When NG108-15 membranes were incubated with [^{3}H]FIT and then separated under denaturing and reducing conditions on polyacrylamide gels, a 58 000-dalton protein was specifically labeled with [^{3}H]FIT (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 benzomorphan derivative has also been used to label δ receptors in NG108-15 cells (Hallermayer et al., 1983). An enkephalin derivative, [D-Ala^2,Leu^5,Cys^6]enkephalin (Bowen et al., 1987), and a photoaffinity peptide, azido-DTLET (Garbay-Jaureguiberry et al., 1984; Zajac et al., 1987), have been shown to irreversibly inhibit δ binding to rat brain membranes. When ^{125}I-labeled azido-DTLET was incubated with NG108-15 membranes, a 33 000-dalton protein was specifically labeled when the membranes were separated under denaturing and reducing conditions on polyacrylamide gels (Icard-Liepkalns & Bochet, 1988). In rat brain membranes, ^{125}I-labeled azido-DTLET labeled proteins with molecular weights of 44 000 and 34 000 (Bochet et al., 1988). 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, irreversible affinity ligands may be facilitated by taking advantage of a molecular characteristic unique to a certain type of opioid receptor.

Disulfide bonds located at a ligand binding site are targets for potential alkylation, after treatment with a reducing agent. The presence of a readily reducible disulfide bond within 1 nm of the acetylcholine binding site of nicotinic acetylcholine receptors has been firmly established (Karlin, 1980). Reduction of this disulfide bond rendered these receptors susceptible to affinity labeling by alkylating agents with specificity for sulfhydryl groups. (Bromoacetyl)choline has proven to be a very specific affinity label for the nicotinic acetylcholine receptor from electroplax (Wolosin et al., 1980; Damle et al., 1978; Moore & Raftery, 1979) and rat brain (Lukas & Bennett, 1980). By use of [^{3}H](bromoacetyl)choline and [^{3}H]MBTA, another specific affinity label, studies determined that these labels specifically bound to 1 of the 2 α subunits in the nicotinic cholinergic receptor. After the sequence of the receptor from *Torpedo californica* electric tissue was determined (Noda et al., 1982), incubating the purified nicotinic cholinergic receptor with [^{3}H]MBTA resulted in the determination of the specific cystinyl residues labeled with this affinity reagent (Kao et al., 1984).



14 β-Bromoacetamidomorphine

FIGURE 1: Structure of BAM.

Opioid receptors have been shown to be sensitive to disulfide reducing agents (Pasternak et al., 1975; Marzullo & Hine, 1980; Nozaki & Cho, 1985; Kamikubo et al., 1988; Gioannini et al., 1988) and sulfhydryl alkylating reagents (Simon & Groth, 1975; Smith & Simon, 1980; Larsen et al., 1981; Childers, 1984; Childers & Jackson, 1984). Agonist binding has been shown to be more sensitive to disulfide reducing agents than antagonist (Pasternak et al., 1975; Nozaki & Cho, 1985). Binding to μ opioid receptors is inhibited with lower concentrations of DTT than δ receptors, and κ binding is relatively insensitive to DTT (Gioannini et al., 1988). μ opioid receptors appear to have an unique disulfide bond at the opioid binding site. The study reported here describes the use of BAM as an alkylating agent specific for μ opioid binding sites, after reduction of a disulfide bond at the binding site.

Experimental Procedures

Materials. Male Sprague-Dawley rats (125–150 g) were purchased form Charles River Laboratories. BAM was synthesized as previously described (Archer et al., 1983), and the structure of BAM is shown in Figure 1. DTT, Ultrol grade, was purchased from Calbiochem. [^{3}H]DAGO (59 Ci/mmol), [^{3}H]DPDPE (34 Ci/mmol), [^{3}H]naloxone (56.1 Ci/mmol), and ^{125}I-labeled β_h-endorphin (1957 Ci/mmol), labeled at the Tyr^{27} residue, were obtained from Amersham. (−)-[^{3}H]-Bremazocine (21.3 Ci/mmol) was purchased from New England Nuclear. Opioid peptides were obtained from Bachem. Liquiscint scintillation fluid was purchased from National Diagnositics.

Opioid Binding to Rat Brain Membranes. Rat brain membranes, excluding cerebellar tissue, were prepared from male Sprague-Dawley rats and washed at 37 °C for 30 min as previously described (Pasternak et al., 1975). Protein concentration was determined by the method of Bradford (1976). For [^{3}H]DAGO, [^{3}H]DPDPE, or [^{3}H]naloxone binding experiments, 0.1–0.3 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 °C. Incubation times of 60 min were used for [^{3}H]DAGO and [^{3}H]naloxone, and a 2-h incubation time was used for [^{3}H]DPDPE. Nonspecific binding was measured by the inclusion or 1 μM DAGO, 2 μM naloxone, or 1 μM DADLE for [^{3}H]DAGO, [^{3}H]naloxone, and [^{3}H]DPDPE, respectively. Binding to κ receptors was measured by determining the binding of 0.2 nM (−)-[^{3}H]-bremazocine in the presence of 400 nM DAGO and 400 nM DADLE, acting as μ and δ blockers, to membranes for 60 min at 25 °C in a final volume of 1 mL. Nonspecific binding was measured by the inclusion of 1 μM etorphine. Binding assays

were terminated by filtering the samples through GF/B glass fiber filters with 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 5 mL of Liquiscint scintillation fluid at a counting efficiency of 35–50%.

For ^{125}I-labeled β_h-endorphin binding assays, 30–50 μg of membrane protein was 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-Labeled 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-labeled β_h-endorphin was aliquoted in 4-mL Nunc polypropylene tubes at 1 μCi in 10 μL/tube and stored at −20 °C 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 1 μCi of ^{125}I-labeled 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 °C, the binding assays were terminated by filtering the samples through GF/B filters that had been soaked in 0.25% poly(ethylenimine) 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 and BAM, 12 different concentrations of each ligand were incubated with the membranes along with the radiolabeled opioids. For the determination of the IC_{50} value for the inhibition of 0.25 nM [^{3}H]DAGO binding in the presence of 20 mM DTT, 0.60 mg of membrane protein was used instead of the 0.25 mg of protein that was used in the absence of DTT. The membranes were incubated with DTT for 30 min at 25 °C before the addition of [^{3}H]DAGO. IC_{50} values were calculated by least-squares fit to a logarithm–probit analysis.

Incubation of Membranes with DTT and BAM. To determine if, after reduction of disulfide bonds with DTT, BAM would bind irreversibly to opioid binding sites, the following experiments were performed. In 1.8 mL of 50 mM Tris-HCl, pH 7.5, 20–30 mg of rat brain membrane protein was incubated with DTT or buffer for 40 min at 25 °C. Except where stated differently, a final concentration of 20 mM DTT and a final volume of 2.4 mL were obtained by the addition of BAM or buffer. The incubation was continued for an additional 30 min. The tubes were then diluted to 40 mL with 50 mM Tris-HCl, pH 7.5, followed by centrifugation at 48000*g* for 15 min. The membranes were subsequently washed three or five additional times, depending on the BAM concentration used in the experiment, before ultimate resuspension in either 4.5 or 9 mL of 50 mM Tris-HCl, pH 7.5. Opioid binding to the membranes was then measured as described above. DTT was titrated from 0.04 to 25.6 mM. BAM was titrated from 50 to 10 000 nM. Control experiments were performed to determine that the washing conditions used in these experiments removed all noncovalently bound BAM from the membranes.

A time–course experiment was constructed in the following manner to determine the time necessary for BAM to alkylate the opioid binding site. In 600 μL of 50 mM Tris-HCl, pH 7.5, 9 mg of rat brain membrane protein was incubated with 30 mM DTT for 40 min at 25 °C. The volume was brought to 900 μL by the addition of 2 μM BAM. The contents of the tube were diluted to 40 mL with 50 mM Tris-HCl, pH 7.5, at time points varying from 30 s to 40 min after the addition of BAM. The membranes were centrifuged at 48000*g* for 15 min and were washed an additional five times before a final resuspension in 4 mL of 50 mM Tris-HCl, pH 7.5. The binding of 1 nM [^{3}H]DAGO to 300 μL of the membranes was then measured.

Experiments were performed to determine if opioids could protect the μ binding site from alkylation with BAM. Morphine at final concentrations ranging from 0.125 to 4 μM was incubated with 20 mg of membrane protein in 50 mM Tris-HCl, pH 7.5, for 15 min at 25 °C. DTT at a concentration of 30 mM was then added to the membranes in 1.8 mL. After incubation of the membranes with morphine and DTT for 30 min, the volume was brought to 2.4 mL by the addition of 400 nM BAM. After an additional 30-min incubation at 25 °C, the samples were diluted with 40 mL of 50 mM Tris-HCl, pH 7.5, centrifuged at 48000*g* for 15 min, and washed four times. 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 200 μL of membranes was measured as described above.

Table I: Comparison of the Inhibition of Opioid Binding to Rat Brain Membranes by Morphine and BAM[a]

radiolabeled opioid	IC_{50} (nM) morphine	BAM
0.25 nM [^{3}H]DAGO	0.91 ± 0.21	4.1 ± 1.1
3 nM [^{3}H]DPDPE	132 ± 7	345 ± 33
0.2 nM [^{3}H]bremazocine + μ and δ blockers	324 ± 53	950 ± 115
2 nM [^{3}H]naloxone	11 ± 3	37 ± 10
1 nM ^{125}I-labeled β_h-endorphin	6.4 ± 0.9	46 ± 7

[a] Rat brain membranes were incubated with 12 different concentrations of morphine or BAM in the presence of the radiolabeled opioid in 50 mM Tris-HCl, pH 7.5, as described under Experimental Procedures. IC_{50} values were calculated by least-squares fit to a logarithm–probit analysis. The results are expressed as the mean IC_{50} value ± SEM from three experiments performed in triplicate.

Table II: Effect of BAM and DTT on [^{3}H]DAGO Binding to Rat Brain Membranes[a]

condition	% control binding
10 μM BAM	93 ± 6
20 mM DTT	106 ± 4
20 mM DTT + 10 μM BAM	10 ± 3

[a] Rat brain membranes, 20 mg of protein, in 50 mM Tris-HCl, pH 7.5, were incubated with 20 mM DTT or Tris buffer for 40 min at 25 °C in 1.8 mL. After this preincubation, 10 μM BAM or buffer was added, and the incubation was continued for an additional 30 min in a final volume of 2.4 mL. The membranes were diluted to 40 mL with Tris-HCl buffer and were washed six times by centrifugation at 48000*g* for 15 min. The membranes were finally resuspended in 4.5 mL of Tris-HCl buffer. The specific binding of 0.25 nM [^{3}H]DAGO to 0.2 mL of membranes was measured. Data are presented as the mean percent of control binding ± SEM from three or more experiments performed in triplicate.

Results

The IC_{50} values obtained for the inhibition of μ, δ, and κ opioid binding to brain membranes by morphine and BAM are compared in Table I. BAM had approximately the same receptor selectivity as morphine, with a 3–5-fold decrease in affinity for the different types of opioid binding sites. The selectivity of BAM for the μ site was at least 70-fold greater than for the δ site and almost 200-fold greater than for the κ site. The addition of a bromoacetamido group to the 14th position of morphine did not have an effect on receptor selectivity and only slightly decreased the affinity of this ligand

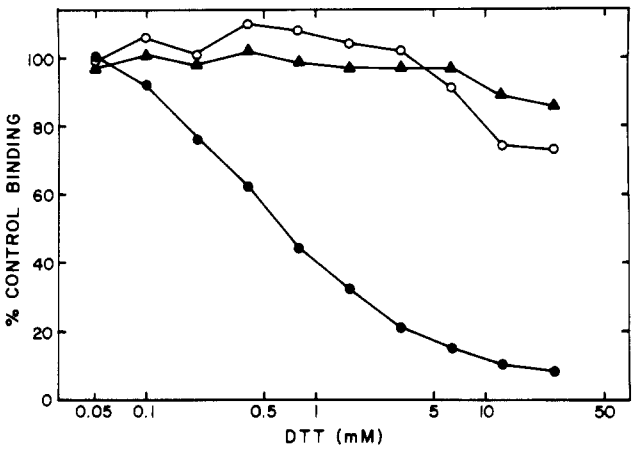


FIGURE 2: Comparison of the inhibition of μ, δ, and κ binding to rat brain membranes as a result of incubating membranes with DTT and BAM. In 1.8 mL of 50 mM Tris-HCl, pH 7.5, 20 mg of membrane protein was incubated with varying concentrations of DTT for 40 min at 25 °C. BAM at a final concentration of 10 μM was added to bring the final volume to 2.4 mL. The final concentrations of DTT ranged from 0.04 to 25.6 mM. Control samples consisted of membranes incubated with 10 μM BAM without the addition of DTT. After a 30-min incubation, membranes were diluted with 40 mL of 50 mM Tris-HCl, pH 7.5, and centrifuged at 48000g for 15 min. After five additional washes, the membranes were resuspended in 4.5 mL of Tris-HCl buffer. The binding of 0.25 nM [^{3}H]DAGO (●), 1 nM [^{3}H]DPDPE (○), and 0.2 nM (−)-[^{3}H]bremazocine in the presence of 400 nM DAGO and 400 nM DADLE (▲) was measured by using 200 μL of resuspended membranes, as described under Experimental Procedures. Points represent the mean percent of control binding from four experiments performed in triplicate.

for the multiple opioid binding sites. As shown in Table II, when brain membranes were incubated with 10 μM BAM, greater than 90% of the 0.25 nM [^{3}H]DAGO binding was recovered by washing the membranes six times by centrifugation with 50 mM Tris-HCl, pH 7.5. Therefore, BAM did not bind covalently to opioid binding sites when membranes were just incubated with a high concentration of the ligand. Inclusion of 20 mM DTT resulted in partial inhibition of [^{3}H]DAGO binding to membranes. The binding of 0.0125 nM [^{3}H]DAGO was inhibited by 75%, while the binding of 6.4 nM [^{3}H]DAGO was inhibited by 36%. In the presence of 20 mM DTT, morphine had a IC_{50} value of 0.60 ± 0.09 nM for the inhibition of 0.25 nM [^{3}H]DAGO binding to membranes. This value was not significantly different than the value, 0.91 ± 0.21 nM (Table I), obtained for the inhibition of 0.25 nM [^{3}H]DAGO binding in the absence of DTT. So, while DTT partially inhibited [^{3}H]DAGO binding, morphine binding to membranes was not significantly altered by inclusion of DTT. Upon washing DTT-treated membranes with Tris-HCl buffer followed by centrifugation, [^{3}H]DAGO binding was restored to control values, as is shown in Table II. Incubating membranes with 20 mM DTT, followed by the addition of 10 μM BAM, resulted in 90% of the [^{3}H]DAGO binding being irreversibly inhibited (Table II). These results suggested that BAM bound covalently at an opioid binding site, but only after the reduction of a disulfide bond at or near the binding site.

DTT was titrated to determine the concentration that is necessary for BAM to bind irreversibly to opioid receptors. The specificity of the irreversible inhibition of opioid binding to membranes was also examined to determine if BAM alkylated one type of opioid binding site or multiple binding sites. As shown in Figure 2, greater than 90% of the 0.25 nM [^{3}H]DAGO binding was inhibited when membranes were incubated with 12 mM or greater concentrations of DTT and 10 μM BAM. Control samples consisted of membranes incubated with 10 μM BAM without the addition of DTT. In the presence of 10 μM BAM, 0.5 mM DTT was necessary for 50% of the μ binding sites to be irreversibly blocked. In contrast to μ binding, δ or κ binding to membranes was not significantly inhibited at DTT concentrations of less than 6 mM. Thus, at 6 mM DTT and 10 μM BAM, 90% of the [^{3}H]DAGO binding was inhibited, while δ binding as measured by 1 nM [^{3}H]DPDPE and κ binding as measured by 0.2 nM (−)-[^{3}H]bremazocine in the presence of μ and δ blockers were not significantly inhibited by BAM alkylation of membranes. Even at 25 mM DTT, only 20% of δ binding and 10% of κ binding were inhibited irreversibly. After reduction of a critical disulfide bond, BAM appears to specifically and completely alkylate the μ binding site.

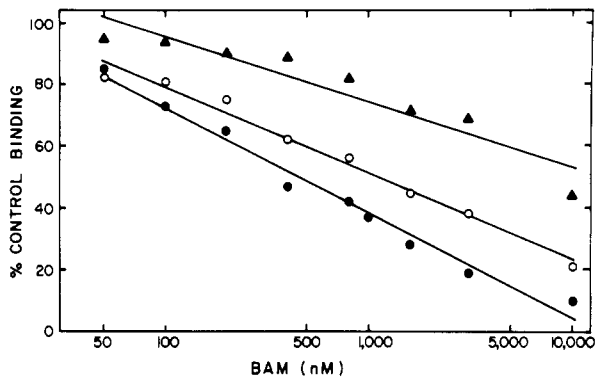


FIGURE 3: Determination of the concentration of BAM necessary for irreversible inhibition of opioid binding. In 1.8 mL of 50 mM Tris-HCl, pH 7.5, 30 mg of membrane protein was incubated with 30 mM DTT for 40 min at 25 °C. Varying concentrations of BAM, ranging from 50 to 10000 nM, were added to the membranes to bring the final volume to 2.4 mL. After an additional 30-min incubation, the membranes were diluted with buffer and washed six times as described in Figure 2 and under Experimental Procedures. The membranes were resuspended in 9 mL of 50 mM Tris-HCl, pH 7.5, and the binding of 0.25 nM [^{3}H]DAGO (●) and 0.8 nM [^{3}H]naloxone (○) was measured by using 200 μL of resuspended membrane. The binding of 0.25 nM ^{125}I-labeled β_h-endorphin (▲) was measured by using 40 μL of resuspended membranes, as described under Experimental Procedures. Points are the mean percent of control binding from four experiments performed in triplicate.

The ability of BAM to inhibit the binding of other ligands known to bind to the μ site was tested by titrating BAM to determine the concentration necessary for irreversible inhibition of μ opioid binding. In the presence of 20 mM DTT and varying concentrations of BAM, an IC_{50} value of 400 ± 45 nM BAM was obtained for the irreversible inhibition of [^{3}H]DAGO binding, as is shown in Figure 3. At 50 nM BAM, only 20% of the μ binding sites were blocked, while 10 μM BAM inhibited greater than 90% of the 0.25 nM [^{3}H]-DAGO binding to membranes. In addition to blocking [^{3}H]DAGO binding, 10 μM BAM resulted in 80% of the 0.8 nM [^{3}H]naloxone binding and 50% of the 0.25 nM ^{125}I-labeled β_h-endorphin binding to membranes being inhibited (Figure 3). These results suggest that most of the binding of 0.8 nM [^{3}H]naloxone was to the μ binding site, while half of the 0.25 nM ^{125}I-labeled β_h-endorphin binding sites were μ sites.

The time–course for BAM to irreversibly block μ binding was determined by incubating 20 mM DTT-treated membranes with 2 μM BAM at times ranging from 30 s to 40 min. Figure 4 shows that after a 10- min incubation at 25 °C, half of the [^{3}H]DAGO binding was inhibited. After a 20-min incubation, maximal inhibition was reached and did not increase further.

Since 0.25 nM [^{3}H]DAGO binding to 20 mM DTT-treated and 10 μM BAM-treated membranes was inhibited by 90% (Table II and Figure 3), experiments were performed to de-

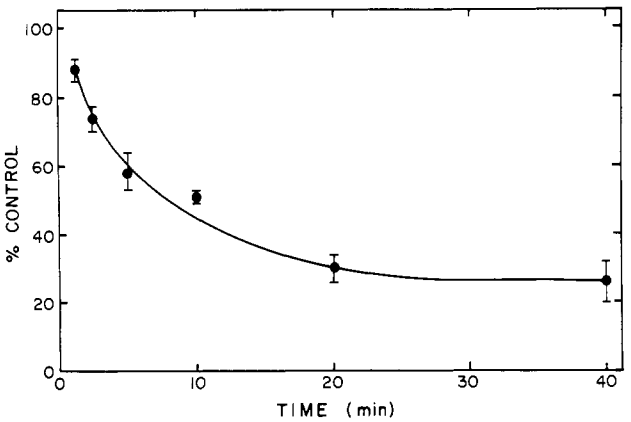


FIGURE 4: Time course for BAM to irreversibly inhibit [^{3}H]DAGO binding to membranes. In 600 μL of 50 mM Tris-HCl, pH 7.5, 9 mg of membrane protein was incubated with 30 mM DTT for 40 min at 25 °C. The volume was increased to 900 μL by the addition of 2 μM BAM. The reaction was terminated by diluting the membrane suspension with 40 mL of 50 mM Tris-HCl, pH 7.5, followed by centrifugation, as described under Experimental Procedures. The reaction was terminated at time points ranging from 30 s to 40 min after the addition of BAM. After six washes, the membranes were resuspended in 4 mL of 50 mM Tris-HCl, pH 7.5. The binding of 1 nM [^{3}H]DAGO was measured to 300 μL of resuspended membranes.

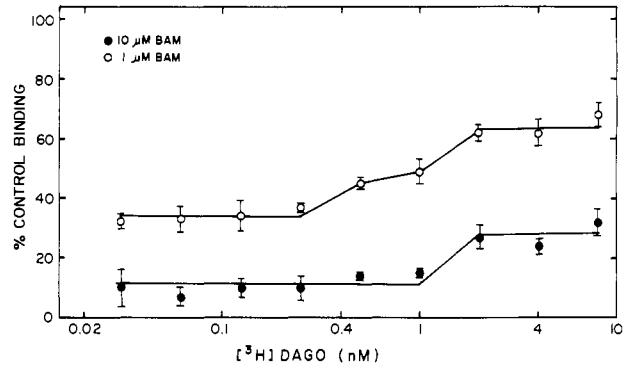


FIGURE 5: Ability of DTT- and BAM-treated membranes to inhibit varying concentrations of [^{3}H]DAGO binding to membranes. In 1.8 mL of 50 mM Tris-HCl, pH 7.5, 30 mg of membrane protein was incubated with 30 mM DTT for 40 min at 25 °C. The volume was increased to 2.4 mL by the addition of 1 (○) or 10 μM (●) BAM, for an additional 30-min incubation. After six washes, the membranes were resuspended in 9 mL of 50 mM Tris-HCl, pH 7.5, and the binding of [^{3}H]DAGO at concentrations ranging from 0.0625 to 8 nM was measured by using 125 μL of resuspended membranes as described under Experimental Procedures. Points represent the mean percent of control binding ± SEM from four experiments performed in triplicate.

termine if this 90% inhibition remained constant regardless of the concentration of [^{3}H]DAGO. As shown in Figure 5, at [^{3}H]DAGO concentrations ranging from 0.04 to 1 nM, the inhibition produced by 10 μM BAM alkylation of membranes remained constant at about 90%. As the [^{3}H]DAGO concentration was increased from 1 to 3 nM, the irreversible inhibition of binding was decreased to about 75% of control binding. A similar pattern was obtained when membranes were incubated with 1 μM BAM instead of 10 μM, though the overall inhibition was less than with 10 μM BAM. The K_d value for [^{3}H]DAGO binding to rat brain membranes was determined to be 0.4 nM by Scatchard analysis (data not shown). Thus, at the K_d value for [^{3}H]DAGO binding or less, the percent of irreversible inhibition of binding by BAM alkylation was not dependent on the concentration of [^{3}H]-DAGO. At higher concentrations [^{3}H]DAGO may be binding slightly to a site not alkylated with BAM.

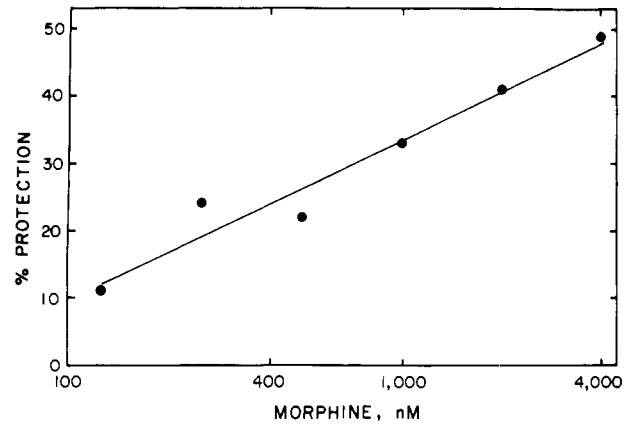


FIGURE 6: Titration of morphine in protecting opioid binding sites from irreversible inhibition with BAM. Morphine, at final concentrations ranging from 125 nM to 4 μM, was incubated with 20 mg of membrane protein for 15 min at 25 °C in 50 mM Tris-HCl, pH 7.5. The volume was increased to 1.8 mL by the addition of 30 mM DTT, and the membranes were incubated for an additional 30 min. The addition of 400 nM BAM increased the volume to 2.4 mL. After a 30-min incubation, the membranes were washed four times and resuspended in 4.5 mL of 50 mM Tris-HCl, pH 7.5. The binding of 0.25 nM [^{3}H]DAGO to 200 μL of membranes was measured as described under Experimental Procedures. Points represent the mean percent protection from five experiments performed in triplicate.

Table III: Ability of Opioids To Protect the μ Opioid Binding Site from Alkylation with BAM[a]

opioid	% protection
morphine	48 ± 2
naloxone	43 ± 5
dextrorphan	1 ± 4
U50,488H	1 ± 4

[a] Rat brain membranes, 20 mg of protein, in 50 mM Tris-HCl, pH 7.5, were incubated with the opioids listed above at a final concentration of 4 μM for 15 min at 25 °C. DTT at a final concentration of 20 mM was then added, and the incubation was continued for an additional 30 min. BAM, 400 nM, was added, and the incubation was continued for 30 min in a final volume of 2.4 mL. The membranes were diluted to 40 mL with Tris-HCl buffer and were washed four times by centrifugation at 48000g for 15 min. The membranes were finally resuspended in 4.5 mL of Tris-HCl buffer. The specific binding of 0.25 nM [^{3}H]DAGO to 0.2 mL of membranes was measured. Incubation with DTT and BAM resulted in a 40 ± 4% inhibition of control binding. Data are presented as the mean percent protection of binding ± SEM from three or more experiments performed in triplicate.

The ability of opioids to protect the binding site from inactivation with BAM was determined by preincubating membranes with opioids before the addition of 20 mM DTT and 400 nM BAM. As shown in Figure 6, morphine in a concentration-dependent manner blocked up to 50% of the irreversible inhibition of [^{3}H]DAGO binding caused by BAM. Due to the high concentrations of BAM and opioids that had to be added to the membranes as a result of DTT decreasing the affinity of the μ binding site, complete protection from BAM alkylation was not possible. When morphine was present at 4 μM, a 10-fold higher concentration than BAM, a 50% protection of the sites from BAM alkylation was obtained. Control experiments determined that, with six washes, 4 μM morphine could be completely washed from the membranes. Table III shows that, in addition to morphine, naloxone at a 10-fold greater concentration than BAM also afforded almost 50% protection of the sites. However, the *d* isomer, dextrorphan, or the κ ligand U50,488H did not protect the site from BAM alkylation. When the μ-selective peptide DAGO or the δ-selective peptide DPDPE was incubated at a final concentration of 100 nM with brain membranes, washing the

membranes with Tris-HCl buffer six times by centrifugation was not sufficient to remove the peptides. Consequently, due to the fact that the opioid peptides remained associated with the membranes, it was not possible to test their ability to protect the μ binding site from alkylation with BAM.

Discussion

These experiments have shown that BAM is a specific irreversible affinity label for μ opioid binding sites, after the reduction of a critical disulfide bond near the binding site. The advantage in using BAM over other affinity ligands to label the μ opioid receptor is the specificity that is gained with this compound. Only BAM takes advantage of a characteristic that is unique to one type of opioid receptor. While in competition studies (Table I) BAM was quite selective for μ binding sites, the advantage in using BAM over other affinity ligands lies in the fact that it will only bind covalently to μ binding sites, after reduction of a disulfide bond at or near the binding site. Opioid binding was reduced by 90% without any effect on δ or κ binding sites (Figure 3). Only at very high concentrations of DTT did a small fraction of δ and κ binding become inhibited. This slight inhibition may be the result of BAM nonspecifically alkylating a number of newly formed sulfhydryl groups, or there may be disulfide bonds near δ and κ binding sites that became reduced only with high concentrations of DTT. These results are in agreement with other studies that have shown that μ opioid binding was inhibited with lower concentrations of DTT than were necessary to inhibit δ binding and that κ binding was almost insensitive to DTT (Gioannini et al., 1988). Only with radiolabeled BAM will it be possible to determine whether BAM is alkylating a number of sites or only μ opioid binding sites.

BAM alkylation of μ binding sites had an IC_{50} value of 400 nM BAM, indicating that fairly high concentrations of BAM were necessary to achieve irreversible labeling of μ sites. High concentrations of BAM may have been necessary because DTT treatment of membranes reduced the μ binding site from a high-affinity site to a low-affinity site (Kamikubo et al., 1988). However, the IC_{50} value for the inhibition of 0.25 nM [^{3}H]-DAGO binding by morphine was not significantly altered by the inclusion of 20 mM DTT. This finding suggests that DTT did not greatly alter the affinity of the μ opioid binding site for the alkaloid. The possibility also exists that BAM may be binding to a sulfhydryl group on the μ opioid receptor but not at the opioid binding site. The binding of BAM to a sulfhydryl group away from the binding site could still alter the receptor in such a manner as to render it unable to bind opioids. The binding of BAM to a distant sulfhydryl group is supported by the finding that morphine and naloxone only partially protected the receptor from BAM alkyaltion. Because of the necessity to use 400 nM BAM to achieve a 50% irreversible inhibition of μ opioid binding, opioid peptides could not be tested for their ability to block BAM alkylation. At concentrations of 100 nM DAGO or DPDPE, six washes by centrifugation were not sufficient to remove all the peptides from the membranes. Alkaloids did not remain associated with membranes as well as opioid peptides.

Evidence that would suggest that BAM was probably not acting as a nonspecific alkylating reagent comes from studies that have used NEM to alkylate sulfhydryl groups (Pasternak et al., 1975; Childers, 1984; Childers & Jackson, 1984). NEM has been shown to decrease [^{3}H]dihydromorphine binding to membranes and suppress the effect of GTP on opioid binding (Childers, 1984). Studies with NEM suggest that there may be free sulfhydryl groups within the vicinity of opioid binding sites in brain membranes. However, BAM did not appear to alkylate these sites since in the absence of DTT, no irreversible inhibition of opioid binding was observed. The BAM alkylation studies described in this paper were performed in 50 mM Tris-HCl, pH 7.5. A pH of greater than 8 is necessary for DTT to optimally reduce disulfide bonds (Cleland, 1964). The pH optimum for BAM reactivity has not been determined. NEM alkylation of membranes was sensitive to pH changes (Childers & Jackson, 1984). In these initial studies, we chose to maintain the buffer conditions identical with the conditions that are used to measure high-affinity opioid binding to membranes. If the pH is altered, BAM alkylation of μ binding sites may be possible with lower DTT and BAM concentrations than were used in this study.

Greater than 90% of [^{3}H]DAGO binding to membranes was inhibited by BAM alkylation. Other affinity ligands that have irreversibly inhibited μ opioid binding to brain membranes have only been successful in achieving a partial inhibition of binding, usually less than 50% (James & Goldstein, 1984; Tam & Liu-Chen, 1986; Recht & Pasternak, 1987). By use of BAM to block virtually all of the μ sites, pharmacological and biochemical studies of the properties of δ and κ binding sites will now be possible without interference from μ binding sites. Knowing that there is a disulfide bond at μ binding sites that can be labeled should facilitate the design of other affinity ligands for μ opioid receptors. With the nicotinic cholinergic receptor, depending on the structure of the affinity label, the affinity reaction resulted in either inhibition (Karlin, 1969; Steinacker, 1979; Walker et al., 1984) or activation (Lester et al., 1980; Walker et al., 1984) of nicotinic receptors. In a similar manner, studies using alkylating affinity labels can be designed to further our understanding of the interaction of μ opioid receptors with second messenger systems and down-regulation of the receptor. Determination of the molecular weight of the μ binding site should now be possible by reacting both membranes and partially purified opioid receptor preparations (Bidlack et al., 1981) with radiolabeled BAM. Purification of the μ binding site will also be facilitated by the use of this affinity label. Finally, BAM should be useful in identifying the location of the μ binding site from the amino acid sequence of the μ opioid receptor.

Registry No. BAM, 82975-77-7; DTT, 3483-12-3.

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