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K303^{6.58} in the μ opioid (MOP) receptor is important in conferring selectivity for covalent binding of β -funaltrexamine (β -FNA)

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Abstract

β -funaltrexamine (β -FNA) is an irreversible μ opioid (MOP) receptor antagonist and a reversible agonist of κ opioid (KOP) receptor. β -FNA binds covalently to the MOP receptor at Lys233^{5.39}, which is conserved among opioid receptors. Molecular docking of β -FNA showed that K303^{6.58} in the MOP receptor and E297^{6.58} in the KOP receptor played distinct roles in positioning β -FNA. K303^{6.58}E MOP receptor and E297^{6.58}K KOP receptor mutants were generated. The mutations did not affect β -FNA affinity or efficacy. K303^{6.58}E mutation in the MOP receptor greatly reduced covalent binding of [³H] β -FNA; however, E297^{6.58}K did not enable the KOP receptor to bind irreversibly to β -FNA. Molecular modeling demonstrated that the ϵ -amino group of K303^{6.58} in the MOP receptor interacted with C=O of the acetate group of β -FNA to facilitate covalent bond formation with Lys233^{5.39}. Replacement of K303^{6.58} with Glu in the MOP receptor resulted in repulsion between the COOH of Glu and the C=O of β -FNA and increased the distance between K233^{5.39} and the fumarate group, making it impossible for covalent bond formation. These findings will be helpful for design of selective non-peptide MOP receptor antagonists.

Keywords

β -funaltrexamine; irreversible binding; mu opioid receptor; kappa opioid receptor

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1. Introduction

Opioid receptors mediate effects of opioid drugs and endogenous ligands. Activation of opioid receptors produces many effects, most notably analgesia, mood changes and respiratory depression. There are three types of opioid receptors, μ , δ and κ (MOP, DOP and KOP receptors, respectively). They belong to the rhodopsin family of the seven-transmembrane receptors (7TMRs) and they are coupled to Gi/Go proteins.

β -FNA, fumarate methyl ester of naltrexamine (see Fig. 1 for structure), was synthesized by (Portoghese et al., 1980). It was found to be an irreversible antagonist of the MOP receptor and a reversible agonist of the KOP receptor both *in vitro* and *in vivo* [reviewed by (Takemori and Portoghese, 1985)].

We have previously reported that β -FNA is covalently attached to K233^{5.39} at the extracellular end of the 5th transmembrane segment (TM5) in the MOP receptor (Chen et al., 1996), which was confirmed recently in the crystal structure of β -FNA-bound MOP receptor (Manglik et al., 2012). However, this residue is conserved between the MOP and KOP receptors and, thus, this residue alone cannot account for the differential activities of β -FNA at MOP and KOP receptors. In the crystal structure of the MOP receptor- β -FNA complex, β -FNA interacts with eight other residues: D147^{3.32}, Y148^{3.33}, M151^{3.36}, W293^{6.48}, I296^{6.51}, H297^{6.52}, V300^{6.55} and Y326^{7.43} (Manglik et al., 2012). Of these eight residues, seven are identical between MOP and KOP receptors, with the only difference at position 6.55, which is a Val in the MOP receptor and an Ile in the KOP receptor. Since the residues interacting with β -FNA are highly conserved, the selective covalent binding of β -FNA to the MOP receptor must be attributed to other residues in the MOP receptor.

We reported previously that β -FNA bound irreversibly to a chimeric KOP receptor/MOP receptor construct containing the N-terminus (NT) to TM5 from the KOP receptor and then the third intracellular loop (IL3) to the C-terminus (CT) from the MOP receptor, despite that β -FNA did not bind irreversibly to the KOP receptor. Conversely, β -FNA did not bind irreversibly to the reciprocal MOP receptor/KOP receptor construct with NT to TM5 from the MOP receptor and then IL3 to CT from the KOP receptor (Chen et al., 1995). These results suggested that some unique element(s) in the region from IL3 to CT of the MOP receptor plays an important role for K233^{5.39} to form a covalent bond with β -FNA.

To further our understanding of the structural basis of differential binding of β -FNA to MOP receptor and KOP receptor, in this study, we examined the impact of a divergent residue in this region. Using the recently published crystal structures of the MOP receptor and KOP receptor (Manglik et al., 2012; Wu et al., 2012), we performed comparative molecular docking of β -FNA in the two receptors. Based on the models, we hypothesize that divergent residues with opposite charges at position 6.58 (K303^{6.58} in MOP receptor and E297^{6.58} in KOP receptor) at the extracellular end of TM6 play distinct roles in positioning the methyl acetate group of β -FNA, resulting in the differential binding of β -FNA to these two receptors. We tested the hypothesis by generating two mutants by mutating Lys303^{6.58} in the MOP receptor to Glu [MOP K303^{6.58}E] and substituting Glu297^{6.58} in the KOP receptor

with Lys [KOP E297^{6.58}K] and examining effects of the mutations on irreversible binding of β -FNA.

2. Materials and Methods

2.1. Materials

β -FNA, [3 H] β -FNA, naloxone, and U50,488H, were provided by the National Institute on Drug Abuse (Bethesda, MD). [35 S]guanosine 5'-O-[γ -thio]triphosphate (GTP γ S) (1250 Ci/mmol), [15, 16- 3 H]diprenorphine ([3 H]DIP) (36–50 Ci/mmol) were purchased from PerkinElmer Life Sciences (Boston, MA); polyethyleneimine, ampicillin, leupeptin hydrochloride, phenylmethylsulfonyl fluoride (PMSF), GDP and GTP γ S were purchased from Sigma-Aldrich (St. Louis, MO). Geneticin (G418) was purchased from Cellgro Mediatech, Inc. (Herndon, VA). Lipofectamine 2000 and BamHI were purchased from Invitrogen (Carlsbad, CA). XhoI and dNTPs were purchased from Promega (Madison, WI). Minimal essential medium (MEM), trypsin and penicillin/streptomycin were purchased from Gibco Life Technologies (Grand Island, NY). Bicinchoninic acid (BCA) assay reagents were purchased from Thermo Fisher Scientific, Inc. (Rockford, IL). The following reagents were purchased from the indicated companies: GF/B glass filters, Brandel, Inc. (Gaithersburg, MD); PFU DNA polymerase, Agilent Technologies (Santa Rosa, CA); EcoRI, Roche (Indianapolis, IN).

2.2. Purification of [3 H] β -FNA

[3 H] β -FNA was purified using high-performance liquid chromatography (HPLC) because of high levels of nonspecific binding of [3 H] β -FNA originally obtained from NIDA. [3 H] β -FNA in methanol was dried under nitrogen flux. The dried [3 H] β -FNA was dissolved in water containing 0.1% trifluoroacetic acid (TFA) and loaded into a C18 reverse-phase column attached to a Hewlett-Packard HPLC system. The mobile phase was a linear gradient of solvent A (0.1% TFA in water) to solvent B (0.1% TFA in acetonitrile). Unlabeled β -FNA was used as an external standard to quantify purified [3 H] β -FNA.

2.3. Numbering schemes of opioid receptors

The numbering schemes used identify amino acid residues in 7TMRs by both their sequence numbers and a generic numbering scheme (Ballesteros and Weinstein, 1995), which has been applied to opioid receptors [for example, (Huang et al., 2001; Manglik et al., 2012)]. This combined scheme facilitates comparison across different receptors. In the generic numbering scheme, amino acid residues in TMs are assigned two numbers (N1.N2). N1 refers to the TM number. For N2, the numbering is relative to the most conserved residue in each TM, which is assigned 50, with numbers decreasing towards the N-terminus and increasing toward the C-terminus. The most conserved residues in the TM6 of the rat MOP receptor and human KOP receptor are Pro295 and Pro289, which are referred to as P295^{6.50} and P289^{6.50}, respectively. K303 in the MOP receptor and E297 in the KOP receptor are referred to as K303^{6.58} and E297^{6.58}, respectively.

2.4. Generation of mutants

Mutants were generated using the overlap polymerase chain reaction (PCR) method (Higuchi et al., 1988). The HA-tagged rat MOP receptor and the FLAG-tagged human KOP receptor, both in the mammalian expression vector pcDNA3 (Xu et al., 2000), were used as the templates for PCR using the following primers:

RMOR_K303 ^{6.58} E_Forward	CTA	CGT	CAT	CAT	CGA	AGC	GCT	G
RMOR_K303 ^{6.58} E_Reverse	CAG	CGC	TTC	GAT	GAT	GAC	GTA	G
RMOR_BamHI_Forward	CTG	CAA	CTG	GAT	CCT	CTC	TTC	TG
HKOR_EcoRI_Forward	TTG	ATG	AAT	TCC	TGG	CCT	TT	
HKOR_E297 ^{6.58} K_Forward	TCA	TCC	TGG	TGA	AGG	CTC	TG	
HKOR_E297 ^{6.58} K_Reverse	CAG	AGC	CTT	CAC	CAG	GAT	GA	

The MOP receptor and KOP receptor PCR overlap products were cloned into BamHI/XhoI sites and EcoRI/XhoI sites, respectively of pcDNA3 vector. DNA sequences were determined to confirm desired mutations and ensure no wanted mutations.

2.5. Establishing cell lines stably expressing each construct

Each of the wildtype and mutant KOP receptor and MOP receptor was transfected into Neuro2A mouse neuroblastoma (N2A) cells and grown in medium containing 0.5 mg/ml G418 (geneticin). Clonal cells stably expressing each of the constructs were established as described previously (Chen et al., 1995). Cells were grown in MEM supplemented with 10% fetal bovine serum, 0.2 mg/ml geneticin, 100 units/ml penicillin, and 100 µg/ml streptomycin in a humidified atmosphere consisting of 5% CO₂ and 95% air at 37°C.

2.6

Cell membrane preparation was done according to a modification of our published of (Wang et al., 2005). Cells were harvested in PBS containing 0.5 mM EDTA and centrifuged at 500 g for 3 min. The cell pellet was suspended in lysis buffer (25 mM Tris-HCl, pH 7.4, 1 mM EDTA and 0.1 mM PMSF), passed through a 26 3/8-gauge needle 10 times and then centrifuged at 46,000 g for 30 min. The pellet was rinsed twice with lysis buffer and resuspended in 50 mM Tris-HCl buffer/0.32 M sucrose (pH 7.4), aliquoted and frozen, and stored at -80°C. All procedures were performed at 4°C. Protein concentration was determined with the BCA assay.

2.7

Saturation binding of [³H]DIP was performed with 50 µg membrane protein per reaction and 11 concentrations of [³H]DIP (from 0.025 to 2nM). Binding was performed in duplicate in 50 mM Tris-HCl buffer containing 1 mM EGTA (pH 7.4) in a final volume of 1 ml. Nonspecific binding was defined with naloxone (10 µM). The reaction mixture was incubated for 1 h at room temperature and terminated by filtration under reduced pressure onto GF/B filters presoaked in 0.1 mg/ml BSA and 0.2% polyethyleneimine. Filters were

washed three times with ice-cold 50 mM Tris-HCl buffer containing 0.15 M NaCl (pH 7.4). Radioactivity on filters was determined by liquid scintillation counting.

2.8. Inhibition of [^3H]DIP binding to receptors by β -FNA

The binding affinity of β -FNA to rMOP receptor and hKOP receptor (wildtype and mutant) was determined by inhibition of [^3H]DIP (~ 0.4 nM) binding to the receptors (Wang et al., 2005) in N2A membranes. Binding was performed using 9 concentrations (0.01 nM – 10 μM) of β -FNA. The reaction was performed in 50 mM Tris-HCl buffer containing 1 mM EGTA and 0.1% (w/v) BSA (pH 7.4) at room temperature for 1 h in duplicate in a volume of 1 ml with 25–50 μg of membrane proteins. Definition of nonspecific binding, filtration of reaction mixture and determination of radioactivities in filters were performed as described above.

2.9

[^{35}S]GTP γ S binding was performed according to our published procedures (Zhu et al., 1997; Wang et al., 2005). Briefly, membranes (containing ~ 5 μg protein) were incubated with 5–7.5 μM GDP and ~ 0.1 nM [^{35}S]GTP γ S in 0.5 ml (final volume) of 50 mM HEPES, 100 mM NaCl, 5 mM MgCl₂, 1 mM EDTA for 1 h at 30°C. Six concentrations of β -FNA ranging from 0.1 nM – 10 μM were used. Nonspecific binding was defined by 1 μM unlabeled GTP γ S. The reaction was terminated by filtration onto GF/B filters to separate bound and free [^{35}S]GTP γ S and filters were washed three times. Radioactivities in filters were determined by liquid scintillation counting.

2.10

Reversible and Irreversible [^3H] β -FNA binding was performed as described (Chen et al., 1995; Chen et al., 1996) Total (irreversible + reversible) binding and irreversible binding were performed side-by-side with the same amount of proteins per tube for calculation of reversible binding. Briefly, membranes were incubated with 1.5–10 nM [^3H] β -FNA for 75 min at 37°C in TEL buffer (50 mM Tris-HCl, 1 mM EGTA, 4 μM leupeptin, pH 7.4) plus 200 mM NaCl and 1 mM PMSF. Definition of nonspecific binding, filtration of reaction mixture and determination of radioactivities in filters were performed as described above.

The irreversible binding reaction was terminated by placing tubes on ice and adding ice-cold trichloroacetic acid (TCA) to 10% to disrupt any non-covalent bonds. The tubes were then spun at $2000 \times g$ for 30 min at 4°C, the supernatant was aspirated, and 10% ice-cold TCA was added to the pellet and sonicated briefly. This reaction was then placed on ice for 20 min before filtration onto presoaked GF/B filters. The washing buffer and filter buffer were both 1% TCA. Radioactivities in filters were determined by liquid scintillation counting.

2.11

Analysis of radioligand binding and [^{35}S]GTP γ S binding data was performed using the Prism 6.0 program of GraphPad Co. (San Diego, CA).

3. Results

3.1. Mutations in the KOP receptor and MOP receptor did not alter K_d values of [^3H]DIP

The first step in characterization of the mutant KOP receptor and MOP receptor was to determine binding affinity of [^3H]DIP, a nonselective opioid antagonist, by saturation binding. The results are shown in Fig. 2A. The mutations did not alter the affinity of [^3H]DIP for the receptors except E297^{6.58}K KOP receptor, which show a slight decrease in affinity. Thus, the mutations do not appreciably change the binding pocket. Additionally, clonal cell lines with similar B_{max} values were chosen for the experiments. The wildtype KOP receptor, E297^{6.58}K KOP receptor and K303^{6.58}E MOP receptor have similar B_{max} values, at ~500–900 fmol/mg protein. The wildtype MOP receptor had a higher B_{max} value (~1500 fmol/mg protein). The data are summarized in Table 1.

3.2. Mutations in the KOP receptor and MOP receptor did not alter the IC_{50} value of β -FNA

Inhibition of [^3H]DIP binding by different concentrations of unlabeled β -FNA was performed (Fig. 2B). β -FNA was able to fully compete with [^3H]DIP for binding to wildtype and mutant KOP and MOP receptors with similar affinities. For all receptors, the mean IC_{50} values were between 4–10 nM, as detailed in Table 1.

3.3. K303^{6.58}E mutation in the MOP receptor greatly reduced the irreversible binding of [^3H] β -FNA

Total (reversible plus irreversible) [^3H] β -FNA binding to the four constructs was performed alongside irreversible [^3H] β -FNA binding at concentrations ranging from 1.5–10 nM. The results are shown in Figs. 3 and 4 and summarized in Table 1. Nonspecific binding (combined reversible and irreversible binding) of [^3H] β -FNA in MOP receptor assays ranged from 6.6% of total binding at 1.6 nM to 16.4% at 10.1 nM. Normally, β -FNA binds reversibly to the KOP receptor, and both irreversibly (covalently) and reversibly to the MOP receptor. In the wildtype MOP receptor, ~60% of the total [^3H] β -FNA binding to the receptor was irreversible binding (Fig. 5). However, in the K303^{6.58}E MOP receptor mutant, only 20% of [^3H] β -FNA bound irreversibly (Fig. 5) and the reversible binding has lower affinity. At 10 nM [^3H] β -FNA, specific irreversible binding to the MOP receptor and K303^{6.58}E MOP receptor was about 3700 and 500 dpm/0.1 mg protein, respectively. [^3H] β -FNA did not bind irreversibly to E297^{6.58}K KOP receptor, similar to wildtype KOP receptor, (Fig. 3 and Fig. 5)

3.4. Mutations in the KOP and MOP receptors did not change the efficacy of β -FNA in the [^{35}S]GTP γ S assay

β -FNA is an antagonist at the MOP receptor and an agonist at the KOP receptor (Takemori and Portoghese, 1985). In order to determine if the mutations changed the efficacy of β -FNA for KOP and MOP receptors, we performed [^{35}S]GTP γ S binding at all four constructs. The results are shown in Fig. 6 and summarized in Table 1. β -FNA up to 10 μM did not activate either the wildtype or mutant MOP receptor, whereas DAMGO at 10 μM increased [^{35}S]GTP γ S binding to 206% and 190% of the basal level, respectively (data not shown). At the KOP receptor, β -FNA enhanced [^{35}S]GTP γ S binding in a dose-dependent manner and

the EC₅₀ and E_{max} for [35S]GTPγS binding was not altered by the E297^{6.58}K mutation. The mean EC₅₀ values for the wildtype and mutant KOP receptors were about 6 and 9 nM, respectively. The E_{max} values were the same for both receptors (Table 1), which were approximately 64% of that of the selective prototypic KOP receptor agonist U50,488H. Therefore, the mutations have no effect on efficacy of β-FNA at the KOP or MOP receptor.

4. Discussion

We found that mutation of K303^{6.58} to Glu at the extracellular end of TM6 of the MOP receptor greatly reduced irreversible binding of β-FNA, indicating that K303^{6.58} is critical for the binding. Since it has been shown that β-FNA forms a covalent bond with K233^{5.39} at the extracellular end of the TM5 in the MOP receptor (Chen et al., 1996; Manglik et al., 2012), this result supports our hypothesis that K303^{6.58} of the MOP receptor helps to position β-FNA for covalent bond formation with K233^{5.39}.

Fig. 7A shows the X-ray crystal structure of MOP receptor with β-FNA docked into the binding site (Manglik et al., 2012). The ε-amino group of K303^{6.58} is only 4 Å away from the oxygen of the C=O of the acetate group of β-FNA, and the favorable attraction between these two groups would facilitate the covalent bond formation between the ε-amino group of K233^{5.39} and the fumarate group of β-FNA. When the positively charged K303^{6.58} was mutated to negatively charged Glu, the carboxyl group of Glu repels the C=O of the acetate group of β-FNA and increases the distance between the ε-amino group of K233^{5.39} and the fumarate group, making it difficult for the covalent bond to form (Fig. 7B). This is the first report of the involvement of K303^{6.58} in the MOP receptor being important for covalent bond formation between β-FNA and the MOP receptor. Thus, in the context of our previous finding that showed substitution of the region of the MOP receptor from IL3 to the C-terminus with that of the KOP receptor abolished irreversible binding of β-FNA (Chen et al., 1995), here we specifically showed that such abolishment is likely due to a negative charge at position 6.58, i.e., E297 of KOP receptor within this region.

The position 6.58 is divergent in the opioid receptors: K303 in MOP receptor, E297 in KOP receptor and W285 in DOP receptor. β-FNA forms wash-resistant binding to the DOP receptor at much higher concentrations than those required for the MOP receptor (Tam and Liu-Chen, 1986). K233^{5.39}, the residue in the MOP receptor that forms a covalent bond with β-FNA, and the eight other residues in the MOP receptor that interact with β-FNA (Manglik et al., 2012) are conserved between MOP and DOP receptors. Therefore, the difference at the 6.58 is likely to contribute to this differential binding of β-FNA to the DOP receptor. Since W285^{6.58} is a neutral residue, it does not interact or repel the C=O group of β-FNA. Therefore, β-FNA binds irreversibly to the DOP receptor, but much less strongly than to the MOP receptor.

The 6.58 residues contribute to ligand binding selectivity of the MOP and KOP receptors. E297^{6.58}K substitution in the KOP receptor greatly reduced affinity of the selective KOP receptor antagonist nor-binaltorphimine (norBNI); conversely, K303^{6.58}E mutation in the MOP receptor profoundly enhanced norBNI binding (Hjorth et al., 1995; Jones et al., 1998; Larson et al., 2000). Molecular modeling showed that the carboxyl group of the E297^{6.58}

interacted with N¹⁷ group of norBNI to enhance affinity of norBNI for the KOP receptor and hence contributed significantly the selectivity of norBNI for the KOP receptor over the MOP receptor (Larson et al., 2000). In addition, for the MOP receptor, K303^{6.58} is among the four amino acid residues found to be critical for DAMGO binding, and is likely contribute to the selectivity of DAMGO for the MOP receptor (Seki et al., 1998).

K303^{6.58}E mutation in the MOP receptor greatly reduced β -FNA irreversible binding, but in the KOP receptor, E297^{6.58}K mutation did not enable the KOP receptor to bind β -FNA irreversibly. A KOP receptor mutant containing the MOP receptor sequence from IL3 to the C-terminus bound β -FNA irreversibly (Chen et al., 1995). Thus, additional residues from this region are involved in allowing positioning of β -FNA for covalent bond formation, which requires further investigation. Along the same line, for DAMGO, Seki and colleagues (Seki et al., 1998) reported that a KOP receptor mutant containing four substitutions [E297K + S310V + Y312W + Y313H], in which Glu297, Ser310, Tyr312 and Tyr313 of the KOP receptor were changed to the corresponding residues in the MOP receptor, Lys, Val, Trp and His, respectively, bound to DAMGO with high affinity and mediated DAMGO-induced inhibition on adenylyl cyclase.

Our finding has important implications for design of selective MOP receptor antagonists. Currently, there is no selective reversible non-peptide MOP receptor antagonist. Two selective MOP receptor antagonists are peptides: [D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH₂ with a disulfide bridge at Cys²-Pen⁷] (CTAP) and [D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH₂ with a disulfide bridge at Cys²-Pen⁷] (CTOP). The non-peptide antagonists naloxone, naltrexone and diprenorphine are not selective for the three opioid receptors. Our study indicates that the abolishment of the covalent attachment did not affect the antagonist property of β -FNA for MOP receptor. As such abolishment is achieved by the mutation of K303^{6.58}, this position may be useful in drug design as a point of interaction for selective epoxymorphinan MOP receptor antagonists that do not form covalent bonds with the receptor.

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Abbreviations

7TMRs	seven-transmembrane receptors
β-FNA	β -funaltrexamine
CT	the C-terminus
DIP	diprenorphine
DOP receptor	δ opioid receptor
FBS	fetal bovine serum
IL3	the third intracellular loop

KOP receptor	κ opioid receptor
MOP receptor	μ opioid receptor
N2A cells	Neuro2A mouse neuroblastoma cells
norBNI	nor-binaltorphimine
NT	N-terminus
PCR	polymerase chain reaction
TFA	trifluoroacetic acid
TM5	5 th transmembrane segment

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Fig. 1.
Chemical structure of β -FNA.

Fig. 2. K303^{6.58}E mutation in the MOP receptor and E297^{6.58}K mutation in the KOP receptor did not change affinity of [³H]diprenorphine and β -FNA

(A) ([³H]DIP) saturation binding to wild type and mutant MOP and KOP receptors.

Saturation binding of [³H]DIP, a nonselective opioid antagonist, to the receptors was performed using 50 μ g of membrane prepared from stably transfected N2A cells.

Nonspecific binding was defined using 10 μ M naloxone. Shown is a representative saturation curve for each receptor. The mutations did not change [³H]DIP affinity for either KOP or MOP receptor mutant compared to the wild type. K_d and B_{max} values are shown in Table 1. **(B) Inhibition of [³H]DIP binding to the wild type and mutated receptors by unlabeled β -FNA.** Inhibition of [³H]DIP (~0.4 nM) binding by β -FNA was performed using 25–50 μ g of membrane prepared from stably transfected N2A cells and varying concentrations (0.01 nM – 10 μ M) of β -FNA. Nonspecific binding was defined using 10 μ M naloxone. The data shown represent the mean $\pm$ S.E.M. of three independent experiments performed in duplicate. IC₅₀ values were determined and are shown in Table 1. Mutations in the MOP and KOP receptors did not affect β -FNA affinity for the receptors.

Irreversible binding

Fig. 3. Irreversible binding of $[^3\text{H}]\beta\text{-FNA}$ to wild type and mutant MOP and KOP receptors
Irreversible binding of $[^3\text{H}]\beta\text{-FNA}$ was performed with various concentrations of $[^3\text{H}]\beta\text{-FNA}$ and 100 μg of membranes prepared from stably transfected N2A cells at 37°C for 75 min. Following incubation, TCA was added to a final concentration of 10% to dissociate non-covalently bound $[^3\text{H}]\beta\text{-FNA}$, centrifuged and resuspended in 10% TCA and samples were filtered over GF/B filters. The data shown are the total, nonspecific and specific irreversible binding from one of three experiments performed at each receptor. Wild type MOP receptor shows a high level of irreversible binding while K303^{6.58}E MOP receptor shows a reduced level of irreversible binding. Wild type KOPR and E297^{6.58}K KOPR do not show any irreversible binding. Note the difference in the Y-axis scale of the MOP receptor figure. The percentage of irreversible binding as compared to total binding is shown in Table 1.

Reversible binding

Fig. 4. Reversible binding of $[^3\text{H}]\beta\text{-FNA}$ to wild type and mutant MOP and KOP receptors
Total (reversible plus irreversible) binding of $[^3\text{H}]\beta\text{-FNA}$ was performed alongside irreversible binding on 100 μg of membranes prepared from stably transfected N2A cells. Reversible binding was calculated by subtracting the irreversible binding from the total binding. Representative data from one of three experiments on each construct is shown. All 4 receptors bind $[^3\text{H}]\beta\text{-FNA}$ with similar affinity, with K_d values shown in Table 1.

Specific binding

Fig. 5. Specific binding of $[^3\text{H}]\beta\text{-FNA}$ to wild type and mutant MOP and KOP receptors
Data from Fig. 3 and Fig. 4 are replotted to show only specific binding [Total (reversible + irreversible), reversible, irreversible]. Wild type MOP receptor shows an appreciable level of irreversible binding, with the K303^{6.58}E MOP receptor mutant showing a diminished level of irreversible binding. Wild type and mutant E297^{6.58}K KOPR do not show any irreversible binding. Representative data from one of three experiments on each receptor is shown.

Fig. 6. E297^{6.58}K mutation in the KOP receptor and K303^{6.58}E mutation in the MOP receptor did not change the efficacy of β-FNA

Agonist activity was assessed using [³⁵S]GTPγS (0.08–0.1 nM) binding with different concentrations of β-FNA, 5 μM GDP and 5 μg membrane prepared from stably transfected N2A cells. Nonspecific binding was defined with 1 μM unlabeled GTPγS. Basal levels were subtracted from each datum and the percentage (%) stimulation over basal was calculated. The basal level of binding was 0.4–0.7 fmol/5 μg membrane protein (1100–2000 dpm/tube). Shown here is the mean ± S.E.M. of three independent experiments performed in duplicate at the wild type and mutant receptors. The $E_{\max}$ and EC_{50} values for wild type KOPR and E297^{6.58}K KOPR are shown in Table 1. The $E_{\max}$ values were ~64% of those of U50,488H. At the MOP receptor, β-FNA up to 10 μM did not activate either the wildtype or mutant receptor, whereas DAMGO at 10 μM increased [³⁵S]GTPγS binding to 206% and 190% of the basal level, respectively (not shown).

Fig. 7. Proposed interaction between residues in MOP receptor and β -FNA

(A) The methyl acetate group of β -FNA interacts with K303^{6,58} in the wild type receptor which positions the double bond in the fumarate in the vicinity of the ϵ -amino group of K233^{5,39} for a covalent bond formation. (B) When K303^{6,58} is mutated to a negatively-charged glutamic acid (E303^{6,58}), there is a repulsion, which changes the position of the double bond in the fumarate in β -FNA preventing a covalent bond formation.

Table 1

Summary of receptor binding and [³⁵S]GTPγS binding data

Saturation binding of [³H]DIP, inhibition by β-FNA of [³H]DIP binding and [³H]β-FNA total and irreversible binding to the wild type and mutant receptors and β-FNA-stimulated [³⁵S]GTPγS binding were performed as described in Materials and Methods. Each experiment was performed in duplicate 3 times and the data shown are expressed as mean ± S.E.M., β-FNA IC₅₀ and EC₅₀ values are expressed as means and 95% confidence intervals.

	[³ H]diprenorphine			[³ H]β-FNA		[³⁵ S]GTPγS	
	K _d (nM)	B _{max} (fmol/mg protein)	β-FNA IC ₅₀ (nM)	K _d (rev binding) (nM)	irreversible/total specific binding (%)	E _{max} (% over basal)	EC ₅₀ (nM)
Wt MOP receptor	0.12 ± 0.02	1571 ± 96	4.08 3.09–5.39	2.04 ± 0.68	63.6 ± 2.97	NA	NA
K303 ⁶⁵⁸ E MOP receptor	0.42 ± 0.11	904 ± 195	10.2 8.14–12.8	9.98 ± 3.64	20.5 ± 6.22	NA	NA
Wt KOP receptor	0.15 ± 0.01	531 ± 23	7.78 6.63–9.13	5.95 ± 0.94	NA	105.8 ± 10.1	5.95 1.11–31.9
E297 ⁶⁵⁸ K KOP receptor	0.13 ± 0.03	851 ± 74	4.29 3.10–5.92	10.3 ± 3.39	NA	107.1 ± 6.6	8.96 3.02–26.6

NA: not determined.