Studies on Inhibition of $\mu$ and $\delta$ Opioid Receptor Binding by Dithiothreitol and N-Ethylmaleimide

His$^{223}$ IS CRITICAL FOR $\mu$ OPIOID RECEPTOR BINDING AND INACTIVATION BY N-ETHYLMALEIMIDE*

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The sensitivity of $\mu$ and $\delta$ receptor binding to dithiothreitol and N-ethylmaleimide was examined to probe receptor structure and function. Binding to both receptor types was inhibited by dithiothreitol ($IC_{50}$ values $= 250 \text{ mM}$), suggesting the presence of inaccessible but critical disulfide linkages. $\mu$ receptor binding was inhibited with more rapid kinetics and at lower N-ethylmaleimide concentrations than $\delta$ receptor binding. Ligand protection against N-ethylmaleimide inactivation suggested that alkylation was occurring within, or in the vicinity of, the receptor binding pocket. Sodium ions dramatically affected the $IC_{50}$ of N-ethylmaleimide in toward both receptor types in a ligand-dependent manner.

Analysis of receptor chimeras suggested that the site of N-ethylmaleimide alkylation on the $\mu$ receptor was between transmembrane domains 3 and 5. Substitution of cysteines between transmembrane domains 3 and 5 and elsewhere had no effect on receptor binding or sensitivity toward N-ethylmaleimide. Serine substitution of His$^{223}$ in the putative second extracellular loop linking transmembrane domains 4 and 5 protected against N-ethylmaleimide inactivation. The H223S substitution decreased the affinity of bremazocine 25-fold, highlighting the importance of this residue for the formation of the high affinity bremazocine binding site in the $\mu$ opioid receptor.

Three major types of opioid receptor, $\delta$, $\kappa$, and $\mu$, have been cloned and characterized extensively (reviewed in Refs. 1 and 2). There is approximately 60% amino acid sequence identity between the opioid receptor types. The $\delta$, $\kappa$, and $\mu$ opioid receptors have unique ligand specificities, anatomical distributions, and physiological functions (3). Morphine, related opioid drugs, and the endogenous opioid peptides activate signal transduction pathways by binding to opioid receptors (4), which are members of the G protein-coupled receptor family (5). G protein-coupled receptors are seven-transmembrane domain (TM) proteins that mediate signal transduction across the plasma membrane. The ligands approach and engage the receptor from the extracellular side, and receptor activation results in the coupling to heterotrimeric G proteins on the intracellular face of the membrane. Opioid receptor types interact with multiple G proteins (6–8) to regulate adenyl cyclase, Ca$^{2+}$ channels, and K$^+$ channels.

It has been known from early studies on the characterization of opioid receptors that specific binding is inhibited by sulfhydryl reagents, such as iodoacetamide, N-ethylmaleimide (NEM), and p-hydroxymercuribenzoate (9–11). Preincubation with opioid ligands protected against receptor inactivation, suggesting that the sensitive sulfhydryl group was located within or near the binding site. Evidence has been obtained that analogs of Leu-enkephalin and morphine, containing activated sulfhydryl groups, form mixed disulfide linkages with opioid receptors (12, 13). The covalently bound agonists caused receptor activation that persisted following extensive washing, yet was naloxone-reversible. The results suggested that the agonists became tethered to the receptor via a mixed disulfide linkage that was in the vicinity of the receptor binding site. Other studies provided evidence that NEM affected opioid agonist binding by at least two mechanisms, direct inhibition (as mentioned above) and indirect inhibition due to uncoupling of receptors from G proteins (14, 15).

Several other, but not all, G protein-coupled receptors are also sensitive to sulfhydryl reagents. Susceptible receptors include the thyrotropin-releasing hormone (16), D1 and D2 dopamine (17, 18), substance P (19), $\alpha_1$ and $\alpha_2$ adrenoreceptor (20), platelet-activating factor (21), leukotriene B$_4$ (22), vasopressin (23), follicle-stimulating hormone (24), and cannabinoid receptors (25). Recently, a cysteine in TM3 of the D2 dopamine receptor has been identified that reacts with sulfhydryl reagents and results in inhibition of binding (26).

The goals of this study were 1) to examine the sensitivity of $\mu$ and $\delta$ receptor binding to reduction with dithiothreitol (DTT), in order to determine whether disulfide linkages were necessary for maintenance of the binding site, and 2) to characterize the sensitivity of $\mu$ and $\delta$ receptor binding to alkylation with NEM and identify the reactive groups involved. Due to the proximity of the NEM-reactive group to the ligand binding site of the receptor, knowledge of its location is essential for construction of accurate molecular models of the binding pocket.

EXPERIMENTAL PROCEDURES

Transfection and Radioligand Binding Assays—Human embryonic kidney 293 cells (ATCC CRL 1737) were transfected with $\mu$ and $\delta$ opioid receptor expression plasmids (obtained from Drs. L. Yu and C. Evans, respectively) using the calcium phosphate method, as described (27). Cells stably expressing opioid receptors were selected in media containing 0.5 mg/ml G418 (Life Technologies, Inc.). Opioid receptor binding assays (28) were conducted in duplicate or quadruplicate on membrane preparations resuspended in 50 mM Tris-HCl, 1 mM Na$_2$EDTA buffer, pH 7.4, utilizing [9$^3$H]bremazocine (DuPont NEN; specific activity,
20–30 Ci/mmol) and 10 μM naloxone to define specific binding. Following a 1-h incubation at 22 °C, binding assays were terminated by filtration through Whatman GF/B filters that had been presoaked in 0.1% bovine serum albumin. Filters were soaked in BCS liquid scintillation mixture (Amersham Corp.) prior to determination of filter-bound radioactivity using a Beckman LS 1801 scintillation counter. Receptor binding data was analyzed by nonlinear regression using the Prism program (GraphPad Software, San Diego, CA). Protein concentrations were determined by the method of Bradford (29), using bovine serum albumin as the standard.

Treatment of Membranes with DTT and NEM—To determine the effect of disulfide bond reduction on μ and δ receptor binding, DTT (1–100 mM) was added immediately prior to performing radioligand binding assays. The effect of NEM alkylation on μ and δ receptor binding was generally determined by preincubating membranes in the absence and presence of varying concentrations of NEM (0.5 μM to 5 mM) at 37 °C for 15 min, followed by the addition of 5 mM reduced glutathione to all samples to quench the reaction. Initially, membranes were centrifuged by centrifugation in order to remove the NEM and glutathione prior to the start of the radioligand binding assays; however, the washes were subsequently found to have no effect on the outcome of the assay. Protection of μ and δ receptor binding against NEM inactivation was assessed by preincubating membranes in the absence and presence of ligands (100 nM or 1 μM) for 10 min at 37 °C, prior to reaction with 0.5 mM NEM for 15 min at 37 °C. Samples were chilled on ice, and glutathione was added to 5 mM. Ligands were removed by centrifugation at 35,000 × g for 20 min. Membranes were resuspended in 50 mM Tris-HCl, 1 mM Na4EDTA buffer, pH 7.4, incubated for 10 min at 37 °C to promote ligand dissociation, and then centrifuged and resuspended two more times prior to initiating the radioligand binding assay.

Construction of Receptor Chimeras—Receptor chimeras were constructed using a two-step polymerase chain reaction process followed by direct subcloning into the pCR3 expression vector, as described previously (30). Receptor chimeras D2M, D3M, D5M, and the reciprocal M2D and M5D were used in these studies. Designations for the chimeras are based on the amino-terminal domain (M represents μ and D represents δ), separated by a number, which refers to the transmembrane helix that is the site of the junction. The chimeras referred to as D2M contains the amino terminus derived from the δ receptor, the site of the 6μ3 junction is in TM2, and the carboxyl terminus is derived from the μ receptor. All chimeric receptor constructs were fully sequenced to verify the location of the junction site and to ensure that no mutations were introduced during synthesis.

Site-directed Mutagenesis—The two-step polymerase chain reaction technique used for site-directed mutagenesis has been described previously (30). Individual μ receptor variants were produced containing serine substitution of cysteines at positions 159, 190, 235, 292, and 321 and histidine at position 223. Mutant receptors are named with the wild-type residue, the position number of the residue, and the substitution residue, using single letter abbreviations for amino acids. The technique used for site-directed mutagenesis has been described previously (30). Mutations were introduced during synthesis.

RESULTS

Effect of DTT on μ and δ Opioid Receptor Binding—The importance of disulfide bonds for the maintenance of active opioid receptor conformations was studied by comparing the effect of the disulfide reducing agent, DTT, on μ and δ opioid receptor binding. [3H]Bremazocine, a ligand of the benzomorphan series with high affinity for μ and δ opioid receptors (30), was used to measure specific binding to both receptor types in the absence and presence of varying concentrations (1–100 μM) of dithiothreitol. Binding to both opioid receptor types was inhibited to approximately 60% of control levels, but only at relatively high concentrations of the disulfide reducing agent (Fig. 1). Nonlinear regression analysis of the DTT inhibition curves yielded extrapolated IC50 values of 230–250 μM for inhibition of both μ and δ opioid receptor binding.

In the course of determining the optimal concentration of glutathione to use to quench NEM reactions, we were surprised to observe that [3H]bremazocine binding to μ and δ receptors was considerably more sensitive to incubation with reduced glutathione than with DTT. The IC50 of glutathione was approximately 15 μM for inhibition of [3H]bremazocine binding to μ and δ receptors, and the slopes of the inhibition curves were very steep (data not shown). Similar results were reported for binding to μ opioid, neurokinin-1, and kainic acid receptors (31). We found, however, that the inhibition of binding by glutathione was due to lowering the pH of the buffer solution, due to the acidic nature of the tripeptide. We suggest, therefore, that the results on glutathione inhibition of binding to neurokinin-1 and kainic acid receptors be interpreted with caution.

Comparison of the Effect of NEM on Wild-type μ and δ Opioid Receptor Binding and Protection by Ligand—The kinetics of NEM inactivation were significantly more rapid for μ opioid receptor binding than for δ receptor binding (Fig. 2). Half-lives of inactivation were calculated by nonlinear regression analysis to be 8 and 56 min for μ and δ opioid receptor binding, respectively. Pseudo-first-order rate constants were 0.09 min−1 and 0.01 min−1 for inactivation of μ and δ opioid receptor binding, respectively. The inhibitory effect of NEM on receptor binding was due primarily to an 8-fold reduction in the maximum number of binding sites, with little change in the affinity of the remaining receptors for bremazocine (Table I).

The ability of agonist and antagonist ligands to protect against NEM inactivation of μ and δ opioid receptor binding was determined. Both receptor types were protected against NEM inactivation by preincubation with ligands, although protection of μ receptor binding was more complete (Fig. 3). All ligands tested were capable of protection, including type-selective peptide agonists (DAMGO and DSLET), alkaloid agonists (morphine and etorphine), and the antagonist, naloxone. The data indicated that the NEM-reactive group on both receptor
FIG. 2. Kinetics of NEM inactivation of μ and δ opioid receptor binding. Membrane preparations from cells stably expressing either μ or δ receptors were incubated at 37°C in the absence and presence of 0.5 mM NEM. All samples were quenched with 5 mM glutathione at the indicated times and then assayed for specific binding of 2 nM [3H]bremazocine. Data points are the means ± S.E. from three or four independent experiments.

TABLE I

| Receptor | Kd (nM) | Bmax (pmol/mg protein) |
|----------|---------|-----------------------|
| δ        | 2.3     | 6.3                   |
| δ + NEM  | 3.2     | 0.8                   |
| δ + DTT  | 3.2     | 5.1                   |

Sodium ions differentially affect the NEM sensitivity of peptide and bremazocine binding to μ and δ opioid receptors. The specific binding of [3H]DAMGO to the μ receptor was slightly more susceptible to inactivation by NEM than the specific binding of [3H]bremazocine when assayed in Tris-EDTA buffer (Fig. 4). The presence of 100 mM NaCl in the buffer, however, differentially altered the sensitivity of DAMGO and bremazocine binding to NEM inactivation. The IC50 of NEM toward inactivation of DAMGO binding to the μ receptor decreased significantly in the presence of 100 mM NaCl from 32 to 3 μM, while the IC50 of NEM toward inactivation of bremazocine increased from 85 to 330 μM (Fig. 4).

Binding to the δ receptor was considerably less sensitive to NEM inactivation than was binding to the μ receptor. [3H]DSLET and [3H]bremazocine binding to the δ receptor were also differentially affected by inclusion of 100 mM NaCl in the buffer (Fig. 5), in a similar manner to that observed with μ receptor binding. The IC50 of NEM toward inactivation of DSLET binding to the δ receptor decreased markedly in the presence of 100 mM NaCl from 450 to 8 μM, while the IC50 of NEM toward inactivation of bremazocine increased from 660 μM to 3.6 mM.

Utilization of μ/δ Receptor Chimeras to Search for the Site of NEM Alkylation on the μ Opioid Receptor—The μ receptor contains 17 cysteines (Fig. 6). A strategy utilizing μ/δ receptor chimeras was devised to determine the location of the NEM alkylation site on the μ receptor, based on the large difference in sensitivity of [3H]bremazocine binding to μ and δ receptors toward NEM inactivation. A panel of chimeric receptors was generated, utilizing junction sites shown in Fig. 6. A schematic illustration of the structures of the μ/δ receptor chimeras is displayed in Fig. 7. D2M, D3M, and D5M contain δ receptor sequences from the amino termini to junction sites in TM2, TM3, and TM5, respectively, followed by sequences derived from the μ receptor from the junction site to the carboxyl termini. M2D and M5D are reciprocal chimeras, with amino-terminal domains derived from the μ receptor, junction sites in TM2 or TM5, respectively, followed by δ receptor-derived sequences to the carboxyl termini.

The ability of NEM to inactivate [3H]bremazocine binding to wild-type μ and δ receptors and μ/δ receptor chimeras was compared (Table I). [3H]Bremazocine binding to wild-type μ receptors was 10 times more sensitive to NEM inactivation...
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Utilization of Truncated and Site-specific μ Receptor Mutants to Determine the Site of NEM Alkylation on the μ Opioid Receptor—Based on the results regarding the NEM sensitivity of [3H]bremazocine binding to μ/δ receptor chimeras, cysteines in TM3, TM4, and TM5 of the μ receptor were individually substituted with Ser and then evaluated for their ability to bind [3H]bremazocine and for their sensitivity toward NEM inhibition of binding. The Ser for Cys substitutions did not affect the affinity of the mutated receptors for bremazocine (data not shown). Furthermore, the NEM IC50 values of the three Ser-substituted μ receptors (0.11–0.28 mM) were all in the same range as that of the wild-type μ receptor (0.16 mM, Table III). These data indicated that Cys159, Cys190, and Cys235 were not the sites of NEM alkylation that resulted in inhibition of [3H]bremazocine binding.

Based on these results, Ser was substituted for other cysteines residing outside of the region between TM3 and TM5, and the mutant receptors were tested for sensitivity to NEM. Ser substitution of either Cys292 or Cys321, located in TM6 and TM7, respectively, did not affect the ability to bind [3H]bremazocine or the sensitivity toward NEM inhibition of binding (Table III). Deletion of 64 amino acids from the amino-terminal domain (ΔN 64), which contains four cysteines at positions 13, 22, 43, and 57 (see Fig. 6), did not affect the affinity of the truncated receptor for [3H]bremazocine (Kd = 1.3 nm versus 0.8 nM for the wild-type μ receptor). It has also been reported previously that this deletion did not affect the binding of [3H]naloxone and [3H]DAMGO to the μ receptor (32). The concentration of NEM required for inactivation of bremazocine binding to the truncated receptor was increased 3-fold relative to the wild-type μ receptor (Table III); however, the IC50 was still in the submillimolar range (0.57 mM). We also tested the effect of removal of 89 amino acids from the amino terminus of the C321S mutant receptor. The deleted region of this construct, referred to as ΔN 89, included the amino-terminal domain and most of putative TM1, including Cys79 (Fig. 6). The affinity of [3H]bremazocine for the ΔN 89 construct decreased 20-fold. The IC50 of NEM for the ΔN 89 receptor, however, was similar to the IC50 of the ΔN 64 receptor (Table III), suggesting that the cysteines in the amino-terminal domain and Cys79 were not the relevant targets for NEM alkylation. Substitution of Cys140 with Ser completely blocked the ability of the mutant receptor to bind [3H]bremazocine (data not shown). Although this mutated receptor could not be tested for NEM sensitivity, Cys140 is thought to be linked by a disulfide bond with Cys217 (Fig. 6); hence, it would not be reactive with NEM.

The data suggested that none of the cysteines that were substituted with Ser or deleted were likely targets for NEM alkylation. Based on the chimeric receptor data, which indicated that the NEM-reactive group resided in the region between TM2 and TM5, the sequence of the μ receptor was reexamined for amino acids other than Cys that might be reactive toward NEM. It has been reported that reaction of lysozyme and ribonuclease with NEM resulted in the alkylation of ε-amino groups and imidazole groups (33). The μ receptor contains a His residue at position 223 in the putative second extracellular loop connecting TM4 and TM5 (Fig. 6). According to our alignment of the opioid receptor sequences, the corresponding amino acids in the δ and κ receptor are Ser204 and Asp216, respectively. Substitution of His223 with Ser in the μ receptor completely abolished the ability of NEM to inhibit [3H]bremazocine binding, even at concentrations 10-fold higher than the IC50 for inhibition of binding to the wild-type μ recep-

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tor (Table III). The H223S substitution also decreased the affinity for [3H]bremazocine dramatically (Table IV). Preliminary data from competition analyses indicated that the affinity of the H223S-substituted receptor for etorphine and naloxone was also decreased 25-fold (data not shown). In addition to the 25-fold decrease in the affinity constant for bremazocine, the cell line that expressed the H223S mutant receptor also had a significantly lower Bmax than the cell line that expressed the wild-type receptor (Table IV).

**DISCUSSION**

The following observations and conclusions were made based on these studies. 1) [3H]Bremazocine binding to μ and δ opioid receptors was inhibited to an equal extent by high concentrations of DTT, implying the presence of relatively inaccessible but critical disulfide linkages for both receptor types. 2) [3H]Bremazocine binding to the μ receptor was considerably more sensitive to treatment with NEM than binding to the δ receptor. This finding suggested that the functional group that was the site of alkylation on the μ receptor was more accessible to and/or reactive with NEM than the relevant group on the δ receptor. 3) Ligand protection against NEM inactivation of binding to μ and δ opioid receptors was consistent with the site of alkylation being within, or in the vicinity of, the receptor binding crevice. 4) Dose-response curves of NEM inactivation of μ and δ receptor binding in the presence of sodium ions suggested that at least two reactive groups were subject to alkylation. Alkylation of μ and δ receptor sites at low concentrations of NEM resulted in inhibition of [3H]DAMGO and [3H]DSLET binding, respectively, with minimal effect on [3H]bremazocine binding. Alkylation of μ and δ receptor sites at much higher concentrations of NEM resulted in inhibition of [3H]bremazocine binding to both receptor types. 5) Analyses of the NEM sensitivity of [3H]bremazocine binding to μ and δ opioid receptor chimeras were consistent with a location of the reactive group in the region between TM3 and TM5. 6) Site-specific substitution of His223 in the μ receptor abolished the inactivation of [3H]bremazocine binding by NEM and led to a dramatic reduction in the affinity for bremazocine. This result suggested that either His223 was the site of NEM alkylation or the H223S substitution caused a conformational change in the receptor that shielded the reactive group from the reagent.

**Effect of DTT on μ and δ Opioid Receptor Binding—**Binding to both μ and δ receptor types was inhibited to a similar extent with high concentrations of dithiothreitol, indicating the role of critical, but inaccessible, disulfide bonds for proper ligand interactions. Similar sensitivity was reported for the inhibition of [3H]naltrindole binding to bovine striatal membranes by DTT (34). In contrast, radioligand binding to several other members
of the G protein-coupled receptor superfamily, such as the cannabinoid, \( \beta_1 \) and \( \beta_2 \)-adrenergic, and \( \alpha_1 \) and \( \alpha_2 \)-adrenergic receptors, was considerably more sensitive to treatment with DTT (20, 25, 35, 36). There is evidence for the presence of a disulfide bond between two conserved Cys residues in the first and second extracellular loops of the \( \beta_2 \)-adrenergic receptor and rhodopsin (36-38). The corresponding residues in the \( \mu \) opioid receptor are Cys\(^{140} \) and Cys\(^{217} \) (Fig. 6). Our finding that the C140S substitution completely blocked \([^{3}H]\)bremazocine binding to the \( \mu \) receptor was consistent with the presence of a disulfide bond between Cys\(^{140} \) and Cys\(^{217} \) that is required for maintenance of an active receptor conformation. The mutagenesis data also suggested that Cys\(^{13} \), Cys\(^{22} \), Cys\(^{43} \), Cys\(^{57} \), Cys\(^{159} \), Cys\(^{190} \), Cys\(^{239} \), Cys\(^{292} \), and Cys\(^{321} \) were not involved in disulfide linkages that were critical for ligand binding.

Comparison of the Effect of NEM on Wild-type \( \mu \) and \( \delta \) Opioid Receptor Binding and Protection by Ligand—It was obvious that radioligand binding to the \( \mu \) receptor was more sensitive to inactivation by NEM than binding to the \( \delta \) receptor, suggesting that the functional group that is the site of alkylation on the \( \mu \) receptor was more exposed and/or more reactive. This finding corroborates previous studies involving selective labeling of rat brain membrane preparations that found that the rank order of receptor sensitivity to NEM was \( \mu > \delta > \kappa \) (39). Our analysis of saturation isotherms indicated that inhibition of bremazocine binding to the \( \delta \) receptor by NEM resulted from a decrease in the total number of binding sites, with no appreciable effect on the affinity of the receptor for the ligand. This observation suggested that alkylated receptors totally lose the ability to bind ligand and that residual receptor binding in the presence of NEM concentrations that gave sub-maximal inhibition was due to the presence of intact, nonalkylated receptors. When high enough concentrations of NEM were used, binding to \( \mu \) and \( \delta \) receptors was totally abolished.

The observation that preincubation with opioid ligands protected against NEM inactivation of binding to \( \mu \) and \( \delta \) receptors was consistent with the site of alkylation being within, or in the vicinity of, the ligand binding crevice of both receptor types. Early studies performed before the realization that there were multiple opioid receptor types also demonstrated ligand protection against N-ethylmaleimide inactivation of receptor binding to rat brain membranes (10, 11).

Sodium Ions Differentially Affect the NEM Sensitivity of Peptide and Bremazocine Binding to \( \mu \) and \( \delta \) Opioid Receptors—The presence of 100 mM NaCl in the buffer affected differentially the NEM sensitivity of peptide agonist and bremazocine binding to \( \mu \) and \( \delta \) receptors. The concentration of NEM needed to reduce \([^{3}H]\)DAMGO binding to \( \mu \) receptors and \([^{3}H]\)DSLET binding to \( \delta \) receptors was dramatically decreased in the presence of sodium ions, while \([^{3}H]\)bremazocine binding to \( \mu \) and \( \delta \) receptors became more resistant to NEM in the presence of 100 mM NaCl.

Differential effects of sodium ions on the ability of NEM to inhibit agonist and antagonist binding have been reported previously (11, 39). The pharmacological profile of bremazocine,
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...however, is not entirely clear. In our studies, [<sup>3</sup>H]bremazocine binding to μ and δ receptors had the characteristics of antagonist binding. In the presence of sodium ions, bremazocine binding was increased slightly (data not shown), and the dose-response curve of NEM was shifted to the right. There is pharmacological evidence that suggests that bremazocine acts as an agonist at κ receptors and as an antagonist at μ and δ receptors (40–43), although it has also been reported that high concentrations of bremazocine caused inhibition of forskolin-stimulated cAMP accumulation in COS cells expressing the δ receptor (44). Additional studies using cloned opioid receptors will be necessary to clarify this issue.

The observation that [<sup>3</sup>H]peptide agonist binding to μ and δ receptors was reduced to <20% of control values at NEM concentrations that had minimal effects on [<sup>3</sup>H]bremazocine binding provided strong evidence for the involvement of at least two NEM-reactive groups. The shift to the left of the NEM dose-response curve for inhibition of [<sup>3</sup>H]DAMGO and [<sup>3</sup>H]DSLET binding may be partially due to NEM alkylation of a GTP-binding protein (15), resulting in receptor uncoupling and a consequent decrease in agonist affinity. [<sup>3</sup>H]Bremazocine binding, in contrast, is not affected by the state of receptor coupling (45).

Localization of the Site of N-Ethylmaleimide Alkylation on the μ Opioid Receptor—Analysis of the N-ethylmaleimide sensitivity of [<sup>3</sup>H]bremazocine binding to a panel of μδ receptor chimeras led to the conclusion that the reactive group on the μ receptor resided in the region between transmembrane domains 3 and 5. Due to the lack of dramatic effects involving substitution or deletion of cysteines, the region between transmembrane domains 3 and 5 was reexamined for other possible reactive side chains. Although NEM is widely regarded as a sulfhydryl-specific reagent, it has been reported that the imidazole group of His can also be alkylated (33, 46).

The H223S substitution (in putative extracellular loop 2 connecting TM4 and TM5) resulted in pronounced effects on basal [<sup>3</sup>H]bremazocine binding and NEM sensitivity of [<sup>3</sup>H]bremazocine binding. The affinity of bremazocine was lowered 20-fold, and bremazocine binding was rendered insensitive toward NEM. There are at least two plausible explanations for these findings. 1) His<sup>223</sup> makes direct contact with bremazocine in the binding site, and NEM alkylation of His or substitution with Ser abolishes the ability of bremazocine to bind to the μ receptor. In this case, the H223S mutant receptor would be insensitive to NEM since the reactive group had been removed. 2) His<sup>223</sup> makes an important contribution to the overall active conformation of the μ receptor. NEM alkylation of His or substitution with Ser would be presumed to disrupt the active conformation, leading to a loss of high affinity bremazocine binding. Again, the H223S mutant receptor would be insensitive to N-ethylmaleimide since the reactive group had been removed, or alternatively, the conformational change resulting from the H223S substitution could conceivably shield other reactive groups (presumably one of the cysteines that was not subjected to deletion or mutagenesis) from interaction with NEM. Investigations are under way to distinguish between these interpretations.

Evidence for essential histidyl residues within opioid receptors has been reported previously (47). In these studies, chemical modification of opioid receptors with two different histidyl-specific reagents resulted in complete inhibition of [<sup>3</sup>H]etorphine binding to rat brain membranes. Further support for His being the actual site of NEM alkylation was obtained by studying the pH dependence of the NEM inactivation of opioid receptor binding. Childers and J. adkson (48) found that the apparent pK<sub>a</sub> value of the N-ethylmaleimide-reactive groups on opioid receptors was between 5.4 and 6.0, which is much closer to the average pK<sub>a</sub> of histidine (pK<sub>a</sub> = 6.5) than cysteine (pK<sub>a</sub> = 8.5) (49).

The data from these studies add to the growing body of knowledge regarding the constituents of opioid receptor binding sites. Previous mutagenesis experiments have highlighted the importance of the Asp in TM2 of the μ and δ receptor for high affinity selective agonist binding (32, 44). Mutation of Asp<sup>147</sup> in TM3 and His<sup>297</sup> in TM6 of the μ receptor inhibited both agonist and antagonist binding (32). These amino acids are also conserved in δ and κ receptors. Regarding receptor selectivity, analysis of μ<sub>κ</sub> and δ<sub>κ</sub> receptor chimeras revealed that the second extracellular loop of the κ receptor was required for high affinity binding of dynorphin-(1–17), dynorphin-(1–13), α-neoendorphin, and dynorphin B (50–52). Evidence has also been provided that the binding site for antagonists in the κ opioid receptor differs substantially from the antagonist site of the μ and δ opioid receptors (53). The amino terminus of the κ opioid receptor was found to be necessary for high affinity naloxone binding and for reversal of κ agonist-mediated inhibition of forskolin-stimulated cAMP accumulation by naloxone. In contrast, Glu<sup>297</sup> in the putative third extracellular loop of the κ receptor plays a major role in binding the κ-selective antagonist, norbinaltorphimine (54). Our group and others (30, 52, 55) have reported recently that a major binding determinant for δ-selective peptides resides in the region spanning TM5 to TM7 of the δ receptor, in excellent agreement with our studies regarding the role of the Arg residues in the putative third extracellular loop (30). Our finding on the importance of the putative first extracellular loop for DAMGO binding using μδ receptor chimeras (30) has also been recently reported independently (55, 56). In contrast, Xue et al. (57) found that the third extracellular loop of the μ receptor was important for agonist selectivity using μκ receptor chimeras. This discrepancy was clarified recently with the important finding that DAMGO distinguishes between μ and δ opioid receptors at a site different from that for the distinction between μ and κ opioid receptors (58).

Until high resolution experimental data is obtained from crystallography, insights from analysis of receptor chimeras and mutagenesis will provide information on the structure and function of opioid receptors, with the aid of molecular modeling and computer simulation. Understanding of the molecular mechanisms involved in receptor activation and G protein coupling triggered by agonist engagement of the opioid receptor binding site remain as long term goals of these studies. It is anticipated that an understanding of opioid receptor structure and function will lead to the development of novel therapeutic agents.

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REFERENCES

1. Reisine, T., and Bell, G. I. (1993) Trends Pharmacol. Sci. 16, 506–510
2. Knapp, R. J., Malatynska, E., Collins, N., Fang, L., Wang, J. Y., Hruby, V. J., Roesele, W. R., and Yamanura, H. I. (1995) FASEB J. 9, 516–525
3. Mansour, A., and Watson, S. J. (1993) Handb. Exp. Pharmacol. 104, 79–106
4. Simon, E. J., and Hiller, J. M. (1994) in Basic Neurochemistry: Molecular, Cellular, and Medical Aspects (Siegel, G. J., Albers, R. W., Agrawolf, B. W., and Katzman, R., eds) pp. 321–339, Raven Press, New York
5. Strader, C. D., Fong, T. M., Tota, M. R., Underwood, D., and Dixon, R. A. F. (1994) Annu. Rev. Biochem. 63, 101–132
6. Laugwitz, K.-L., Oefermann, S., Spicher, K., and Schultz, G. (1993) Neuron 10, 233–242
7. Carter, B. D., and Medzhitov, G. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 4062–4066
8. Pratner, P. L., McGinn, T. M., Erickson, L. J., Evans, C. J., Loh, H. H., and Law, P. Y. (1994) J. Biol. Chem. 269, 21293–21302.

M. Shahrestanifar, W. W. Wang, and R. D. Howells, unpublished observations.
His^{223} Is Critical for μ Opioid Receptor Binding

9. Simon, E. J., Hiller, J. M., and Edelman, I. (1973) Proc. Natl. Acad. Sci. U. S. A. 70, 1947–1949
10. Simon, E. J., and Gotoh, J. (1975) Proc. Natl. Acad. Sci. U. S. A. 72, 2404–2407
11. Pasternak, G. W., Wilson, H. A., and Snyder, S. H. (1975) Mol. Pharmacol. 11, 340–351
12. Kodama, H., Shimohigashi, Y., Ogasawara, T., Koshizaki, T., Kurono, M., Matsueda, R., Soejima, K., Kondo, M., and Yagi, K. (1989) Biochem. Int. 19, 1159–1164
13. Kanematsu, K., Naito, R., Shimohigashi, Y., Ohno, M., Ogasawara, T., Kurono, M., and Yagi, K. (1990) Chem. Pharm. Bull. 38, 1438–1440
14. Mullikin-Kilpatrick, D., Larsen, N. E., and Blume, A. J. (1983) J. Neurosci. 3, 145–152
15. Childers, S. R. (1984) J. Pharmacol. Exp. Ther. 230, 684–691
16. Sharif, N. A., and Burt, D. R. (1984) J. Neurochem. 42, 209–214
17. Siddhu, A., Kassiss, S., Kebabian, J., and Fishman, P. H. (1986) Biochemistry 25, 6695–6701
18. Chozet, P. L., and Strange, P. G. (1992) Biochem. J. 281, 377–380
19. Sharma, P. M., and Musacchio, J. M. (1992) Eur. J. Pharmacol. 218, 9–19
20. Reader, T. A., Briere, R., and Grondin, L. (1996) Neurochem. Res. 21, 9–27
21. Ng, D. S., and Wong, K. (1986) Eur. J. Pharmacol. 134, 47–52
22. Falcone, R. C., and Aharony, D. (1990) J. Pharmacol. Exp. Ther. 255, 565–571
23. Pavo, I., and Fahrenholz, F. (1990) FEBS Lett. 272, 205–208
24. Santos-Delana, J. A., Grasso, P., and Rechert, L. E. (1991) Biochem. Biophys. Res. Commun. 176, 1256–1261
25. Liu, R., Hubbard, J. R., Martin, B. R., and Kalimi, M. Y. (1993) Mol. Cell. Biochem. 121, 119–126
26. Javitch, J. A., Li, X., Kackab, J., and Karlin, A. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 10355–10359
27. Shahrestanifar, M., and Howells, R. D. (1994) Regul. Peptides 57, 269–277
28. Howells, R. D., Liu-Chen, L-Y. (1995) J. Biol. Chem. 270, 248–254
29. Wang, W. W., Shahrestanifar, M., Jin, J., and Howells, R. D. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 12436–12440
30. Liu, Y. F., and Quirion, R. (1992) J. Neurochem. 59, 1024–1032
31. Surratt, C. K., Johnson, P. S., and Quirion, R. (1988) Anal. Biochem. 176, 248–255
32. Gioannini, T. L., Liu, Y. F., Park, Y-H., Hiller, J. M., and Simon, E. J. (1989) J. Mol. Recog. 2, 44–48
33. Vauquelin, G., Bottari, S., Kanarek, L., and Strosberg, A. D. (1997) Biochemistry 36, 4462–4469
34. Dohlman, H. G., Caron, M. G., DeBlasi, A., Frielle, T., and Lefkowitz, R. J. (1990) Biochemistry 29, 2335–2342
35. Dixon, R. A. F., Sigal, I. S., Candelore, M. R., Register, R. B., Scattagoria, W., Rands, E., and Strader, C. D. (1987) EMBO J. 6, 3269–3275
36. Karnik, S. S., Sakum, T. P., Chen, H.-B., and Khonana, H. G. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 8495–8463
37. Ofiri, D., and Simon, E. J. (1992) Receptor 2, 109–119
38. Römer, D., Büscher, H., Hill, R. C., Maurer, R., Petcher, T. J., Welle, H. B. A., Bakel, H. C. C., and Akkerman, A. M. (1980) Life Sci. 27, 971–978
39. Römer, D., Büscher, H., Hill, R. C., Maurer, R., Petcher, T. J., Zsürger, H., Benson, W., Finner, E., Milskowki, W., and Thies, P. W. (1982) Life Sci. 31, 1217–1220
40. Von Voigtlander, P. F., and Lewis, R. A. (1982) Prog. Neuro-psychopharmacol. & Biol. Psychiatry 6, 467–470
41. Gambro, M. C., Pettilio, P., and Tawani, A. (1983) Life Sci. 33, 461–464
42. Kong, H., Raynor, K., Yasuda, K., Moe, S. T., Portoghese, P. S., Bell, G. I., and Resine, T. (1993) J. Biol. Chem. 268, 23055–23058
43. Richardson, A., Demoflo-ul-Mason, C., and Barnard, E. A. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 10198–10202
44. Means, G. E., and Feneey, R. E. (1971) Chemical Modification of Proteins, pp. 110–114, Holden-Day, San Francisco
45. Roy, B. P., and Ng, A. Y. (1982) Biochem. Biophys. Res. Commun. 109, 518–526
46. Childers, S. R., and Jackson, J. L. (1984) J. Neurochem. 43, 1163–1170
47. Stryer, L. (1995) Biochemistry, 4th Ed., W. H. Freeman, New York, NY
48. Wang, J. B., Johnson, P. S., Wu, J. M., Wang, W. F., and Uhl, G. R. (1994) J. Biol. Chem. 269, 25966–25969
49. Xue, J.-C., Chen, C., Zhu, J., Kunapuli, S., DeRie, J. K., Yu, L., and Liu-Chen, L-Y. (1994) J. Biol. Chem. 269, 30195–30199
50. Meng, F., Hovarost, M. T., Thompson, R. C., Taylor, L., Watson, S. J., and Akil, H. (1999) J. Biol. Chem. 274, 12730–12736
51. Kong, H., Raynor, K., Yano, H., Takeda, J., Bell, G. I., and Resine, T. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 5042–5046
52. Hjorth, S. A., Thirstrup, K., Grandy, D. K., and Schwartz, T. W. (1995) Mol. Pharmacol. 47, 1089–1094
53. Fuku, K., Kato, S., and Mori, K. (1995) J. Biol. Chem. 270, 6702–6709
54. Onogi, T., Minami, M., Katao, Y., Nakagawa, T., Aok, Y., Toy, T., Katsurama, S., and Satoh, M. (1995) FEBS Lett. 357, 93–97
55. Xue, J.-C., Chen, C., Zhu, J., Kunapuli, S., de Rie, J. K., Yu, L., and Liu-Chen, L-Y. (1995) J. Biol. Chem. 270, 12977–12981
56. Minami, M., Onogi, T., Nakagawa, T., Katao, Y., Aoki, Y., Katsurama, S., and Satoh, M. (1995) FEBS Lett. 364, 23–27