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Naloxone Activation of μ-Opioid Receptors Mutated at a Histidine Residue Lining the Opioid Binding Cavity

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SUMMARY

The μ-opioid receptor is the principal site of action in the brain by which morphine, other opiate drugs of abuse, and endogenous opioid peptides effect analgesia and alter mood. A member of the seven-transmembrane domain (TM) G protein-coupled receptor (GPCR) superfamily, the μ-opioid receptor modulates ion channels and second messenger effectors in an opioid agonist-dependent fashion that is reversible by the classic opiate antagonist naloxone. Mutation of a histidine residue (His297) in TM 6 afforded agonist-like G protein-coupled signal transduction mediated by naloxone and other alkaloid antagonists and enhanced the intrinsic activity of documented alkaloid partial agonists, including buprenorphine. The intrinsic activities of all opioid peptide agonists and antagonists tested were not altered at the His297 mutant receptors. Consistent with a role for the TM 6 histidine in maintaining high affinity binding sites for opioid agonists and antagonists, opioid ligand-dependent protection of this residue from a histidine-specific alkylating agent indicated that the His297 side chain is positioned in or very near the binding cavity. The TM 6 His297 mutants identify a discrete region of the receptor critical for determining whether a specific drug pharmacophore triggers receptor activation. Because many GPCRs possess a similarly positioned TM histidine residue, our findings with the μ-opioid receptor may extend to these receptors and potentially serve as a model for rational design of therapeutic GPCR partial agonists and antagonists.

The amino acid sequences encoded by μ-, δ-, and κ-opioid receptor cDNAs (1–8) include three residues typically charged at physiological pH that are predicted to lie within the receptor TM. Such TM residues within the lipophilic environment of the cell membrane are inherently key in contributing to ligand recognition or signal transduction among GPCRs and are expected to be oriented toward a relatively hydrophilic central cavity (9–11). Although the TM 2 aspartic acid is virtually invariant among GPCRs, the TM 3 aspartic acid is conserved among GPCRs that bind amine-containing ligands (11). These residues, as well as a third potential charge in the form of a modestly conserved TM 6 histidine residue (12, 13), are critical for high affinity agonist binding at the μ receptor (14). To visualize the role of such residues in forming intramolecular μ-opioid receptor contacts as well as interactions with opioid ligands, we modeled the seven putative α-helical TM regions of the rat μ-opioid receptor based on the crystallographic coordinates of bacteriorhodopsin (15, 16) and the GPCR rhodopsin (17). Evidence from Thirstrup *et al.* (18) implies that opioid receptors adopt a similar TM conformation. Our molecular modeling scenarios suggested that the TM 6 His297 side chain was directed toward the receptor interior, possibly forming contacts with alkaloid and peptide ligands or other TM residues within the opioid binding cavity. Moreover, the direct covalent linkage of TM 6 to the third intracellular loop, a region critical for G protein interactions in other GPCRs (19), suggested that His297 mutants could have altered requirements for receptor activation.

Here, we elucidate the features of the His297 side chain required for μ-opioid receptor function and, by demonstrating that μ receptor ligands protect the TM 6 histidine from alkylation by diethylpyrocarbonate, demonstrate that His297 is in the vicinity of opioid binding sites. The classic alkaloid antagonist naloxone, traditionally useful in revealing opioid receptor-linked processes by its ability to reverse the action of opioid agonists, served as a partial agonist at His297 mutant μ receptors. A continuum of increasing agonist activity at His297 mutant receptors was observed for the structure-activity progression of naloxone to naltrexone to diprenorphine to buprenorphine. Taken with results obtained from testing the same ligands at the wild-type μ receptor, we provide for the first time evidence associating a specific amino acid side-chain lining the μ-opioid receptor

ABBREVIATIONS: TM, transmembrane domain; GPCR, G protein-coupled receptor; PBS, phosphate-buffered saline; DAMGO, [D-Ala^2, *N*-MePhe^4, Gly-ol^5]enkephalin; CTOP, D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DEPC, diethylpyrocarbonate; PTX, pertussis toxin.

ligand binding cavity with the ability of the receptor to discriminate between alkaloid agonists and antagonists. The analogous TM 6 histidine is present in other GPCRs, meaning that a role for this histidine in defining the agonist properties of a drug may extend to these GPCRs.

Experimental Procedures

Site-directed mutagenesis, expression of mutant receptors, and pharmacological characterization. The construction and subcloning of mutant cDNA fragments into the full-length rat μ receptor coding region via *Fsp*I and *Pvu*I sites were conducted as described previously (14), as was the pharmacological characterization of mutant receptors. COS-7 cells were transfected by the calcium phosphate method with 20 μg of wild-type or His297 mutant DNA/10^7 cells. The DNA/$Ca_3(PO_4)_2$ suspension was added to 150-mm plates (Nunc, Naperville, CT) containing 30 ml of Dulbecco's modified Eagle's medium/10% fetal bovine serum and cells at 30% confluence. $Ca_3(PO_4)_2$ and untransfected DNA were removed after 24 hr by replacing the medium with Dulbecco's modified Eagle's medium, and the culture was incubated for 48 hr. Transfected cells were tested for receptor expression by radioligand binding and immunostaining (14). Radioligand binding screening experiments that yielded detectable binding by a μOR mutant were followed by more detailed saturation and displacement experiments. Saturation analyses used 0.1–15 nM [^{3}H]naloxone (Amersham, Arlington Heights, IL) for wild-type and H297Q receptors and 0.5–50 nM for the H297N receptor, expressed on the surface of intact COS cells. Scatchard analysis of [^{3}H]naloxone binding yielded B_{max} values for the wild-type, H297N, and H297Q receptors of 34 ± 3, 26 ± 5, and 89 ± 8 fmol/mg of COS cell protein, respectively. These values are lower than typically found in the literature because the Bradford analysis of SDS/NaOH-treated protein samples was of whole COS cells. Displacement of 2 nM [^{3}H]naloxone was tested with 0.1–10,000 nM final concentrations of nonradioactive μ-selective ligands, including DAMGO (Sigma Chemical, St. Louis, MO), PL017 ([*N*-MePhe^3,D-Pro^4]morphiceptin; Peninsula Laboratories, Belmont, CA), CTOP (Peninsula Laboratories), morphine sulfate (Research Biochemicals, Natick, MA), buprenorphine HCl (Research Biochemicals), and sufentanyl citrate (Janssen Pharmaceutica, New Brunswick, NJ). Data analyses were performed with the LIGAND software (20) and according to the method of Cheng and Prusoff (21).

Immunocytochemical studies. COS cells transfected with pCDNA1, μORF (wild-type), or mutant μOR cDNAs were incubated for 72 hr on 22-mm glass coverslips, fixed with 4% paraformaldehyde in PBS (1×= 137 mM NaCl, 2.7 mM, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) at 4° for 1 hr, and washed three times with PBS at 25°. Cells were incubated with 0.1% H_2O_2 for 30 min to suppress endogenous peroxidase. Fixed cells were preincubated with PBS, 0.5% Triton X-100, and 3% normal goat serum for 5 min, followed by incubation with μOR primary antiserum (or control sera) diluted 1:5000 for 72 hr at 4°. The μOR antisera was directed against the carboxyl-terminal 18 amino acid residues, and its preparation has been described thoroughly(14). Primary antiserum was removed, coverslips were washed with PBS, and bound IgG was detected with the use of biotinylated goat anti-rabbit antiserum and avidin-conjugated peroxidase (Vectastain ABC kit; Vector Laboratories, Burlingame, CA). Control experiments included staining of paraformaldehyde-fixed brain sections, staining of a Western blot containing purified authentic μOR protein, use of preimmune sera, omission of secondary antibodies, and preincubation of 500 μg/ml concentration of the carboxyl-terminal peptide or nonspecific octadecameric peptide with μOR primary antisera overnight at 4° before application to cells.

Alkylation studies. Harvested cells were washed and resuspended in 50 mM Tris·HCl, pH 7.4, divided evenly among five tubes, isolated through centrifugation (5 min at 4000 × *g*), resuspended in 5 ml of 50 mM CH_3COONa, pH 6.0, at 25°, and immediately adjusted to final concentrations of 0, 100, 250, 500, and 1000 μM DEPC. After 10 min of shaking on a rotating wheel at 25°, DEPC was quenched with 25 ml of ice-cold 50 mM Tris·HCl, pH 7.4, and cells were isolated by centrifugation, washed twice with 25 ml of ice-cold 50 mM Tris·HCl, pH 7.4, and immediately assayed as described previously (14) for radioligand binding. For ligand protection experiments, cells in the pH 6.0 buffer above were incubated with 1 μM DAMGO, morphine, naloxone, or no drug for 5 min at 25° followed by the addition of 100 μM or 1000 μM DEPC and then treated and analyzed as above. A "no-DEPC" control reaction used the 5-min morphine preincubation step without subsequent DEPC treatment; as indicated in Fig. 4B, the protecting drug was completely removed before [^{3}H]-ligand binding.

Oocyte preparation. Stage 5 and 6 *Xenopus laevis* oocytes were maintained at 19° in ND96 solution (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 2.5 mM sodium pyruvate, 50 units/ml penicillin, and 50 μg/ml streptomycin in 5 mM HEPES, pH 7.6. The medium was changed daily. The animal hemispheric pole of each oocyte was injected with 15 nl of a 1:1 mixture of pCDNA-based plasmids (1 ng/nL concentration each) encoding the wild-type or mutant μ receptor and the inwardly rectifying atrial potassium channel (22). Oocytes were incubated for 30 min with 2 mg/ml collagenase (clostridiopeptidase A, type IA-S; Sigma) 24 hr after injection and then soaked in 96 mM NaCl, 2 mM KCl, and 1 mM $MgCl_2$ in 5 mM HEPES, pH 7.6, for 30 min, followed by exhaustive washing with complete ND96 solution. In PTX studies, the cytoplasm of oocytes robustly responding to normorphine was injected with 11 nl of 1 ng/nl PTX (23).

Electrophysiological recording. Oocytes that had been manually defolliculated before impalement were superfused with 96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, and 1 mM $CaCl_2$ in 5 mM HEPES, pH 7.6, at 1.0 ml/min. The resting membrane potential was typically −35 to −45 mV, with an input resistance of 0.5 to 1.5 MΩ. On stabilization of the voltage-clamped oocyte at a holding potential of −60 mV, the ND96 solution was exchanged for a "high potassium" recording medium (96 mM KCl, 2 mM NaCl, 1 mM $MgCl_2$, and 1 mM $CaCl_2$ in 5 mM HEPES, pH 7.6). The high potassium concentration and a holding potential reset to −80 mV enhanced the current signal. After reestablishment of a base-line in the high potassium medium, the oocyte was tested for μ receptor-mediated K^+ channel gating with 20 μM normorphine. The μ receptor-selective ligands were dissolved in the high potassium medium and applied to a robustly responding oocyte until a maximum response was obtained.

Oocytes coinjected with μ receptor and K^+ channel cDNAs responded with inward potassium currents in the presence of an agonist. No current was detected for oocytes lacking either cDNA. The current elicited with 20 μM normorphine varied from 1 to >500 nA among oocytes. Normorphine (20 μM) was applied periodically throughout the experiment to evaluate and correct for the sensitivity of the oocyte; typically, a small increase in sensitivity was followed by monotonic decrease.

Data analysis. A logistic function, $I = I_{max}\cdot[A]^n/([A^n] + EC_{50}{}^n)$, where I is current, [A] is agonist concentration, EC_{50} is concentration of agonist that causes a half-maximal response, and *n* is Hill coefficient, was fitted to the concentration-response data. In all experiments, the Hill coefficient approximated unity. Both the EC_{50} and I_{max} values depended on the agonist; because normorphine and DAMGO consistently elicited a uniform, maximum current response, the intrinsic activities of the other opiate alkaloids and opioid peptides were defined with reference to one of these two full agonists. When normorphine and a partial agonist or an antagonist were mixed, the data were described by an extension of the equation above that assumes that both agents compete for the same site but activate with different dissociation constants, $EC_{50,A}$ and $EC_{50,B}$, and with different maxima, I_A and I_B: $I = (I_A\cdot EC_{50,B}\cdot [A] + I_B\cdot EC_{50,A}\cdot [B])/([A]\cdot EC_{50,B} + [B]\cdot EC_{50,A} + EC_{50,A}\cdot EC_{50,B})$.

When $I_B = 0$, this equation describes a competitive antagonist, *B*. For consistency, the term EC_{50}, as defined here, is used throughout, regardless of the intrinsic activity of a drug. In experiments in which two drugs were tested separately as well as in mixtures, all the data were analyzed simultaneously. Parameter estimates were obtained by nonlinear regression using MLAB (Civilized Software, Bethesda, MD).

Results

Features of the TM 6 His297 side chain critical for maintaining high affinity binding of peptide and alkaloid agonists and antagonists. Alanine replacement of His297 previously yielded a receptor with little binding affinity for the radiolabeled agonists and antagonists that were tested (14). To define features of the histidine imidazole side chain important for μ receptor recognition of various opioid receptor ligands, eight additional point mutations at position 297 were generated, replacing histidine with either the π-electron donor phenylalanine, basic residues lysine or arginine, acidic residues aspartic acid or glutamic acid, hydrogen bond donors glutamine or asparagine, or aliphatic residue leucine. A screen for high affinity radioligand binding revealed that only the glutamine (H297Q) and asparagine (H297N) mutants displayed marked displaceable binding of the μ-selective peptide agonist [^{3}H]DAMGO (Fig. 1). The remaining seven mutants also failed to demonstrate significant binding of a second enkephalin analog, [^{3}H][D-Ala^2,D-Leu^5]enkephalin (data not shown). Except for a clear preference for glutamine (≈50% of wild-type), binding of the classic alkaloid antagonist [^{3}H]naloxone was reduced 2.5–10-fold regardless of the amino acid substitution (Fig. 1). The pattern of binding of [^{3}H]diprenorphine, another alkaloid antagonist, by the nine mutant receptors was indistinguishable from that in Fig. 1 for [^{3}H]naloxone (data not shown). To address the possibility that poor radioligand binding by a mutant receptor was due to poor receptor expression, the expression and cell surface targeting of each mutant receptor were verified immunocytochemically (Fig. 2). Comparable

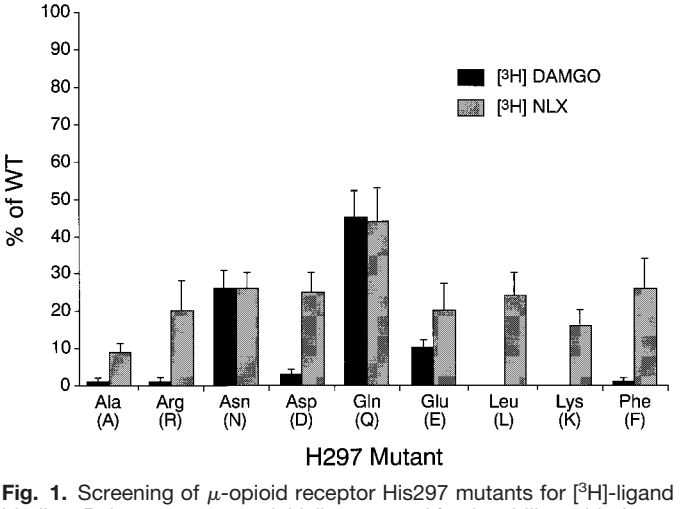

Fig. 1. Screening of μ-opioid receptor His297 mutants for [^{3}H]-ligand binding. Point mutants were initially assessed for the ability to bind 5 nM [^{3}H]DAMGO (*black bars*) and 5 nM [^{3}H]naloxone (*NLX*; *gray bars*). Site-directed mutants included His297 replacement with alanine (*A*), arginine (*R*), asparagine (*N*), aspartic acid (*D*), glutamine (*Q*), glutamic acid (*E*), leucine (*L*), lysine (*K*), or phenylalanine (*F*). All values are based on comparison with the wild-type (*WT*) receptor. Values are mean ± standard error from four experiments.

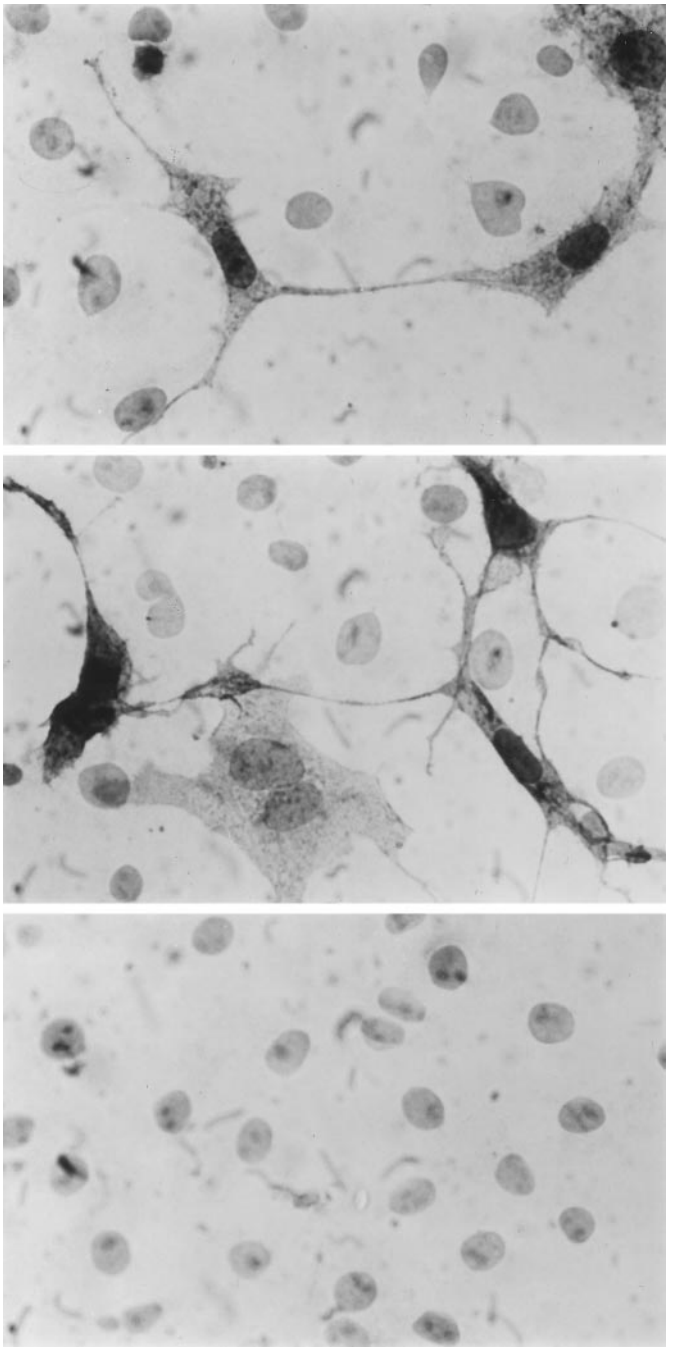

Fig. 2. Immunostaining of COS cells transiently transfected with wild-type and mutant μ-opioid receptor cDNAs. Representative fields of cells transfected with one of three selected cDNA constructions are shown. *Top*, wild-type μ receptor. *Center*, H297R mutant μ receptor. *Bottom*, pCDNA1 plasmid "vector-alone" negative control, entirely lacking the μ receptor coding region. In no case did preimmune sera exhibit immunostaining (data not shown).

levels of cDNA transfection efficiency and immunoreactivity were observed for the wild-type receptor and all His297 mutants. The fact that naloxone binding was somewhat uniform among the nine mutant μ receptors tested (Fig. 1) further indicates that the expression levels of these receptors were similar. B_{max} values from Scatchard analysis of [^{3}H]naloxone binding to the wild-type, H297Q, and H297N μ receptors were within 1 order of magnitude (see Experimental Proce-

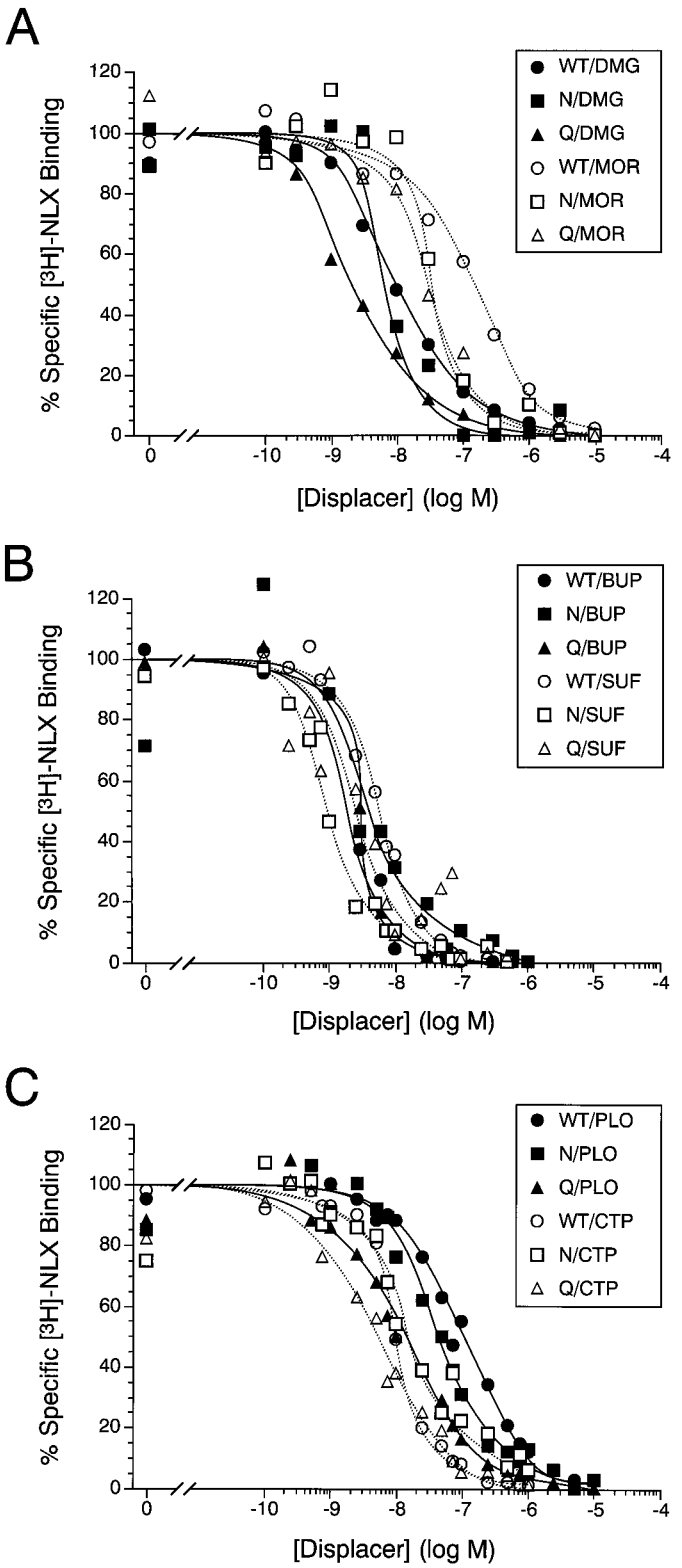

Fig. 3. Displacement of [^{3}H]naloxone binding to His297 mutant receptors. A, Nonradioactive DAMGO (*DMG*) or morphine (*MOR*) was tested as a displacer at 0.1–10,000 nM concentrations with the wild-type (*WT*), H297N (*N*), or H297Q (*Q*) receptors. B, Nonradioactive buprenorphine (*BUP*) or sufentanyl (*SUF*), as in A. C, Nonradioactive PL017 (*PLO*) or CTOP (*CTP*), as in A.

TABLE 1

Affinities of opiate alkaloid and opioid peptide agonists and antagonists for wild-type, H297N, and H297Q μ receptors

	Wild-type	H297N	H297Q
		K_d nM	
Naloxone	1.2 ± 0.1	6.7 ± 0.1	2.0 ± 0.6
		K_i nM	
DAMGO	2.7 ± 0.3	5.8 ± 0.5	2.9 ± 0.6
PL017	38.3 ± 0.5	42.9 ± 5.2	7.9 ± 0.6
Morphine	35.8 ± 3.0	46.7 ± 8.0	24.4 ± 3.9
Sufentanyl	1.3 ± 0.3	0.8 ± 0.1	1.1 ± 0.3
Buprenorphine	1.6 ± 0.3	2.2 ± 0.4	2.1 ± 0.4
CTOP	3.7 ± 0.3	9.9 ± 2.5	2.0 ± 0.4

Values are mean ± standard error from at least four experiments.

dures), indicating similar receptor numbers at the cell surface.

The H297Q and H297N mutant receptors bound radioligands sufficiently to assess affinities for nonradioactive alkaloid and peptide agonists and antagonists (Fig. 3 and Table 1). The glutamine substitution at position 297 was preferred for all opioid peptides tested; DAMGO and PL017 (agonists) and CTOP (an antagonist) exhibited 2–5-fold higher affinities for the glutamine mutant than that of asparagine. Interestingly, PL017 was also bound by the H297Q receptor with an affinity 5-fold above that of the wild-type receptor (Table 1). The wild-type histidine residue was preferred by the alkaloid antagonist naloxone; affinity for the drug decreased 2- and >5-fold with glutamine and asparagine substitution, respectively. In contrast, the opiate alkaloid agonists morphine, sufentanyl, and buprenorphine demonstrated no marked side chain preference. The preference for a histidine, glutamine, or asparagine side chain may correlate with the drug class (alkaloid or peptide agonist or antagonist), with the caveat that more representatives from each class must be tested.

His297 is positioned near or within the opioid binding cavity. The accessibility of ligands to His297 and the effect of alkylation of this side chain on ligand binding were tested by measuring the sensitivity of the wild-type μ receptor to DEPC, an agent observed to carboxyethylate histidine residues selectively at pH 6.0 (24). To address specifically His297, the lone TM histidine residue, DEPC studies directly compared the wild-type, H297N, and H297Q receptors. Wild-type and mutant μ receptors were exposed to various concentrations of DEPC, which was thoroughly washed out of cell membranes before radioligand binding assays. Pretreatment of receptors with 100 or 250 μM DEPC reduced [^{3}H]DAMGO binding at the wild-type μ receptor relative to mutants lacking the His297 residue (Fig. 4A); a similar binding profile for [^{3}H]naloxone binding was observed, although this ligand was uniformly more tolerant to receptor pretreatment with DEPC (data not shown). We expected subtle differences of DEPC susceptibility between the wild-type and His297 mutant receptors for two reasons: (a) some of the authors[1] of the current study and Shahrestanifar *et al.* (25) have observed that the μ receptor His223 residue is important for ligand binding, and on the basis of the predicted extracellular loop location of this residue, His223 would be expected to be more accessible to DEPC than His297. Alkylation of His223 would

[1] B. K. Seidleck, C. J. Blaschak, and C. K. Surratt, unpublished observations.

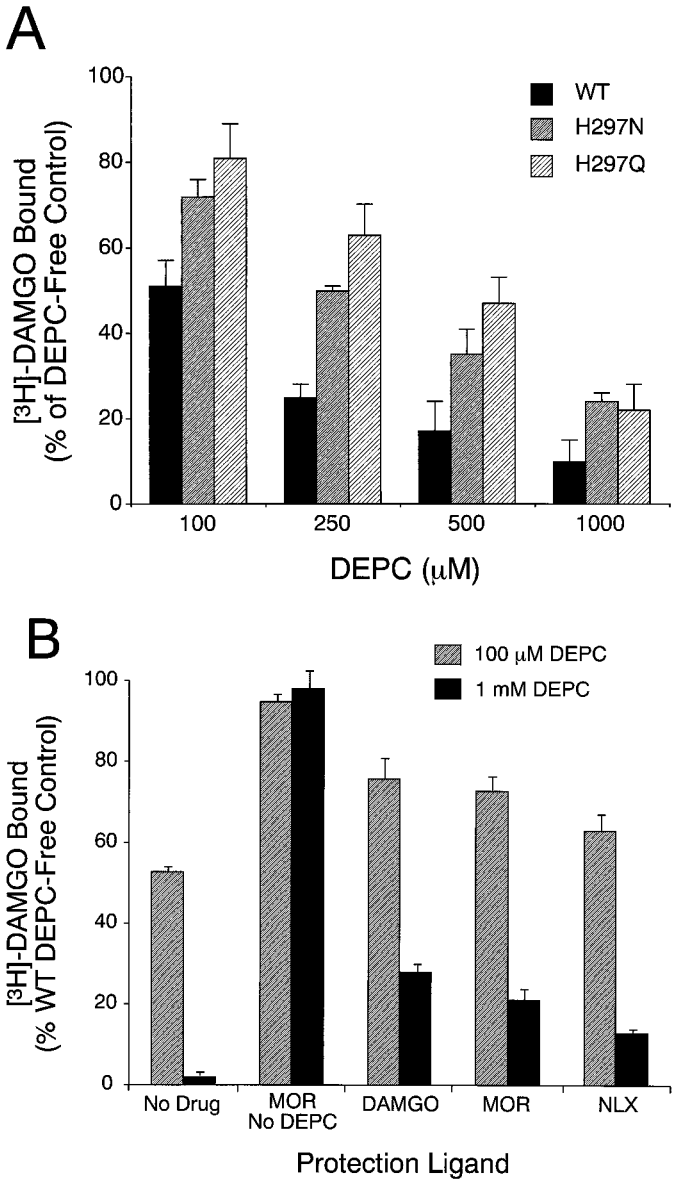

Fig. 4. A, DEPC alkylation of wild-type and His297 mutant μ receptors. The effect of DEPC pretreatment of wild-type (*WT*), H297N, and H297Q receptors on [^{3}H]DAMGO binding (5 nM) is reflected relative to a control lacking DEPC. B, Ligand-mediated protection of the wild-type receptor from DEPC alkylation. A "No DEPC" control reaction included the morphine preincubation step without subsequent DEPC treatment, verifying that the protecting drug was completely removed before [^{3}H]-ligand binding. *MOR*, morphine; *NLX*, [^{3}H]naloxone. Values are mean ± standard error from five experiments.

therefore be expected to partially reduce ligand binding at all three receptors. (b) DEPC selectivity for histidine residues is very concentration and pH dependent; any DEPC concentration would afford some nonspecific alkylation, and the "window" for observing a His297-specific effect would be necessarily small. Nevertheless, the His297-specific alkylation event was clearly detected (Fig. 4A).

Pretreatment of the wild-type receptor with 1 mM DEPC essentially abolished subsequent [^{3}H]DAMGO binding; the addition of an opioid peptide or opiate alkaloid ligand before the alkylation step protected the receptor to varying degrees (Fig. 4B). This protection was not due to residual ligand from the pretreatment step blocking the radioligand because full [^{3}H]DAMGO binding was achieved in the "no-DEPC" control reaction (Fig. 4B). The order of protective ability was DAMGO (28 ± 2) > morphine (21 ± 3) > naloxone (13 ± 1), as would be predicted when considering the similarity of the protection ligand (peptide agonist > alkaloid agonist > alkaloid antagonist) to the [^{3}H]DAMGO binding site. Again, the lack of full receptor protection by the ligands against DEPC was not unexpected, a likely effect of incomplete shielding of His297 by the protecting ligand. Considering the small and hydrophobic DEPC molecule within a relatively large GPCR cavity (assuming a μ receptor transmembrane domain array similar to rhodopsin; Ref. 17), the DEPC molecule may be available for both suprafacial and antarafacial attack by His297. The results indicate the His297 residue lines the interior face of the receptor TM domains that encompass the relatively hydrophilic ligand binding cavity, as opposed to the residue solely participating in adjoining TM domain (TM 5 and TM 7) interactions and being deeply ensconced within the lipid milieu of the membrane. Although it is both unclear and perhaps unlikely that His297 directly participates in ligand binding, the ligand-dependent protection from alkylation places the residue in the vicinity of the ligand binding sites, before and/or after the agonist-induced conformational change characteristic of the GPCR family.

Naloxone and other alkaloid antagonists mediate G protein-linked activation of a His297 mutant μ-opioid receptor. The influence of the internally directed His297 residue of TM 6 on μ receptor ligand recognition (Fig. 1) in a drug class-specific manner (Table 1) suggested that His297 might contribute to the structural underpinning of the receptor that defines opioid peptide and opiate alkaloid agonist and antagonist binding sites. TM 6 is directly connected to the third intracellular loop, a region critical among GPCRs for binding G proteins (19); thus, disruption of a ligand cavity-spanning His297 interaction may alter the position of TM 6 and consequently alter the geometry of receptor intracellular loops that bind G proteins. In such a mutant receptor, the ligand requirements for agonism may not be identical to those for the wild-type receptor.

Indeed, the classic opiate antagonist naloxone mediated opening of an inwardly rectifying K^+ channel (22) in the presence of the asparagine-bearing mutant H297N but not the wild-type receptor (Fig. 5A). The H297Q mutant μ receptor was also tested for naloxone activation and yielded results similar to H297N in this respect (Tables 2 and 3). Opening of the K^+ channel, transiently coexpressed with a μ-opioid receptor in *X. laevis* oocytes, is typically driven by direct G protein coupling (26) to the agonist-bound opioid receptor. At this point, additional opioid antagonists were screened as potential agonists at His297 mutant receptors. Increasing intrinsic activity (defined as the maximum K^+ channel current elicited by an opioid ligand relative to the full agonist DAMGO) at H297N was observed for the alkaloid antagonist structure-activity series proceeding from naloxone to naltrexone to diprenorphine (Fig. 5A); mean ± standard error peak amplitudes were 5.5 ± 1.0%, 11.8 ± 1.7%, and 19.3 ± 3.8% of the initial normorphine peak, respectively. The wild-type receptor registered either no response or an insignificant peak amplitude (<1% of that for normorphine) in the presence of each antagonist (Fig. 5A). The alkaloid "antagonist"-driven K^+ channel activity in the presence of His297 mutants was still mediated by a G protein,

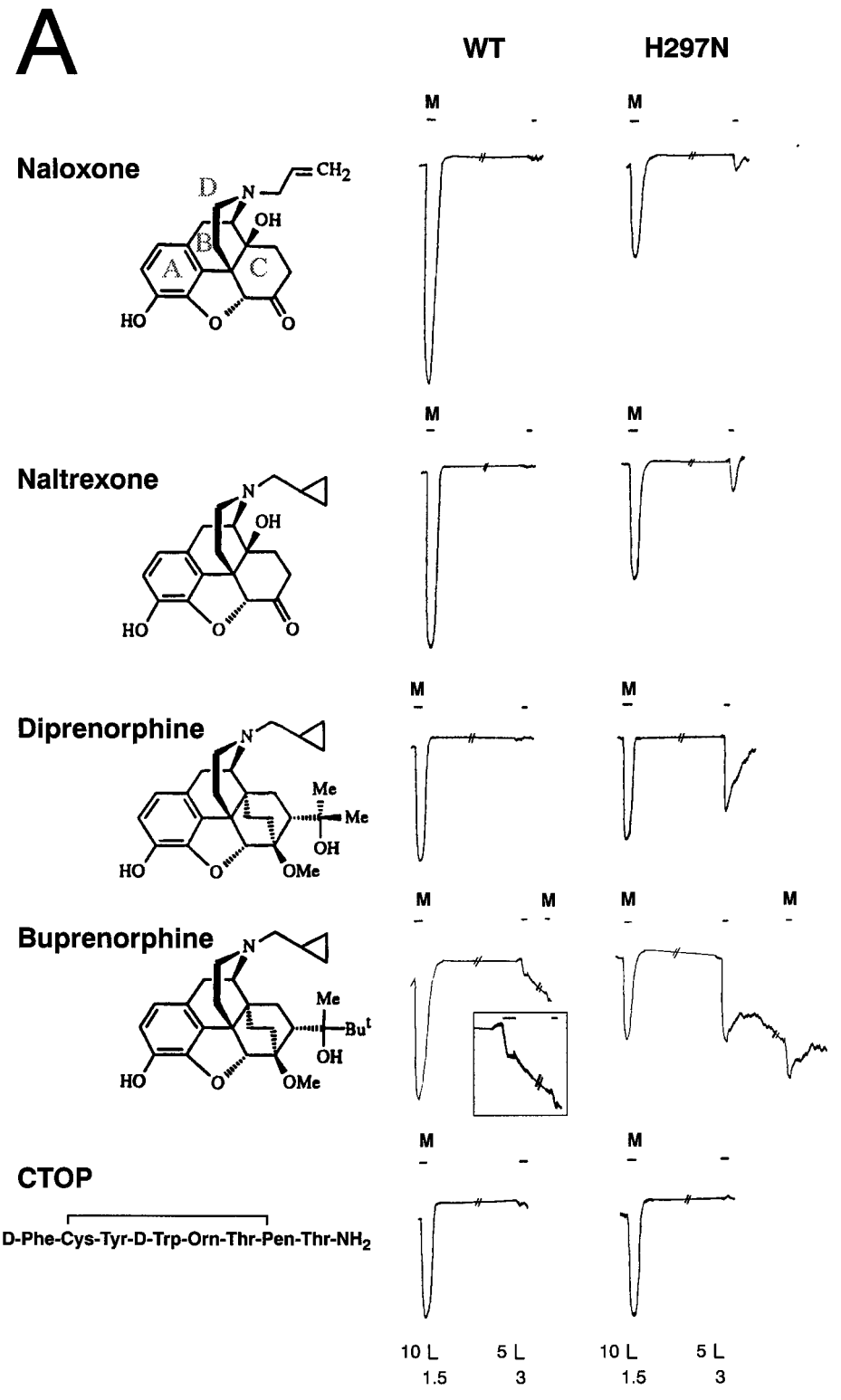

Fig. 5. A, Modulation of K^+ channels coexpressed with wild-type (*WT*) or H297N mutant μ receptors in oocytes. Individual traces represent a typical result from at least six experiments. Normorphine (20 μM; *M*) was used to screen for acceptable oocytes, an agonist easily washed out of the oocyte membrane. To measure relative responses in the presence of 1 μM naloxone, naltrexone, diprenorphine, buprenorphine, or CTOP, only oocytes yielding maximum responses of ≈100 nA to normorphine were used in the study. *Short horizontal bars above the traces*, duration of drug application. *Bars not labeled with M*, drug represented in the left column. *Horizontal component of the scales at the bottom*, minutes elapsed. *Vertical component*, current amplitude (in nA). Note that for illustrative purposes, the scales indicate that the sensitivity has been doubled on application of the drug under study, for both wild-type and mutant receptors, relative to the sensitivity during recording of normorphine peaks. *Double break in the tracing*, unrepresented (and uneventful) minutes elapsed between drug additions. *Inset*, magnification of the small peaks obtained with buprenorphine and subsequent normorphine application. Six-membered rings of the alkaloid structures (*left*) are typically designated and referred to in the text as A, B, C, and D (*gray letters within naloxone structure*), with the piperidine (nitrogen-bearing heterocycle) ring D coming

TABLE 2

EC_{50} values for opiate alkaloids and opioid peptides at wild-type, H297N, and H297Q μ receptors

	Wild-type	H297N	H297Q
		EC_{50} nM	
DAMGO	36 ± 5	46 ± 7	25 ± 5
PL017	140 ± 40	220 ± 60	90 ± 20
Morphine	86 ± 22	600 ± 260	200 ± 40
Naloxone	4 ± 1	30 ± 5	8 ± 2

Values are mean ± standard error from at least three experiments.

TABLE 3

Intrinsic activities of opiate alkaloids and opioid peptides at wild-type, H297N, and H297Q μ receptors

	Intrinsic activity × 10^2		
	Wild-type	H297N	H297Q
	% of full agonist control		
PL017[a]	88 ± 3	78 ± 3	93 ± 3
Morphine[a]	58 ± 6	84 ± 6	83 ± 3
Naloxone[b]	0.2 ± 0.1	6 ± 1	11 ± 3

Values are relative to one of two full agonists, DAMGO [a] or normorphine [b] and are mean ± standard error from at least three experiments. For naloxone, intrinsic activities were determined by the 1 μM naloxone/20 μM normorphine response ratio, not by concentration-response curves.

demonstrated by elimination of the drug-dependent K^+ current in the presence of PTX (Fig. 5B). Moreover, the "antagonist"-mediated channel opening was achieved without prior exposure to normorphine or other opiate agonists (Fig. 5C). The μ-selective peptide antagonist CTOP did not exhibit similar agonist properties at H297N (Fig. 5A) and effectively antagonized normorphine at this receptor.

The continuum of increased "antagonist" intrinsic activity at the H297N μ receptor, extending from naloxone to naltrexone to diprenorphine, also extended beyond diprenorphine to buprenorphine, a documented μ receptor partial agonist (27). The increased intrinsic activity of buprenorphine was quantified at 43 ± 6.4% of the normorphine peak amplitude at the H297N μ receptor compared with 6.8 ± 0.6% at the wild-type receptor (Fig. 5A). Removal of the extremely lipophilic buprenorphine was not complete, as demonstrated by the altered base-line and reduced response (and reduced receptor availability) to a second normorphine application; the second normorphine addition nevertheless confirmed the K^+ channel was still functional (Fig. 5A).

The relatively slow off-rate for the more lipophilic drugs, including buprenorphine, diprenorphine, and CTOP, prevented a conventional assessment of EC_{50} values and drug intrinsic activities at the mutant receptors; such values were obtained for the less lipophilic opiates and opioid peptides that were tested (Tables 2 and 3). For DAMGO, PL017, and naloxone, EC_{50} values (Table 2) largely mirrored the pattern of binding affinity (Table 1) when comparing the H297N and H297Q receptors with the wild-type μ receptor, whereas the EC_{50} value for morphine was several-fold higher when His297 was replaced with asparagine. The intrinsic activities of the peptide agonists DAMGO and PL017 were similar among wild-type, H297N, and H297Q receptors (Table 3). In contrast, the intrinsic activity of all opiate alkaloids tested

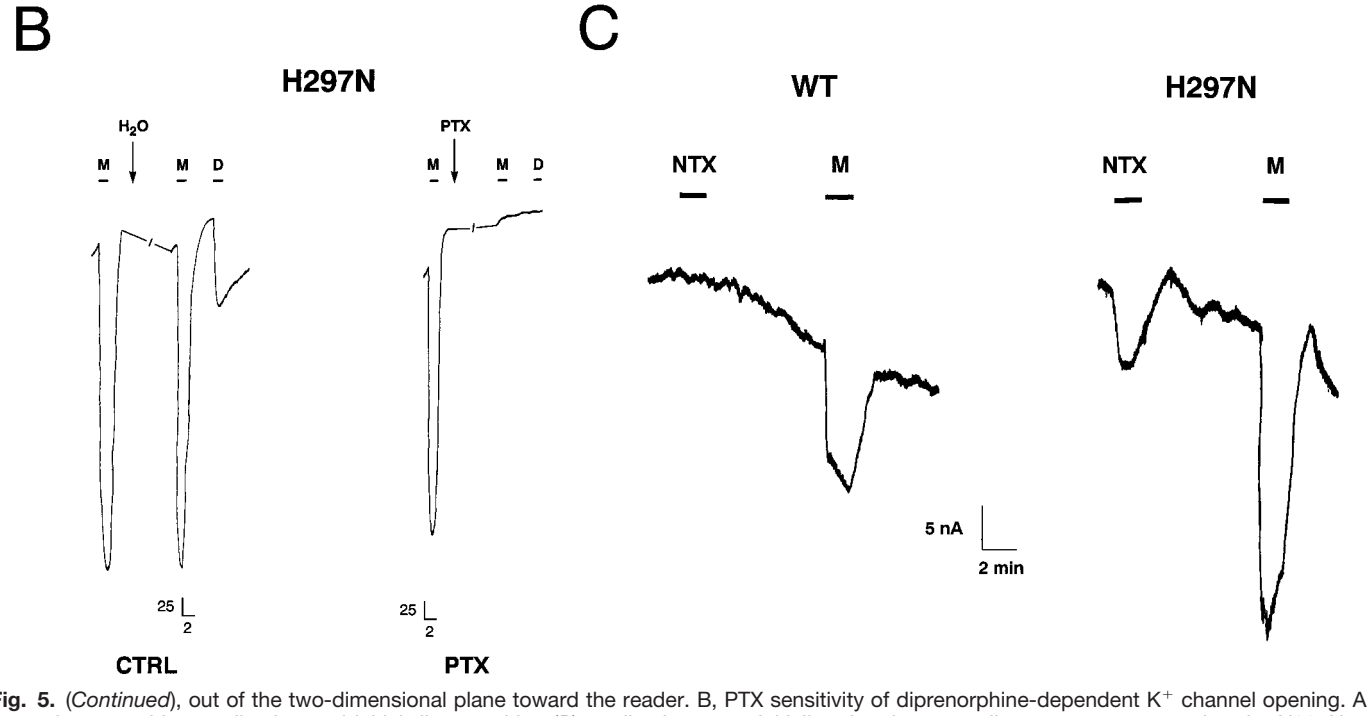

Fig. 5. (*Continued*), out of the two-dimensional plane toward the reader. B, PTX sensitivity of diprenorphine-dependent K^+ channel opening. A second normorphine application and initial diprenorphine (*D*) application to an initially robustly responding oocyte coexpressing the H297N μ receptor and K^+ channel was attempted 45 min after PTX injection (*right tracing*). A control (*CTRL*) oocyte was injected with an equal volume of distilled water and also tested after 45 min. *Horizontal component of scale*, time (in min). *Vertical component*, current amplitude (in nA). C, "Antagonist"-mediated opening of a K^+ channel via the H297N μ receptor. The wild-type (*WT*; *left*) and H297N mutant (*right*) μ receptors were treated with 1 μM naltrexone (*NLX*) as described in the legend to Fig. 3 but without prior normorphine treatment. Naloxone and diprenorphine also selectively activated His297 mutant receptors but not the wild-type receptor in naive oocytes (data not shown). The subsequent normorphine application (*M*) reproducibly yielded a considerably smaller peak for the wild-type receptor compared with H297N. The data reflect a typical result from 10 trials. *Horizontal scale bar*, time (in min). *Vertical scale bar*, current amplitude (in nA).

increased at the mutant receptors (Fig. 5 and Table 3). This effect was most pronounced for the classic antagonist naloxone, for which a 50-fold increase was observed when glutamine was substituted for histidine (Table 3). DAMGO and normorphine displayed full agonism at all three receptors and are not shown in Table 3 because they served as the points of reference for assessing intrinsic activity. Based on the data in Fig. 5A and Table 3, asparagine or glutamine replacement of the wild-type histidine residue at position 297 augmented the agonist properties of all opiate alkaloid drugs tested, allowing traditional antagonists to function as partial agonists while increasing the activity of established partial agonists.

Discussion

The molecular mechanism used by opioid receptors in distinguishing agonists from antagonists has remained elusive for decades. Myriad structural analogs of morphine, meperidine, and other small molecule templates have been synthesized and tested in the search for therapeutically useful antagonists and potent but nonaddictive analgesics. The wave of cDNA clones encoding the μ-, δ-, and κ-opioid receptors isolated within the past 5 years has provided the tools with which to approach structure-function studies of opioid drug/receptor interactions in a new way—from the perspective of the opioid receptor.

Charged and polar amino acid residues encoded by G protein-coupled receptor cDNAs and predicted for the relatively nonpolar transmembrane domains are useful starting points in identifying important residues for receptor signal transduction; there must be a compensatory benefit that outweighs the otherwise energetically unfavorable consequence of introducing a polar residue within the hydrophobic environment of the cell membrane lipid bilayer. The TM 6 histidine residue encoded by rat and human μ-, δ-, and κ-opioid receptor cDNAs represents the sole putative positive charge of the lipid-anchored portion of the receptor. To elaborate on the contribution of the TM 6 histidine side chain to μ receptor ligand binding and G protein-coupled signal transduction, this side chain was replaced with one of nine amino acids that alters charge, ring π electrons, hydrogen bonding potential, or steric bulk. In screening the nine His297 mutant receptors for high affinity binding of the radiolabeled peptide agonists DAMGO and [D-Ala^2,D-Leu^5]enkephalin, only the glutamine (H297Q) and asparagine (H297N) substitutions clearly displayed binding affinities within 1 order of magnitude of the wild-type receptor. The ^{3}H-labeled alkaloid antagonists naloxone and diprenorphine were also bound more efficiently by the glutamine-substituted receptor compared with the other eight mutant receptors, but only by a factor of 2.

The affinity constants (Table 1) obtained for binding of nonradioactive alkaloid opiates and opioid peptides at the H297N and H297Q receptors suggest that although no single property of the histidine side chain accounts for its role, side chain length and hydrogen bonding potential at this position may be critical in retaining ligand binding at or near wild-type levels. The ϵ-nitrogen atom of glutamine and the δ-nitrogen atom of asparagine may mimic roles of ϵ- or δ-nitrogen atoms of the histidine side chain in this respect. The hydrogen bonding potential may be especially important; the leucine substitution mutant (H297L), lacking this potential but essentially isosteric with the asparagine side chain of the H297N receptor, was considerably less effective than H297N in binding certain ligands. Although the aspartic acid and glutamic acid side chains offer δ- and ϵ-oxygen atoms for hydrogen bonding, respectively, their anionic character likely creates a charge repulsion that accounts for their ranking immediately after H297Q and H297N. In keeping with a multifaceted contribution by His297, the hydrophobicity of the substituted amino acids may be significant. In ranking the 20 naturally occurring amino acids by hydrophobicity (28), positions 14–18 are held by histidine, glutamine, lysine, asparagine, and glutamic acid, respectively. Except for lysine, this ranking of mutant receptors also holds for DAMGO binding and DAMGO-mediated K^+ channel opening. The steric hindrance of the longer lysine side chain may override its hydrogen bonding benefits as well as its similar hydrophobicity to histidine. The preference for mid to upper range hydrophilicity at position 297 again suggests that His297 is neither openly exposed to the extracellular space nor sequestered as an immobile, hydrophobic strut. Regardless of which His297 features are required for high affinity ligand binding and proper G protein-coupling function, there is no definitive evidence for direct ligand contact with His297.

The importance of the μ receptor TM 6 His297 residue in maintaining agonist and antagonist binding, coupled with the biochemical confirmation of its proximity to the opioid binding cavity via "footprinting" of opioid ligands with an alkylating agent (Fig. 4), led us to investigate the ligand requirements for mediating G protein-linked signal transduction at His297 mutant receptors. The μ receptor opens voltage-gated potassium channels (26, 29) via direct G protein coupling, an event postulated to contribute significantly to the observed physiological effects of systemically administered opiates (30). Classic opiate alkaloid antagonists mediated opening of a G protein-coupled inwardly rectifying potassium channel in the presence of the H297N (Fig. 5A and Table 3) and H297Q (Table 3) mutant μ receptors in a PTX-sensitive fashion (Fig. 5B), the latter indicating a G_o or G_i protein-linked event. Quantitative analysis of this increase in agonist potential at H297N and H297Q μ receptors revealed that only the opiate alkaloid drugs tested demonstrated increased intrinsic activity, with no increase observed for the peptide agonists DAMGO and PL017 (Table 3). Intriguingly, EC_{50} values for morphine were substantially higher for the mutant receptors (Table 2); this cannot be explained by poorer binding at the mutant receptors (Table 1), as is the case for naloxone. We are surveying the binding affinities and agonist properties of several opiate alkaloids at these three receptors to test whether H297N and H297Q can truly discriminate between opiate ligands at this level.

This "antagonist" activation of a μ-opioid receptor mutated at a residue verified to reside near or in the ligand binding cavity represents a novel observation among opioid receptors. Intriguingly, mutation of a TM 4 serine residue common to the μ-, δ-, and κ-opioid receptors increased the intrinsic activity of both alkaloid and peptide antagonists to that of full agonists for all three receptors (31). The authors ascribe a role for this residue in maintaining intramolecular contacts within each receptor that underpin its globular structure as opposed to the residue being in the vicinity of ligand binding sites; such a role is likely played by a great number of resi-

dues in the protein. It is possible that mutation of His297 yields a similar "global" disruption of intramolecular contacts, such that the position of TM helices other than, or in addition to, TM 6 is altered. Contrary to that scenario, a large body of findings based on studies with chimeric opioid receptors suggests that the vicinity of TM 6 is especially critical for recognition of opiate alkaloid antagonists. Hybrid receptors composed of μ and κ (32), μ and δ (33), and δ and κ (34) polypeptides identified receptor regions critical for high affinity alkaloid antagonist binding to segments encompassing TM 5 through the carboxyl terminus (34), the lower half of TM 5 through the carboxyl terminus (33), or the intracellular region immediately before TM 6 through the carboxyl terminus (32). Interestingly, the latter μ/κ chimera (32) contained the minimum sequence of μ receptor (of the available chimeras assayed in the study) necessary for high affinity binding of the μ-selective irreversible alkaloid antagonist β-funaltrexamine (35), even though the Ring C cross-linking substituent seems to form a covalent bond with a TM 5 lysine residue (Lys233, common to all three opioid receptors) predicted for the extracellular border and preceding the "μ portion" of the chimera (36). Molecular modeling scenarios in which β-funaltrexamine is anchored to the μ receptor at this TM 5 lysine residue place the ligand in the vicinity of the TM 6 His297 side chain.[2] A μ/δ chimeric receptor also displayed high affinity binding of the μ agonists morphine and codeine when only TM 5–7 of the μ receptor amino acid sequence was present (37). These reports are consistent with a role for either His297 or TM 6 in receptor recognition of the key pharmacophores of opiates that determine intrinsic activity of the drug.

The diverse array of Ring C and D functional groups (Fig. 5A) from one opiate to the next renders unlikely a scenario in which His297 uniquely interacts with the combination of pharmacophores for each drug. A perhaps more likely scenario is that mutation of His297 weakens or disrupts an intramolecular contact within the binding cavity that subtly alters the position of TM 6, allowing a Ring C or D pharmacophore to more efficiently trigger the conformational change in the μ receptor, which in turn activates G protein-coupled signal transduction. A second and closely related explanation for the general increase in opiate alkaloid agonism at the H297N and H297Q receptors is that the equilibrium between opiate agonist and antagonist binding sites has been shifted to favor the agonist site. The observation that mixtures of opiate partial agonists or antagonists with full agonists competed for a single site suggested that the opiate agonist and antagonist binding sites overlap. A subtle structural change in the ligand binding cavity induced by mutation of His297 could result in a compensatory subtle shift of the opiate toward the agonist side of the overlapping sites; the mutation would serve to increase the intrinsic activity of the drug by reducing the affinity of the drug more for the antagonist site than for the agonist site.

It remains to be seen whether the role of the μ-opioid receptor TM 6 histidine in governing signal transduction is shared by the δ- and κ-opioid receptors and other members of the GPCR superfamily that possess the analogous histidine residue. The contribution or necessity of this residue for δ or κ receptor activation has not been reported. A TM 6 histidine residue is predicted or confirmed for many 7 TM GPCRs (13), occurring at or within an α-helical turn of the position analogous to His297 in the μ-opioid receptor. Of 285 GPCRs surveyed, histidine was present at this position in ≈30%, almost double that of the next most prevalent side chain (glutamine). Such conservation, when considering the structural diversity of the GPCR ligands, again argues that His297 functions less as a direct ligand contact and more as a contributor in governing signal transduction. Although mutagenesis of the TM 6 histidine in the neurokinin 1 (38), dopamine D_2 (39), and angiotensin type 1 (40) receptors selectively abolished high affinity binding of aromatic antagonists, the effects of these mutations on receptor signal transduction have not been reported. Consistent with our findings for the μ-opioid receptor, glutamine was found to substitute most effectively for histidine in the neurokinin 1 and angiotensin type 1 receptor binding studies (a substitution not addressed in the dopamine D_2 receptor study). A common motif for maintaining the nonpeptide antagonist site is suggested among these receptors and involves the ϵ-nitrogen atom of a TM 6 amino acid side chain. The TM 6 His297 residue of the μ-opioid receptor is additionally required for defining activation properties of opiate alkaloids (Fig. 5A and Table 3) (i.e., in addition to side chain length, other features of the imidazole side chain, which may include a full positive charge, ring π electrons, or similar hydrophobicity, are necessary for the proper intermolecular or intramolecular contact in the ligand binding cavity). It will be interesting to see whether activation of the neurokinin, dopamine, angiotensin, and other opioid receptors is similarly dependent on these requirements.

Further mapping of the interior of opioid drug-μ receptor complexes via site-directed mutagenesis should clarify the boundaries and elucidate the commonalities of agonist and antagonist binding sites. Molecular modeling of diprenorphine, buprenorphine, and related opiate ligands with the μ-opioid receptor in the vicinity of TM 6 may guide the synthesis of drug structure-activity families containing variants of the critical antagonist/partial agonist pharmacophore toward developing drugs superior to buprenorphine in combating physical dependence on abused opiates and mitigating opiate withdrawal symptoms. This work may provide a template for other 7 TM signal transducing receptors that share a similarly positioned histidine residue and facilitate a similar rational design of useful therapeutics.

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