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ACCELERATED COMMUNICATION

The Conserved Aspartate Residue in the Third Putative Transmembrane Domain of the δ-Opioid Receptor Is not the Anionic Counterpart for Cationic Opiate Binding but Is a Constituent of the Receptor Binding Site

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SUMMARY

Opioids are cationic compounds that mediate their biological action through three highly homologous receptors (μ, δ, and κ) known to belong to the G protein-coupled receptor (GPR) family. The third putative transmembrane domain of opioid receptors contains a conserved aspartate residue that is typically found in biogenic amine binding GPRs and is generally believed to form an ion pair with the cationic neurotransmitters. Using site-directed mutagenesis, we investigated the possibility of an identical role for this residue (Asp^{128}) in the mouse δ-opioid receptor. Removal of the carboxylate group via an aspartate-to-alanine mutation did not modify binding affinity of a representative set of opioid compounds, including bremazocine, diprenorphine, naloxone, Tyr-D-Thr-Gly-Phe-Leu-Thr, [D-Ala^2,D-Leu^5]enkephalin, cyclic[D-$penicillamine^2$,D-$penicillamine^5$]enkephalin, deltorphin II, (±)-4-[(a-*R**)-a-[(2*S**,5*R**)-4-allyl-2,5-di-methyl-1-piperazinyl]-3-hydroxybenzyl]-*N*,*N*-diethylbenzamide, and naltrindole. It nevertheless decreased receptor expression level and affected the binding of three agonists ([D-Ala^2, D-Leu^5]enkephalin, Tyr-D-Thr-Gly-Phe-Leu-Thr, and (±)-4-[(a-*R**)-a-[(2*S**,5*R**)-4-allyl-2,5-di-methyl-1-piperazinyl]-3-hydroxybenzyl]-*N*,*N*-diethylbenzamide) when the receptor was under Na^+-induced low affinity state. On the other hand, the aspartate-to-asparagine mutation strongly impaired the binding of all of the above ligands and highlighted differential modes of interaction for alkaloids and peptides. Finally, removal of the homologous carboxylate group in the mouse μ receptor had distinct effects because it dramatically reduced the binding potency of some, but not all, tested ligands. Taken together, these results demonstrate that (i) the direct ligand/receptor interaction previously demonstrated for the β-adrenergic receptor does not take place in the δ receptor, (ii) Asp^{128} nevertheless contributes to stabilization of the spatial conformation of the binding pocket, and (iii) these conclusions cannot be extended to the closely related μ receptor.

Opiates have a dual action on the nervous system by mediating both strong analgesia and euphoria. They act through three classes of membrane receptors (μ, δ, and κ) that display distinct anatomic distributions (1) and pharmacological profiles (2) and are therefore differentially involved in opiate action. Under physiological conditions, opioid receptors interact with a family of structurally related peptides derived from three large precursor proteins known as proopiomelanocortin, preproenkephalin, and prodynorphin (3). Opioid peptides and their receptors form a neuromodulatory system that plays a major role in the control of nociceptive information (4) and rewarding pathways (5). Alternatively, they modulate locomotion, cognition, neuroendocrine physiology, autonomic functions, and immune response (6).

Since the elucidation of morphine structure and the analysis

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ABBREVIATIONS: BW373U86, (±)-4-[(a-*R**)-a-[(2*S**,5*R**)-4-allyl-2,5-di-methyl-1-piperazinyl]-3-hydroxybenzyl]-*N*,*N*-diethylbenzamide; DADLE, [D-Ala^2,D-Leu^5]enkephalin; DAGO, [D-Ala^2,$MePhe^4$,Gly-ol^5]; DTLET, Tyr-D-Thr-Gly-Phe-Leu-Thr; GPR, G protein-coupled receptor; PBS, phosphate-buffered saline; Tm, transmembrane domain; mDOR, mouse δ-opioid receptor; mMOR, mouse μ-opioid receptor; NTI, naltrindole; DOR-IR, δ-opioid receptor immunoreactivity.

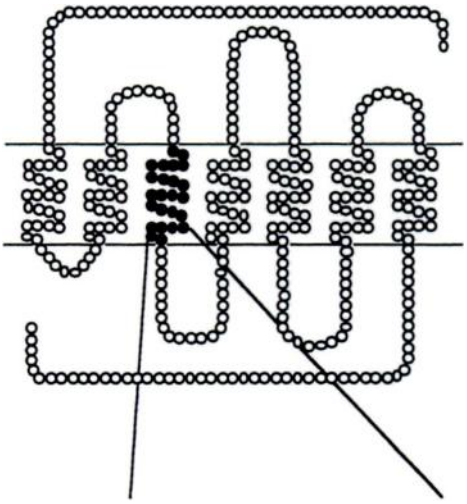
of other naturally occurring morphine-like molecules, with the general term of alkaloids, chemical synthesis has provided a wide diversity of compounds with high affinity for the receptors, variable degree of selectivity toward receptor classes, and agonist, antagonist, or mixed agonist/antagonist activities (7). Also, the molecular characterization of endogenous opioid peptides has allowed synthesis of more stable peptide analogues with increased selectivity, particularly for μ and δ receptors (8). With the use of this vast ligand repertoire, structure/function studies have demonstrated that a basic amine in alkaloids or the protonated amino terminus of opioid peptides is essential for ligand recognition at the receptors. The strict requirement of a positively charged nitrogen atom applies to binding to each receptor class and is considered to be a common property of opioid ligands (7, 9). It has therefore been postulated that a negatively charged amino acid side chain of the receptor may directly interact with the cationic ligands via an electrostatic bound.

The recent cloning of an mDOR cDNA (10, 11) provided the first primary structure of an opioid receptor and demonstrated that this receptor is a member of the GPR superfamily, which exhibits a seven-Tm topology. An aspartic acid residue found in Tm III of the δ receptor (Asp^{128}) attracted our attention because it is fully conserved in GPRs activated by cationic neurotransmitters. It is not found in GPRs that bind endogenous peptides, with the exception of opioid receptors and the closely related somatostatin receptors (Fig. 1). The role of this residue in the binding of biogenic amines has been extensively studied by single amino acid replacement. Displacement or removal of the carboxylate group dramatically affects agonists and antagonists binding in several GPRs (12–20). Strader *et al.* (21) demonstrated that the side chain of this aspartate residue in the β-adrenergic receptor forms an ion pair with the amine group of the ligand. By modifying both the carboxylate group in the receptor and the cationic amine group in the ligand into hydrogen donor/acceptor partners, they restored full receptor/ligand interaction. Thus, there is a great body of evidence that an essential function of this aspartate residue is to interact with amines.

The equivalent aspartate residue in the mDOR therefore appears to be a likely candidate for a direct receptor/ligand interaction, and in the present study our aim was to verify that this residue provides the counterion for the protonated amine of opioid ligands. Unexpectedly, point mutations at position 128 demonstrated that this is not the case, and we present the first evidence that the conserved aspartate residue in Tm III of opioid receptor is not equivalent to that of biogenic amine receptors and does not interact directly with opioid ligands. It nevertheless contributes to the general conformation of the binding site and participates in structural changes underlying high and low affinity states of the receptor. We also demonstrate that this residue is different in its involvement in ligand binding to mMOR.

Experimental Procedures

Materials. Fluorescein-conjugated concanavalin A; avidin D/biotin blocking kit, normal goat serum, biotinylated goat anti-rabbit



	mouse δ-opioid	AVLSIDYYNMFTSIFTLTMMSV
	mouse SSTR1 somatostatin	LVLSVDAVNMFTSIYCLTVLSV
Biogenic amine binding GPRs	human β2 adrenergic	FWTSIDVLCVTASIETLCVIAV
	human α2A adrenergic	IYLALDVLFCTSSIVHLCAISL
	human m1 muscarinic	LWLALDYVASNASVLNLLLISF
	rat 5HTA serotonine	LFIALDVLCCTSSILHLCAIAL
	rat D2 dopamine	IFVTLDVMMCTASILNLCAISI
	human H2 histamine	IYTSLDVMLCTASILNLFMISL
Peptide binding GPRs	rat NK1 tachykinin	FHNFFPIAALFASIYSMTAVAF
	human AT1 angiotensin II	IASASVSFNLYASVFLLTCLSI
	cow ET1 endothelin	LFPFLQKSSVGITVLNLCALSV
	canine CCK-B	AVSYLMGVSVSVSTLSLVAIAL
	rat bradykinin	VVNTMIYMNLYSSICFLMLVSI
	rat neurotensin	GYYFLRDACTYATALNVASLSV

Fig. 1. Schematic representation of the transmembrane topology of mDOR shows comparison of Tm III amino acid sequences of mDOR and other representative GPRs. Sequences of Tm III from several GPRs are aligned. *Reverse text*, aspartate residue conserved in all known biogenic amine receptors.

IgG, and Vectashield mounting media were purchased from Vector; lissamine rhodamine-conjugated streptavidin was obtained from Jackson ImmunoResearch; DTLET, DADLE, cyclic[D-penicillamine^2,D-penicillamine^5]enkephalin, D-Ala^2-deltorphin II, naltrindole, DAGO, and naloxone were obtained from Sigma Chemical Co.; D-Phe-Cys-Tyr-D-Trp-Orn-Pen-Thr-NH_2 and dermorphin were purchased from Peninsula (St. Helens, Merseyside, England, UK); bremazocine and naloxonazine were purchased from Research Biochemicals International (Natick, MA); levorphanol was provided by B. Illien (Faculté de Pharmacie, Strasbourg, France); BW373U86 was kindly provided by K. J. C. Chang (Burroughs Wellcome Co., Research Triangle Park, NC); [^{3}H]DTLET (specific activity, 60 Ci/mmol) was obtained from CEA Saclay (Saclay, France); [^{3}H]NTI (specific activity, 44.5 Ci/mmol) was provided by A. Borsodi (Szeged, Hungary); and [^{3}H]diprenorphine (specific activity, 37 Ci/mmol) and [^{3}H]DAGO (specific activity, 60 Ci/mmol) were obtained from Amersham.

Mutagenesis of mDOR and mMOR. mDOR (11) and mMOR cDNAs (22) were subcloned in *Hin*dIII/*Bam*HI and *Eco*RI sites of the mammalian expression vector pCDNAI/Amp (Invitrogene), respectively. mDOR and mMOR single-stranded DNAs were prepared from the recombinant expression vectors with the use of the helper phage M13KO7 (Promega) and the *Escherichia coli* strain NM 522 (Stratagene) and were used as templates to prepare the site-directed mutants. Sequences of the oligonucleotides were CTCTCCATTGCCTACTACA, CTCTCCATTAACTACTACAAC, and ATCTCAATAGCCTACTACAAC to obtain the mDOR D128A (GAC-to-GCC), mDOR D128N (GAC-to-AAC), and mMOR D147A (GAC-to-GCC) mutations, respectively. Mutagenic oligonucleotides were annealed to the single-stranded templates and elongated with the Klenow fragment and ligase. Heteroduplex plasmid DNA was then used to transform the repair-deficient *E. coli* strain BMH71–18 mutS (Clontech). Transformants were selected by growth on LB plates supplemented with ampicillin (100 μg/ml), and mutations were confirmed by manual DNA sequencing (Sequenase kit; United States Biochemicals) in both directions. Sequences were also confirmed by automated DNA sequencing (373A DNA, Applied Biosystems) with the use of fluorescently labeled nucleotides (Taq Dye Deoxy Terminator Cycle Sequencing Kit, Perkin-Elmer).

Expression of wild-type and mutant opioid receptors in COS cells and ligand binding. COS-1 cells (1.5×10^6 cells/140-mm dish) were transfected with purified plasmids (35 μg/dish) using the DEAE-dextran method. After 72 hr growth in Dulbecco's modified Eagle's medium with 10% fetal calf serum, the cells were harvested, and membranes were prepared as previously described (11). For binding experiments, 20 μg (mDOR), 40 μg (mDOR D128A, mDOR D128N), and 50 μg (mMOR, mMOR D147A) of membrane proteins were diluted in 50 mM Tris·HCl, pH 7.4, and incubated with opioid ligands in a final volume of 0.5 ml. For saturation experiments, variable concentrations were used of [^{3}H]DTLET (0.05–6.4 nM for mDOR/mDOR D128A), [^{3}H]NTI (0.05–10 nM for mDOR/mDOR D128N), and [^{3}H]diprenorphine (0.05–6.4 nM for mDOR/mDOR D128A and for mMOR/mMOR D147A). Incubations were done for 30 min at 37° for [^{3}H]DTLET and 1 hr at 25° for the other radiolabeled ligands. Nonspecific binding was determined in the presence of 2 μM naloxone. For competition studies, membrane preparations were incubated for 1 hr at 25° with [^{3}H]diprenorphine (0.8 nM for mDOR and mDOR D128A and 1 nM for mMOR and mMOR D147A) or [^{3}H]naltrindole (1 nM for mDOR D128N) in the presence of various concentrations of opioid competing ligands. K_d, K_i, and B_{max} values were calculated using the EBDA/Ligand program (G. A. McPherson, Biosoft, Cambridge, UK).

Immunocytochemistry of transfected COS cells. Cells were transfected as above and transferred to two-chamber glass Labtek slides (Nunc) 48 hr after transfection at a density of 10^5 cells/chamber. After 24 hr growth, cells were washed three times with PBS, fixed with 2% paraformaldehyde in PBS for 2 hr at room temperature, washed three times with PBS, and stored at 4° in PBS. Cells were then processed for immunocytochemistry using immunofluorescence. All further steps were performed at room temperature unless otherwise stated. Cells were rinsed in 0.05 M PBS for 5 min, rinsed in 0.05 M PBS for 15 min, incubated in fluorescein-conjugated concanavalin A diluted to 2.5 μg/ml in PBS for 1 hr, rinsed with PBS for 1 hr, and then pretreated as follows: incubated in 7% avidin D in PBS for 20 min, rinsed in PBS for 10 min, incubated in 7% biotin for 20 min, rinsed in PBS for 15 min, and incubated in a blocking buffer consisting of PBS with 2% gelatin and 10% normal goat serum for 1 hr at 37°. Slides were then incubated for 1 hr at room temperature followed by 36-hr incubation at 4° in affinity-purified DT-1 (23) diluted to 3 μg/ml in a diluent consisting of PBS with 0.1% bovine serum albumin, 10% normal goat serum, and 0.1% Triton X-100. Slides were then processed as follows: rinsed with PBS containing 0.1% Triton X-100 for 1 hr, incubated in goat anti-rabbit IgG diluted 1:200 in diluent for 1 hr, incubated in lissamine rhodamine-conjugated streptavidin diluted 1:1000 in PBS containing 0.1% Triton X-100 for 1 hr, rinsed in PBS for 1 hr, rinsed in PBS for 15 min, dipped briefly in distilled water, and coverslipped with Vectashield mounting media. Both fluorochromes were viewed simultaneously with a Bio-Rad confocal microscope with COMOS 6.01 software.

Results

Expression of mDOR and mDOR mutants in COS cells. To assess the role of D128 in ligand binding at the mDOR receptor, we replaced the aspartic acid residue with an alanine (D128A) or an asparagine (D128N) residue. These mutations led to removal or neutralization of the carboxylate group, respectively. The mutant receptors were expressed in COS cells, and their expression was monitored by immunocytochemistry (Fig. 2). Transfected COS cells were double-labeled with concanavalin A to label the plasma membrane and an anti-mDOR antibody (DT-1; Ref. 23). No DOR-IR is detectable on mock-transfected control COS cells, whereas COS cells transfected with the wild-type receptor show DOR-IR in the plasma membrane as well as in the cytoplasm. COS cells transfected with the both mutant D128A and D128N receptors exhibit immunostaining similar to that of the wild-type receptor-expressing cells, suggesting that modification of the aspartate residue does not impair expression of the receptor protein at the cell surface. We quantified expression levels of receptors by measuring [^{3}H]DTLET and [^{3}H]diprenorphine (mDOR and mDOR D128A) or [^{3}H]NTI (mDOR D128N) binding to the mutant receptors (Table 1). Scatchard analysis indicates that the wild-type receptor and the D128N mutant are expressed at comparable levels, whereas expression of mutant D128A is lower.

Effect of the mutation D128A on mDOR pharmacology. Saturation experiments indicate that [^{3}H]DTLET, a δ-selective peptide agonist, and [^{3}H]diprenorphine, a nonselective alkaloid antagonist, bind with identical affinity to both the wild-type and the D128A mutant receptors (Table 1). Thus, removal of the carboxylate group in the receptor binding site does not affect interaction of the receptor with these two structurally and functionally distinct opioid ligands. [^{3}H]diprenorphine was then used as the radiolabeled ligand in competition studies to establish whether the mutation impairs receptor binding of other opioid ligands. We tested a set of representative opioids that belong to both alkaloid and peptide ligand types, exhibit variable degree of selectivity toward the δ receptor, and display agonist or antagonist activity. Again, no significant discrepancy was found in the binding potency of every tested ligand between the

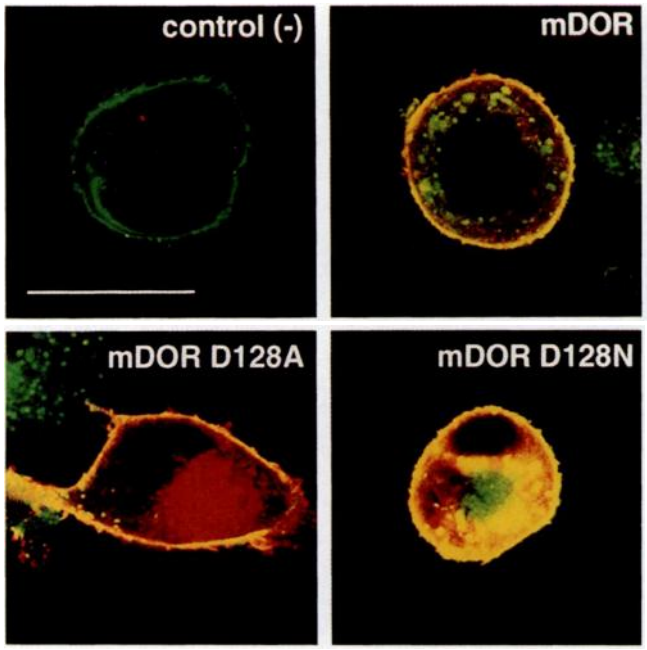


Fig. 2. Expression of mDOR and mDOR mutants in transiently transfected COS cells. To investigate the expression and localization of wild-type and mutants receptors of mDOR, we transfected cells with pCDNAI/Amp [*control (−)*], mDOR, mDOR D128A, and mDOR D128N. Cells were double-labeled with fluorescein-conjugated concanavalin A to label the plasma membrane (*green*) and with an anti-δ-opioid receptor antibody (DT-1; Ref. 32), followed by rhodamine-conjugated streptavidin (*red*). *Yellow*, colocalization. *Control (−)* shows no DOR-IR; COS cells transfected with mDOR show DOR-IR in the plasma membrane as well as in the cytoplasm. COS cells transfected with either mDOR D128A and mDOR D128N exhibit immunostaining similar to that of the wild-type receptor-expressing cells. Shown is immunocytochemistry of a representative cell for each type of transfected cells (*scale bar*, 25 μm).

wild-type and mutant receptors (Table 2). This results strongly suggests that the D128A mutation generally does not affect opioid binding.

Effect of the mutation D128A on agonist binding to mDOR under the Na^+-induced low affinity state. We examined the effect of Na^+ on the binding potency of δ-selective peptide agonists at mDOR and mDOR D128A (Table 3). Increases of 50- and 18-fold in K_i values are found at the wild-type receptor for DTLET and DADLE, respectively. This demonstrates that mDOR transiently expressed in COS cells shifts from high to low affinity state for agonists in the presence of sodium ions, as expected based on other studies (24–26). We also tested BW373U86, a potent δ-selective alkaloid agonist, and found no effect of sodium on the K_i value, which is in accordance with previous studies underscoring a distinct mode of interaction of this agonist with δ receptor compared with classic agonists (27). When examining the binding potency of the three agonists to mDOR D128A in the presence of sodium chloride, we found that K_i values for DTLET, DADLE, and BW373U86 increased by 150-, 2800-, and 8-fold at the mutant receptor (Table 3). These results demonstrate that the tested agonists bind less efficiently to the D128A mutant receptor, as they do to the wild-type receptor when sodium ions are included in the assay. Therefore, unlike what is observed with the receptor in a high affinity state, the carboxylate group of residue Asp^{128} contributes to agonist binding when the receptor is under a low affinity conformation.

Effect of the mutation D128N on mDOR pharmacology. We also examined the effect of a more conservative substitution, namely, aspartate into asparagine. [^{3}H]Diprenorphine binding on mDOR D128N transfected COS cell membranes appeared undetectable. [^{3}H]NTI, however, still bound to the mutant receptor with nanomolar affinity (Table 1) and was used in additional competition experiments (Table 2). The binding potency of δ-selective peptide agonists was dramatically decreased, with enkephalin-derived linear (DTLET and DADLE) and cyclic (cyclic[D-penicillamine^2,D-penicillamine^5]enkephalin) peptides or the frog potent agonist deltorphin II affected equally (K_i >5–7 μM). Alkaloids also appeared to be significantly affected by this mutation, although to a lesser extent. Thus, 50- and 25-fold decreases in binding potency are found for nonselective (naloxone) and δ-selective (BW373U86 and NTI) compounds, respectively. Interestingly, the alkaloid bremazocine behaves differently, displaying a shift in K_i value comparable to that observed for peptides (K_i >5 μM). In conclusion, the mutation of aspartate into asparagine has distinct consequences on ligand binding compared with the mutation into alanine, in that replacement of the carboxylate group by the amide counterpart induces major modifications of the receptor pharmacology .

Effect of the mutation D147A on mMOR pharmacology. The conserved aspartate residue in Tm III of mMOR, found at position 147, was replaced by an alanine residue. The analysis of [^{3}H]diprenorphine binding on COS transfected cell membrane preparations indicates that it binds with identical potency at the wild-type and mutant receptors. As for mDOR D128A, the expression level of the mutant receptor is significantly lower relative to the wild-type receptor because B_{max} values decrease by 8-fold (Table 1). The aspartate-to-alanine mutation at the μ receptor therefore appears to have an effect similar to that of the δ receptor regarding [^{3}H]diprenorphine binding. Further investigation of the pharmacological profile of the mMOR D147A receptor, however, shows diverse effects of the mutation depending on the ligand under study (Table 4). Antagonist binding remained essentially unaffected as affinities of the nonselective ligands naloxone and diprenorphine were not changed and the K_i value for the μ-selective ligand naloxonazine increased only by 2.3-fold. Alkaloid agonists were significantly affected, with 14- and 35-fold increases in K_i values for the μ-selective compound levorphanol and the nonselective ligand bremazocine, respectively. Finally, as was previously observed for the mDOR D128N mutant, a major modification of binding potencies was found for peptidic ligands (>100-fold decrease in affinity). Thus, the synthetic enkephalin-derived (DAGO) and somatostatin-derived (D-Phe-Cys-Tyr-D-Trp-Orn-Pen-Thr-NH_2) peptides, as well as the naturally occurring frog peptide dermorphin, bound to the mMOR D147A receptor with affinities above micromolar values. In this mutation of the μ receptor, we can therefore discriminate alkaloids from peptides, as well as agonists from antagonists. Taken together, these results indicate that replacement of aspartate by alanine in the μ receptor strongly modifies the receptor pharmacology in a manner that is distinct from both the aspartate-to-alanine or the aspartate-to-asparagine mutation in the δ receptor.

TABLE 1

Radioligand binding on mDOR, mMOR, and mutant receptors transiently expressed in COS cells

Saturation experiments were done to evaluate affinity of radiolabeled ligands and binding site levels in transfected COS cell membrane preparations. [^{3}H]Diprenorphine was used to label the wild-type receptors as well as mDOR D128A and mMOR D147A mutant receptors. [^{3}H]DTLET was used to confirm B_{max} values for mDOR and mDOR D128A. For mDOR D128N, [^{3}H]naltrindole was used because diprenorphine binding was undetectable. Nonspecific binding was determined in the presence of 2 μM naloxone. Experiments were done in triplicate, and values are the mean ± standard error of three experiments. n.d. = not determined.

	[^{3}H]Diprenorphine		[^{3}H]DTLET		[^{3}H]Naltrindole	
	K_d	B_{max}	K_d	B_{max}	K_d	B_{max}
	nM	pmol/mg	nM	pmol/mg	nM	pmol/mg
mDOR	0.6 ± 0.06	9.22 ± 2.5	1.05 ± 0.12	8.88 ± 1.75	0.43 ± 0.05	9.67 ± 0.23
mDOR D128A	0.49 ± 0.08	2.34 ± 0.29	1.21 ± 0.09	1.43 ± 0.29	n.d.	n.d.
mDOR D128N	n.d.	n.d.	n.d.	n.d.	2.2 ± 0.53	7.31 ± 0.02
mMOR	1.56 ± 0.08	9.73 ± 1.71	n.d.	n.d.	n.d.	n.d.
mMOR D147A	1.53 ± 0.04	1.16 ± 0.34	n.d.	n.d.	n.d.	n.d.

TABLE 2

Pharmacological profiles of mDOR, mDOR D128A, and mDOR D128N receptors expressed in COS cells

Competition experiments were performed on transfected COS cell membrane preparations with variable concentrations of unlabeled ligands to displace 0.8 nM [^{3}H]diprenorphine (mDOR and mDOR D128A) and 1 nM [^{3}H]naltrindole (mDOR D128N). Experiments were done in duplicate and repeated two or three times for each compound.

Ligand	K_i		
	mDOR	mDOR D128A	mDOR D128N
		nM	
Nonselective alkaloid agonist			
Bremazocine	2.79	3.21	>5000
Nonselective alkaloid antagonist			
Diprenorphine	0.6 (K_d)	0.49 (K_d)	>30 (K_d)
Naloxone	30.6	22.1	1330
δ-Selective peptidic agonist			
DTLET	1.04 (K_d)	1.21	>7000
DADLE	10	13.4	>7000
DPDPE	31.7	29.8	>7000
Deltorphin II	9.23	8.88	>5000
δ-Selective alkaloid agonist			
BW373U86	1.63	1.52	44
δ-Selective alkaloid antagonist			
Naltrindole	0.42	0.51	11.1

TABLE 3

Differential Na^+ effect on agonist binding at mDOR and mDOR D128A mutant receptors

Na^+ was used to induce modification of the receptor conformation from high to low affinity state for agonist binding. Potencies of DTLET, DADLE, and BW373U86 to compete with 1 nM [^{3}H]diprenorphine binding were determined in the absence or presence of 120 mM NaCl. Experiments were repeated twice and in duplicate for each compound. Presented are ratios of K_i values obtained with and without NaCl to emphasize the extent of decrease in affinity.

Agonist	K_i (+NaCl/−NaCl)	
	mDOR	mDOR D128A
DADLE	18	150
DTLET	50	2800
BW373U86	1.3	8

TABLE 4

Pharmacological profile of mMOR and mMOR D147A receptors expressed in COS cells

Binding experiments were performed on transfected COS cell membrane preparations. Competitions with [^{3}H]diprenorphine binding (1 nM) were performed with variable concentrations of unlabeled ligands, and experiments were repeated two or three times in duplicate for each compound.

Ligand	K_i	
	mMOR	mMOR D147A
	nM	
Nonselective alkaloid agonist		
Bremazocine	4.42	153
Nonselective alkaloid antagonist		
Diprenorphine	1.56 (K_d)	1.54 (K_d)
Naloxone	13	20.5
μ-Selective peptidic agonist		
DAGO	42.8	>10,000
Dermorphin	28.4	>1,000
CTOP	10.2	>1,000
μ-Selective alkaloid agonist		
Levorphanol	7.96	111
μ-Selective alkaloid antagonist		
Naloxonazine	36.2	83.3

Discussion

The carboxylate group of Tm III Asp^{128} does not form a salt bridge with cationic opioid ligands. The role of the conserved aspartate residue, present in Tm III of the β-adrenergic receptor, has been elucidated by Strader *et al.* (21). By modifying both the receptor and the ligand, the authors showed full activity of catechol esters and ketones at the mutant D113S receptor, whereas these compounds are totally inactive at the wild-type receptor. This experiment provided a clear demonstration of the existence of a direct interaction between the ligand and the amino acid at position 113 and conferred to the aspartate residue the role of counterion for the amine-containing cationic ligand. By analogy, the same role has been assigned to this residue in other receptors, and the hypothesis of the general existence of a Tm

III aspartate/cationic ligand ion pair in all catecholamine GPR binding sites has been further supported by the observation of impaired ligand binding after modification of the aspartate residue side chain at other receptors. Thus, replacement of the aspartate by an asparagine in α-adrenergic (15), m1 (16) and m2 (19) muscarinic, H1 (18) and H2 (17) histaminergic, and μ-opioid (20) receptors results in severe ligand binding impairment, as does replacement of the aspartate by alanine in H1 (18), m2 (19), or μ (20) receptor. In these latter three studies, substitution by a glutamate residue was also assessed and appeared to be less detrimental to ligand recognition, further strengthening the need for a negative charge in the receptor binding site. Accordingly, the conserved Tm III aspartate residue has become a compulsory docking point for amine ligands in GPR three-dimensional modeling studies (28). In our model of the mouse δ receptor,[1] as well as in another recently reported model of the human δ receptor (29), opioid ligands are easily docked in an energetically favorable position with the amino group of alkaloids or the amino terminus of peptides located 3–4 Å from the Asp^{128} residue. This distance is compatible with the existence of a salt bridge between opioids and the conserved Tm III aspartate residue. Modeling, therefore, also supports the possibility of a ligand/receptor electrostatic interaction for the δ receptor.

Our results from the aspartate-to-alanine mutation shed a new light on the generally accepted dogma. Full maintenance of the pharmacology after removal of the carboxylate group demonstrates that the negative charge is not needed to achieve high affinity binding. Therefore, the direct ligand/receptor interaction demonstrated for catecholamines at the β_2-adrenergic receptor does not take place in the mouse δ receptor. Nevertheless, Asp^{128} may be required in δ receptor function, as discussed below.

Asp^{128} lies in a critical area of the receptor binding site. In contrast to carboxylate elimination, carboxylate replacement by an amide group profoundly modifies the receptor pharmacology. It appears that introduction of the amide group strongly disturbs the local environment at position 128. An explanation for this result might be a slight increase in the size of the side chain at position 128 (increment of 1–1.5 Å) that would prevent ligands from adopting an appropriate orientation in the binding pocket. Alternatively, we replaced a negatively charged side chain that acts as a proton acceptor exclusively by a neutral amide group with dual hydrogen acceptor or donor properties. This newly functionalized side chain may now interact with another constituent of the binding site that is otherwise critical for opioid binding and normally does not interact with Asp^{128}. Misorientation of this residue (which remains to be determined) would then prevent the establishment of a major ligand/receptor interaction. Any of these possible interpretations leads to the conclusion that the aspartate residue is one of the structural elements that form the binding site and that modification of the side chain disrupts the binding pocket conformation.

Structural modification of the receptor at position 128 is more detrimental to peptidic than to alkaloid opioid ligands. All tested ligands display a strong decrease in affinity toward the mutant D128N receptor. One salient feature of this mutation is that the structural modification does not affect opioid ligands equally well. In general, peptides are more affected than alkaloids, suggesting that a major interaction for peptides has been disrupted in the mutant D128N receptor, whereas the mutation-induced structural modification is less critical for the alkaloid compounds naloxone, naltrindole, and BW373U86. This observation is in accordance with recent pharmacological studies of μ/κ (30) and μ/δ (31) chimeric opioid receptors demonstrating different binding domains for peptide and nonpeptide ligands. Our data show that a single point mutation at the receptor is sufficient to discriminate two classes of opioid ligands. The fact that bremazocine binding is distinct from that of other alkaloids tested in this study suggests a specific mode of interaction of this particular nonpeptidic opiate with the receptor.

Asp^{128} moves within the binding site when the receptor shifts from high to low affinity state. Numerous reports have demonstrated that agonist but not antagonist binding to opioid receptors is attenuated in the presence of sodium ions or nonhydrolysable GTP analogues (24–26, 32, 33). This observation suggests the existence of high and low affinity states of the receptor that would be distinguishable only by agonists. A recent study has shown that the same observation applies to the cloned mDOR receptor expressed in Chinese hamster ovary cells (26). Our attempts to induce low affinity state with GppNHp and GTPγS failed, suggesting that allosteric regulation of receptor conformation by nonhydrolysable GTP analogues is not achieved when the receptor is transiently expressed in COS cells (data not shown). In contrast, we show that sodium ions efficiently induce a change of mDOR from a high to a low affinity state in this expression system. Our investigation of Na^+-induced low affinity receptor pharmacology demonstrates that the aspartate-to-alanine mutation is detrimental to agonist binding, and this result is different from that obtained with the receptor under the high affinity state. The aspartate residue therefore is not equivalent in the two receptor conformations. Consequently, Asp^{128} may exist under different orientations within the binding pocket, depending on the receptor conformation.

The conserved aspartate in Tm III is not equivalent in δ- and μ-opioid receptors. The absence of modification of the δ receptor pharmacology after the aspartate-to-alanine mutation, in contrast to results on catecholamine receptors, prompted us to examine whether the same mutation in the μ receptor would lead to the same observation. The mMOR D147A mutant behaves differently than both the mDOR D128A and mDOR D128N mutants. Our analysis of the role of the Tm III conserved aspartate residue in the μ receptor leads to two observations: (i) the aspartate residue is functionally comparable in the δ and μ receptors with regard to recognition of nonselective alkaloid antagonists as removal of the carboxylate group does not impair their binding; and (ii) the aspartate residue is not equally implicated in ligand recognition as far as subtype-selective ligands are concerned, as opioid peptide binding is dramatically impaired in mMOR D147A mutant yet unaffected in mDOR D128A mutant. This particular class of compounds therefore highlights a receptor subtype-specific local environment for this residue. We conclude that the Tm III aspartate amino acid residue is not equivalent in the two opioid receptor types despite their strong sequence homology.

[1] B. Maigret, unpublished observations.

Where is the receptor anionic counterpart for cationic opioid ligands? Our finding that the conserved Tm III aspartate residue is not the negatively charged counterpart of opioid ligands raises the question of which amino acid residue of the receptor is the likely partner for direct interaction with the positively charged part of opioid ligands. Kong *et al.* (34) investigated the role of the Tm II Asp^{95}, which is the only other negatively charged residue located in putative α-helices of the δ receptor. In their study, the replacement of aspartate by asparagine resulted in reduced binding for δ-selective peptide agonists but, interestingly, no change in affinity for some alkaloid molecules. Their results combined with the results of our study strengthen the hypothesis that there is no universal negatively charged attachment point for opioid peptides in the δ receptor binding pocket. Other possible anionic determinants might then be aspartate or glutamic residues found in the extracellular loops of the receptor. Alternatively, the amine group of opioid ligands may be stabilized by nonionic interactions. An interesting observation comes from the analysis of the recently solved three-dimensional structure of acetylcholinesterase, an enzyme that binds acetylcholine, a small positively charged amine neurotransmitter. In this study, the authors suggested the existence of a "nonclassic" anionic site, in which the chelation of quaternary ammoniums is mainly achieved through multiple aromatic interactions (35). Such amine/aromatic interactions between a ligand and its binding site have also been described for the phosphotyrosine recognition domain SH2 of the v-*src* oncogene, whose crystal structure has been obtained (36), or suggested to occur in the neurokinin-1 receptor after site-directed mutagenesis studies (37). Similarly, nonionic interactions may take place in the δ-opioid receptor binding site and constitute the predominant interaction network in the formation of a stable ligand/receptor complex.

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