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Antagonist efficacy in MORS196L mutant is affected by the interaction between transmembrane domains of the opioid receptor.

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Abbreviations:

MOR- μ-opioid receptor
DOR- δ-opioid receptor
DAMGO: D-Ala^2,MePhe^4,Gly-ol^5]enkephalin
CTOP: D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-amide
DPDPE: [D-Pen^2-D-Pen^5]enkephalin
TIPPψ: Tyr-Ticψ[CH_2NH]Phe-Phe-OH, Tic=1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid
GPCR: G protein-coupled receptors
CHO cells: Chinese hamster ovary cells
HEK293 cells: human embryonic kidney 293 cells
DMEM: Dulbecco's modified Eagle's medium
HEPES: N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
IBMX: isobutylmethylxanthine
TM: transmembrane

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ABSTRACT

In a previous study, we demonstrated that antagonists such as naloxone or naltrexone acted as full agonists at MOR/DOR chimeric receptor (μδ2, where the DOR sequence from 1st extracellular loop to the carboxyl terminus was spliced to the MOR sequence) when a conserved serine residue in TM4 was mutated to leucine. However, when Ser^{196} in the TM4 of MOR was mutated to Leu, antagonists exhibited partial agonistic properties. Since molecular modeling studies suggested transmembrane movement during receptor activation, the observed partial agonistic properties could be due to TM1 and TM7 interaction. Hence, MOR/DOR chimeric mutant receptors with the MOR TM1 and TM7 sequence ($\mu\delta_2\mu_7$S196L) or with MOR TM1 and TM6/7 sequence (($\mu\delta_2\mu_{67}$S196L) were constructed to test such hypothesis. Using four tests of opioid receptor activation, we found that the opioid antagonists were full agonists in chimeric mutant receptor if the TM1 and TM7 were from different opioid receptors. Additionally, when two of the TM7 amino acid residues of MORS196L receptor mutants were mutated (T327A and C330S) resulting in a mutant receptor with DOR TM7 sequence, opioid antagonists naloxone exhibited full agonistic properties. These data suggest that the efficacy of opioid antagonists in the Ser^{196} mutant can be affected by the interaction between TM1 and TM7.

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Introduction

There are three pharmacologically distinct types of opioid receptor: δ, μ and κ. However, it wasn't until the cloning of these receptors (Evans et al., 1992; Kieffer et al., 1992; Chen et al., 1993; Meng et al., 1993; Yasuda et al., 1993) that they were shown to share many structural features with an extensive list of rhodopsin-like receptors in the G-protein coupled receptor (GPCR) superfamily, *i.e*. serotonin, adrenergic, muscarinic, dopamine and somatostatin receptors (Probst et al., 1992). Receptors in this family have seven putative α-helical transmembrane (TM) spanning domains with a glycosylated extracellular amino-terminus and an intracellular carboxyl-terminus. Though the extracellular domains vary greatly between receptors, there is high primary amino acid sequence homolgy within TM regions. The opioid receptors share 73-78% (Minami and Satoh, 1995) amino acid identity within the TM regions as compared to a 60% overall amino acid identity. Opioid receptors are coupled to the pertussis-toxin sensitive G-proteins, $G_{\alpha i}/G_{\alpha o}$ to produce an inhibition of adenylyl cyclase activity (Sharma et al., 1975), inhibition of N-, P- and L-type calcium channel activity (Jin et al., 1993b; Piros et al., 1995; Bourinet et al., 1996), activation of G-protein coupled inwardly rectifying potassium channels (GIRK) (North et al., 1987; Henry et al., 1995), activation of inositol trisphosphate turnover leading to an increase in intracellular calcium (Jin et al., 1993a; Smart and Lambert, 1996) , and activation of the mitogen activating kinase pathway (Berhow et al., 1996; Fukuda et al., 1996).

As described previously, the presence of a single Ser^{196} to Leu substitution in the TM4 region in a chimeric receptor: $\mu\delta_2$S196L in which the DOR sequence from 1st

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extracellular loop to the carboxyl terminus was spliced to the MOR sequence, (***Figure 1***), allows the opioid antagonists naloxone, naltriben and H-Tyr-Ticψ[CH_2NH]Phe-Phe-OH (TIPPψ; Tic=1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid) to act as full agonists (Claude et al., 1996). The effect was specific to the presence of the mutation as back-mutation of the leucine residue to the endogenous serine residue ($\mu\delta_2$) abolished the agonist activity of antagonists. However, when the analogous serine to leucine substitution was made in the wild-type μ-opioid receptor (MORS196L), opioid antagonists had only partial agonist activity. Such partial agonistic properties of antagonists were also observed in the *in vivo* antinociception assays with the knock-in mutation of Ser^{196} to Ala (Wang et al., 2003).

The reason behind the difference in antagonist activities in the wild-type and the receptor chimera is unclear. It has been proposed that the seven transmembrane regions of GPCRs form a ring-like structure with interactions between TM1 and TM7 (Suryanarayana et al., 1992; Lui et al., 1995). Therefore, the observed difference could stem from incompatible interactions between TM1 of MOR with TM7 of DOR resulting in a destabilization of the $\mu\delta_2$S196L chimeric opioid receptor inactive conformation. Activation of opioid receptors by ligands has been suggested to require the presence of a protonated amine group on the ligand that will interact with an aspartic acid residue on the receptor and movement of TM6 and TM7 (Ronai et al., 1993). A distinguishing difference between many classical opioid agonists and their competitive antagonists is the presence of a shielding bulky moiety around the protonated amine group of the opioid antagonists (Reisine and Pasternak, 1996). Therefore, a destabilization of the TM1 and TM7 interaction may allow the sterically

hindered antagonists greater access to the activation domains resulting in the full activation of the $\mu\delta_2$S196L chimeric opioid receptor.

To study the impact of transmembrane interactions on the efficacy of opiate antagonist in MORS196L, two additional chimeric receptors and three point-mutation in TM7 of MORS196L were generated. The receptor chimeras: $\mu\delta_2\mu_{67}$S196L and $\mu\delta_2\mu_7$S196L, have compatible TM1 and TM7 from MOR in an attempt to reproduce the transmembrane packing conformation of MOR. In an attempt to disrupt wild-type transmembrane packing, the amino acids in TM7, Thr^{327} and Cys^{330} of the MORS196L were converted to the corresponding amino acids Ala and Ser observed in DOR. The magnitude of both opioid agonists and antagonists activation of these receptor chimeras and mutants were determined using four functional assays of agonist activity: maximal inhibition of adenylyl cyclase activity, maximal activation of the G-protein coupled inwardly rectifying potassium channel (GIRK1), ability to induce the superactivation of the adenylyl cyclase after chronic agonist treatment and the ability to produce down-regulation of the receptor after chronic agonist treatment. Our results demonstrated that opioid antagonists were full agonists at the opioid receptors when the sequences of TM1 and TM7 were derived from different receptors.

MATERIALS AND METHODS

Construction of the $\mu\delta_2$S196L, $\mu\delta_2\mu_{67}$S196L, $\mu\delta_2\mu_7$S196L and the $\mu\delta_2$ chimeric opioid receptors. The $\mu\delta_2$S196L (a.a. MOR1-120/DOR103-372) and $\mu\delta_2$ opioid receptors were constructed as described previously (Claude et al., 1996).

The $\mu\delta_2\mu_{67}$S196L (a.a. MOR1-120/DOR103-261/MOR280-398) and $\mu\delta_2\mu_7$S196L (a.a. MOR1-120/DOR103-305/MOR324-398) chimeric receptors were created by ligation of the $\mu\delta_2$S196L opioid receptor with the $\delta\mu$5 (DOR1-261/MOR280-398) chimeric receptor or the $\delta\mu$6 (DOR1-305/MOR324-398) opioid receptor, respectively. The $\delta\mu$5 chimeric opioid receptor was created by introducing the Mlu-1 restriction site at Thr^{279}/Arg^{280} of MOR and Thr^{260}/Arg^{261} of DOR using the Promega pAlter-1 site-directed mutagenesis system. An additional oligodeoxynucleotide primer was used in the DOR reaction to remove an Mlu-1 site at nucleotide 1085. The DOR site-directed mutagenesis product was digested with EcoRI and Mlu-1 while the MOR site-directed mutagenesis product was digested with Mlu-1 and XbaI. Resulting fragments were isolated and ligated into the pcDNA3 plasmid vector digested with EcoRI/XbaI. The $\delta\mu$6 chimeric opioid receptor was created using PCR primers which introduced a NheI site at $Ala^{323}Leu^{324}Gly^{325}$ of the MOR and $Ala^{305}Leu^{306}Gly^{307}$ of the DOR. In generation of the DOR fragment, T7 RNA polymerase primer sequence was used as the upstream primer and an oligonucleotide with the following sequence: GTGCATTGCGCTAGCCTACGCCAACAGCAGC was used as the downstream primer. DOR subcloned into the XhoI site of pcDNA3 was used as template cDNA. For generation of the MOR fragment, the upstream primer was an oligonucleotide primer with the following sequence: GCTGTTCGTGTAGGCTAGCGCCAATGCAGAAGTG while the SP6 RNA polymerase primer sequence was used as the downstream primer. MOR subcloned into the HindIII site of the pRc/CMV was used as template cDNA. PCR products were subcloned into the pCRII (Invitrogen) plasmid vector before further digestion with the appropriate endonucleases. The DOR PCR product

was digested with EcoRI and NheI while the MOR PCR product was digested with NheI and XbaI. Fragments were isolated and ligated into the pcDNA3 plasmid vector prepared by digestion with EcoRI/XbaI. The $\mu\delta_2\mu_{67}$S196L chimeric receptor and $\mu\delta_2\mu_7$S196L chimeric receptor were created by digesting the $\mu\delta_2$S196L chimeric receptor with EcoRI and BglII and isolating the resulting fragment. The $\delta\mu$5 chimeric receptor or $\delta\mu$6 chimeric receptor was digested with BglII and XbaI for the $\mu\delta_2\mu_{67}$S196L or $\mu\delta_2\mu_7$S196L chimeric receptor, respectively. Fragments were ligated into the pcDNA3 plasmid vector (Invitrogen) prepared with EcoRI and XbaI. $\mu\delta_2$S196L, $\mu\delta_2$, $\mu\delta_2\mu_{67}$S196L and $\mu\delta_2\mu_7$S196L chimeric receptor cDNA was stably expressed in Chinese hamster ovary (CHO) cells using the calcium phosphate transfection method described by Chen and Okayama (Chen and Okayama, 1988). Selection of the stable colonies with geneticin (G418) and maintenance of the clonal cell lines were carried out as described previously (Claude et al., 1996).

Construction of the MORS196LT327A, MORS196LC330S and MORS196LCSTA mutants. The mutations of the Thr^{327} to Ala (T327A) or the Cys^{330} to Ser (C330S), or the combination of these two mutations (CSTA) in the TM7 of MOR were carried out using the Promega pAlter-1 site-directed mutagenesis system. The sequence of the oligodexoynucleotide used to generate the T327A mutation was as follow: GGATTCAGGCAGCTATTCGCGTAACCCAAAGC, and the sequence for generating the C330S mutation was as follow: GAACTGGATTCAGG CTGCTGTTCGTGTAAC. The combination mutation was obtained by using the MORC330S subcloned in the pAlter-1 vector as the template, and the oligodeoxynucleotide with the sequence of AACTGGATTCAGGCTGCTATTCGCGTAACCCAAAGC was used. These

oligodexoynucleotides were designed either to eliminate a PVUII sites (T327A and C330S) or to add a PVUII site (CSTA) in the receptor so as to facilitate the identification of the mutants. After confirming the mutation with DNA sequencing, the TM7 mutants were spliced into the MORS197L mutant receptor in pCDNA3 by digesting the plasmids with Eco47III and Xba1. The resulting mutant receptors in pCDNA3 plasmids were used to transfect the human embryonic kidney cells (HEK293) for stable colonies selection with the G418 antibiotic.

Intracellular cAMP measurements. The ability of opioid agonists and antagonists to inhibit the adenylyl cyclase activity was determined by measuring the intracellular cAMP contents of the CHO and HEK293 cells. Two methods were used in the intracellular cAMP measurements. The first method was performed with CHO cells as previously described (Childers and LaRiviere, 1984). Briefly, CHO cells stably expressing the chimeric opioid receptors were seeded onto 24-well tissue culture plates. Cells were assayed at 80-90% confluence. Before the assay, incubation media was replaced with 0.5 ml/well 0.5 mM isobutylmethylxanthine (IBMX) in Krebs-Hepes Ringers Buffer (KRHB) (110 mM NaCl, 25 mM glucose, 55 mM sucrose, 10 mM Hepes, 5 mM KCl, 1 mM $MgCl_2$ and 1.8 mM $CaCl_2$, pH 7.4). Cells were preincubated with IBMX in KRHB for 10 minutes at room temperature. After preincubation, the KRHB/IBMX solution was replaced with KRHB containing opioid ligand, 0.5 mM IBMX and 10 mM forskolin. Plates were incubated for 15 min. at 37^{o}C. Reaction was stopped by the removal of the KRHB solution and addition of 0.4 ml boiling 25 mM Tris at pH 7.0. Samples were transferred to glass culture tubes and centrifuged for 5 minutes at 1000 x g. Basal levels of adenylyl cyclase activity were

determined in the absence of forskolin and opioid ligand while forskolin stimulated levels were determined in the absence of opioid ligand. Levels of intracellular cAMP were determined by competition binding with 0.8 pmole [^{3}H] cAMP (30-50 Ci/mmole, Amersham) using adrenal cortex extract as the protein kinase A source (Childers and LaRiviere, 1984). Non-specific binding was determined in the presence of 500 pmole of cAMP. cAMP content of cells was determined from a standard curve for cAMP binding using cAMP concentrations ranging from 0.0375 to 100 pmole.

For the intracellular cAMP level in HEK293 cells expressing various MORS196A mutants, the **A**mplified **L**uminescent **P**roximity **H**omogenous **A**ssays (AlphaScreen™) for cAMP supplied by Perkin Elmer Life Sciences were used. The day before the assays, HEK293 cells were plated into 96-well plates. Various concentrations of opioid agonists and antagonists were diluted with KRHB buffer containing 10 μM forskolin and 0.5 mM IBMX. After removal of the growth medium, the 96-well plates were placed on ice and 100 μl of the drug solution was added to the well, with 4 wells were used per drug concentration. After sealing the plates with HotSeal™(Diversified Biotech, Boston, MA), the plates were incubated at 37ºC for 15 minutes. Reactions were terminated by placing the plates in a water bath at 85ºC to 90ºC for 5 minutes to lyse the cells and release the intracellular cAMP. After centrifuging the plates at 500 x g for 2 minutes, the amount of cAMP in 4 μl of the supernatant were determined using the donor beads coated with strepavidin, acceptor beads coated with anti-cAMP antibodies, and biotinylated cAMP in the AlphaScreen™ assay system. The cAMP concentrations, from 10^{-11} to 10^{-4}M, were used to construct the standard curve. The donor beads (final 20 μg/ml), the acceptor beads (final 15 μg/ml) and biotinylated

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cAMP (final 10 nM) were diluted in the 1 x control buffer (5 mM HEPES pH 7.4 and 0.3% Tween-20 (60%) and Hank Buffered Salts Saline (40%)). 4 µl of the reaction supernatant or standard cAMP solutions were pipetted into duplicate wells of a 384-well opti-plate (Perkin Elmer Life Sciences) with a Biomek 2000 (Beckman-Coulter, Fullerton, CA) in a dimly lit room at 4ºC. The plate was then sealed with TopSeal™ adhesive sealing films (Perkin Elmer Life Sciences) and incubated in the dark at 4ºC for 2 hours. Then 12 µl of the donor beads were pipetted into the wells and the mixtures were incubated in the dark at 4ºC for 18 to 24 hours. After equilibrating to room temperature in the dark (4 hours), the content of the cAMP in each well were determined by exciting the donor beads at 680 nm generating a singlet O_2 resulting in the fluorescence emission of the acceptor beads at 520-620 nm. The fluorescence of the wells was quantitated with the Fusion™ (Perkin Elmer Life Sciences) plate reader and the amount of cAMP in each samples were extrapolated from the standard curve.

Radioligand Binding Assays. Opioid receptor binding assays were performed with membranes prepared from CHO or HEK293 cells stably expressing the chimeric or mutant opioid receptors. Membrane preparation and binding assays were carried out as described (Law et al., 1994). Protein concentrations were determined by the method of Lowry (Lowry et al., 1951). Receptor density (B_{max}) and K_D values for [^{3}H] diprenorphine binding was calculated using the LIGAND program and K_i values were determined using the Cheng and Prussof equation: $K_i = K_{app}/1+([L^*]/K_D^*)$. Each competition binding curve was determined by at least 11 concentrations of opioid ligand. IC_{50} values and maximal inhibitory levels were determined by curve fitting of concentration-response and competition binding curve results using the Prism

program (GraphPad, San Diego, CA). The assessment of the ability of opioid ligands to reduce specific [^{3}H] diprenorphine binding was performed as described previously (Prather et al., 1994a). Binding with 2 nM [^{3}H] diprenorphine (20-50 Ci/mmole, Amersham) was performed in KRHB. Non-specific binding was determined in the presence of 10 μM naloxone.

***Xenopus* oocyte Preparation and Injection.** The cRNAs for the $\mu\delta_2$S196L, $\mu\delta_2\mu_{67}$S196L and $\mu\delta_2\mu_7$S196L opioid receptor constructs were prepared using linearized template cDNA obtained by restriction digestion with XbaI (Roche Diagnostics, Indianapolis, IN). The GIRK1 cDNA (from Dr. Henry Lester (Dascal, 1993)) was subcloned into the EcoRI/XhoI sites of pcDNA3 (Invitrogen, Carlsbad, CA) and linearized with XhoI. *In vitro* transcription of capped cRNA was performed using T7 RNA polymerase and the mMessage mMachine (Ambion, Inc., Austin, TX) The quantity and quality of cRNA was determined by agarose gel electrophoresis and measurement of absorbance spectra. cRNA was aliquoted and stored at -70^{o}C. *Xenopus laevis* (*Xenopus* One, Ann Arbor, MI) oocytes were prepared for injection by collagenase treatment with 175 units/ml collagenase Type I (Life Technologies, Grand Island, NY) in collagenase buffer (82.5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 2.5 mM sodium pyruvate and 5 mM HEPES pH 7.4) supplemented with 50 mg/ml gentamicin (Sigma, St. Louis, MO) followed by manual defolliculation in Modified Barth's solution (90 mM NaCl, 1 mM KCl, 1.64 mM $MgSO_4$, 1.48 mM $CaCl_2$ and 20 mM HEPES, pH 7.4) supplemented with 50 mg/ml gentamicin. Oocytes were maintained at 18 $\pm$ 1^{o}C until injection. Oocytes in Modified Barth's solution were

injected into the vegetal pole with 50 nl of cRNA using a Drummond microinjector for final concentration of 0.8-1.0 ng GIRK1 channel cRNA and 1.5 ng of opioid receptor cRNA per oocyte. After injection, oocytes were incubated at 18 $\pm$ 1^{o}C in ND96 (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 5 mM HEPES, pH 7.5) supplemented with 2.5 mM sodium pyruvate and 5% (v/v) heat inactivated horse serum (Life Technologies, Inc,). Pertussis toxin treatments were performed by microinjection of 0.0225 ng per 50 nl of the active pertussis toxin A protomer (List Biologicals, Campbell, CA), for a final concentration of 25 ng/ml of oocyte. Vehicle injections were performed with 50 nl pertussis toxin buffer (0.25 mM Tris pH 8.0, 0.001% CHAPS, 2.5 mM NaEDTA). Oocytes were incubated for an additional 4-8 hours at 18 $\pm$ 1^{o}C before electrophysiological recording.

***Xenopus* oocyte Electrophysiology.** Five to seven days after oocyte injection, standard two-electrode voltage-clamp recordings were performed at room temperature using the Dagan TEV-200 Voltage Clamp. Perfusion of oocytes was performed with ND96 for clamping at -80 mV and high K^+ buffer (Dascal et al., 1993) for K^+ current recording and drug addition. Drugs were applied for 0.75 - 1 minute in high K^+ buffer. Each oocyte was only exposed to one dose of drug. Results are reported as mean $\pm$ SEM. Significance was determined using the Student's t-test, ANOVA with Fisher PLSD post-hoc on StatView for Macintosh (Abacus, Berkeley, CA).

RESULTS

Antagonists exhibited partial agonistic activities in μδ$_2$μ$_{67}$S196L and μδ$_2$μ$_7$S196L but full agonistic properties in μδ$_2$S196L

Our previous studies with the MORS196L mutant receptor indicated that opioid antagonists such as naloxone or naltrexone exhibited partial agonistic properties (Claude et al., 1996). Such partial agonistic activities exhibited by antagonist are in direct contrast to those observed with the μδ$_2$S196L chimeric receptor where antagonists have full agonistic properties. We hypothesize that interaction between TM1 and TM7 or between TM1 and TM6/7 could influence the antagonist efficacy. Hence in order to test such hypothesis, chimeric receptors having TM1 and TM7 from two different receptors were constructed. The μδ$_2$μ$_7$S196L and μδ$_2$μ$_{67}$S196L were constructed from the μδ$_2$S196L chimeric receptor by replacing DOR TM6 and/or TM7 region to carboxyl terminus with analogous regions of MOR and each contains the TM4 Ser^{196} to Leu mutation ***(Figure 1)***. The chimeric receptor cDNA were stably expressed in CHO cells and clonal cell lines expressing similar levels of receptor were selected for further study ***(Table 1)***. Radioligand binding performed on membranes prepared from CHO clonal cell lines stably expressing the chimeric receptors (μδ$_2$, μδ$_2$S196L, μδ$_2$μ$_7$S196L or μδ$_2$μ$_{67}$S196L) demonstrate that each of the chimeric receptors exhibit DOR selectivity ***(Table 2)***. They had a higher affinity for DOR selective ligands, [D-Pen^2-D-Pen^5]enkephalin (DPDPE) (peptide agonist), naltriben (alkaloid antagonist) and TIPPψ (pseudopeptide antagonist) than for MOR selective ligands, DAMGO (peptide agonist) and CTOP (peptide antagonist) ***(Table 2)***.

Though the chimeric receptors $\mu\delta_2\mu_{67}$S196L and $\mu\delta_2\mu_7$S196L had a slightly lower affinity for DPDPE than either the $\mu\delta_2$S196L or $\mu\delta_2$ opioid receptors, they were considered DOR selective as their for DPDPE was ten times greater than the affinity of DPDPE at MOR stably expressed in CHO cells (Chakrabarti et al., 1995). Moreover, the chimeric receptors have a low affinity for the MOR antagonist CTOP. Additionally, the affinity of the non-selective opioid antagonist, naloxone, at $\mu\delta_2$S196L, $\mu\delta_2\mu_{67}$S196L and $\mu\delta_2\mu_7$S196L was characteristic of its affinity for DOR stably expressed in CHO cells (Prather et al., 1994b). The affinities of ligands for the $\mu\delta_2$S196L opioid receptor were similar to the $\mu\delta_2$ receptor indicating that the presence of the Ser^{196} mutation did not affect ligands binding affinities and selectivities (***Table 2***). These results demonstrated that neither the S196L mutation nor the replacement of DOR TM6 and TM7 with the MOR transmembrane regions appeared to greatly affect the selectivity of the chimeric opioid receptors.

The ability of opioid receptors to inhibit adenylyl cyclase activity in clonal cells expressing opioid receptors is well characterized making this a useful tool for evaluating the potency and efficacy of the opioid antagonist response at the $\mu\delta_2$S196L, $\mu\delta_2\mu_{67}$S196L and $\mu\delta_2\mu_7$S196L chimeric opioid receptors. Thus, the ability of the antagonists, naloxone, naltriben and TIPPψ to inhibit adenylyl cyclase activity in CHO cell lines stably expressing the chimeric receptors was compared to that of the full agonist, DPDPE. As the absolute level of maximal inhibition of adenylyl cyclase activity obtained in a stably expressing transfected clonal cell has been shown to vary between cell lines and is dependent on receptor density (Law et al.,

2000b), CHO cell lines stably expressing the receptors were chosen that exhibited similar level of maximal DPDPE inhibition. All three opioid antagonists were able to inhibit forskolin-stimulated accumulation of intracellular cAMP at cells expressing the $\mu\delta_2$S196L, $\mu\delta_2\mu_{67}$S196L and $\mu\delta_2\mu_7$S196L receptors while only TIPPψ retained trace agonist activity at the $\mu\delta_2$ opioid receptor, as described previously ((Claude et al., 1996); ***(Figure 2)***). This, again, indicated the specificity of TM4 Ser to Leu substitution for agonistic activity exhibited by opioid antagonists. Classical opioid antagonists, naloxone, naltriben and TIPPψ, had similar potencies to inhibit forskolin-stimulated accumulation of intracellular cAMP at $\mu\delta_2$S196L, $\mu\delta_2\mu_{67}$S196L and $\mu\delta_2\mu_7$S196L opioid receptors ***(Table 3)***. Such inhibition of the adenylyl cyclase activity by antagonists was not cell line dependent, as similar inhibition was observed also when these mutant opioid receptor chimeras were expressed in HEK293 cells (*data not shown*). In cells expressing the $\mu\delta_2$S196L receptor, 1 μM each of naloxone, TIPPψ and naltriben produced the same maximal inhibition of the forskolin-stimulated accumulation of intracellular cAMP as DPDPE. However, at the $\mu\delta_2\mu_{67}$S196L and $\mu\delta_2\mu_7$S196L opioid receptors, naloxone, naltriben and TIPPψ demonstrated significantly less inhibition of forskolin-stimulated accumulation of intracellular cAMP as compared to DPDPE ***(Figure 2)***. These results suggest that the opioid receptor antagonists, naloxone, naltrexone and TIPPψ, were as efficacious as the DOR selective agonist DPDPE at the $\mu\delta_2$S196L chimeric opioid receptor, but not at the $\mu\delta_2\mu_7$S196L and $\mu\delta_2\mu_{67}$S196L chimeric opioid receptors.

Interaction of the transmembrane domains account for the partial agonistic properties in S196L mutants.

When comparing the amino acid sequences of the $\mu\delta_2$S196L and the two chimeric receptors, it was apparent that the major difference between these receptors was within TM7 and the carboxyl tail domains. Earlier modeling studies have suggested the interaction between TM1 and TM7 of the GPCRs (Suryanarayana et al., 1992; Lui et al., 1995). Since the TM1 in the $\mu\delta_2$S196L chimeric receptor is derived from MOR while the TM7 is from DOR, it is likely that the interaction between non-compatible TM1 and TM7 domains in this chimeric receptor contributed to the observed full agonistic properties exhibited by antagonists. On the other hand, TM1 and TM7 of both μδ2μ7S196L and μδ2μ67S196L chimeric receptor are from MOR. Their tight interaction could reduce the antagonist efficacy at these receptors. If this is the case, mutation of the amino acids on TM7 of μδ2μ7S196L and μδ2μ67S196L to the corresponding DOR amino acids, together with the S196L mutation, should result in the antagonists exhibiting full agonistic properties. When the amino acid sequences of DOR and MOR were compared, two amino acid residues were different within the TM7 domains used to construct the receptor chimeras. The Thr^{327} and Cys^{330} in MOR correspond to the Ala^{309} and Ser^{312} in DOR. Instead of using the $\mu\delta_2$ receptor chimera, we tested this hypothesis with the MORS196L mutants so as to eliminate any contribution by the difference in carboxyl tail sequences between MOR and DOR. The MORS196L receptor mutant and the T327A or C330S or the combination mutant (CSTA) of MORS196L receptor mutant was stably expressed in

HEK293 cells. As summarized in ***Table 1***, the stable clones of HEK293 cells expressed similar level of the mutant receptors, and the receptors exhibited similar affinity for the [^{3}H]diprenorphine.

When the antagonist naloxone activities in these mutant receptors were compared to those exhibited by DAMGO, a MOR selective agonist, it was apparent that Thr^{327} and Cys^{330} contributed to the antagonist's efficacy. As summarized in ***Table 4***, the potency of the agonist DAMGO appeared to be increased by the S196L mutation, and also by the C330S mutation. Such increase was not observed with the T327A mutation or the combination of the T327A and the C330S mutations (CSTA). Most significantly, as observed with the stable CHO cell lines, naloxone did not inhibit the forskolin-stimulated intracellular cAMP production in the HEK293 cells stably expressing the wild-type MOR (***Figure 3, Table 4***). Naloxone inhibited the cAMP production in a dose-dependent manner in HEK293 cells expressing the MORS196L mutant receptor (***Table 4***). When the level of naloxone maximal inhibition was compared among the S196L mutants, there was significant difference between the MORS196LC330S, the MORS196LCSTA and the MORS196L (***Figure 3***). There was an increase in the maximal inhibition level observed with naloxone in HEK293 cells expressing the MORS196LT327A mutant receptor when compared with that expressing the MORS196L mutant receptor. The naloxone remained a partial agonist in the cells expressing this mutant receptor. However, naloxone exhibited full-agonistic activity in cells expressing MORS196L with either C330S or both Thr^{327} and Cys^{330} residues were mutated to generate the CSTA mutant (***Figure 3***). In contrast, the efficacy of the partial agonist, nalorphine, increased to that of full agonist DAMGO

when either Thr^{327} or Cys^{330} in the MORS196L were mutated to the corresponding amino acids of DOR (***Figure 3***). Thus, the mutation of these two amino acids in MOR to the corresponding residues in DOR resulted in a phenotype resembling that of $\mu\delta_2$S196L receptor chimera, where naloxone could activate the receptor in manner similar to that of an opioid agonist.

Chronic antagonist effects in cells expressing the S196L mutant receptors.

It has been determined that chronic exposure to a full agonist, but not a partial agonist or antagonist, will induce the loss of specific receptor binding sites *i.e.* down-regulation (Law et al., 1983). Therefore, to confirm that the antagonists were acting as full or partial agonists, the ability of these ligands to induce down-regulation after chronic treatment was determined. CHO cells stably expressing $\mu\delta_2$S196L and chronically treated with 1 μM DPDPE, naloxone or TIPPψ for 24 hours demonstrated about a 60% loss in specific [^{3}H]-diprenorphine binding sites indicating that all three opioid ligands were acting as full agonists at the $\mu\delta_2$S196L opioid receptor ***(Figure 4)***. While chronic treatment with 1 μM DPDPE produced about a 35% reduction in [^{3}H]-diprenorphine binding sites in CHO cells stably expressing the $\mu\delta_2\mu_{67}$S196L or $\mu\delta_2\mu_7$S196L opioid receptors, both naloxone and TIPPψ produced a statistically significant up-regulation of [^{3}H]-diprenorphine binding sites ***(Figure 4)***. This up-regulation of receptor binding sites after chronic partial agonists or antagonist exposure has been observed in HEK293 cells expressing wild-type opioid receptor

(Petaja-Repo et al., 2002). Therefore, DPDPE was a full agonist at all of the chimeric receptors while naloxone and TIPPψ were full agonists only at the μδ$_2$S196L chimeric opioid receptor.

Another test for the agonistic properties of the ligands is the ability to produce superactivation of the adenylyl cyclase activity during chronic treatment. Earlier studies by Sharma et al (Sharma et al., 1977) reported a parallel increase in the adenylyl cyclase activity and a decrease in agonist inhibition during chronic agonist treatment. The increase in the adenylyl cyclase activity was observable only after the removal of the agonist by repeated washings or by the addition of antagonist. Hence if mutation of the amino acids within TM7 of MORS196L increases the agonistic properties of naloxone, then naloxone should not be able to induce the increase in the adenylyl cyclase activity after chronic agonist treatment. When the HEK293 cells expressing the wild-type MOR were treated with 1 μM DAMGO for 4 hours and the forskolin-stimulated intracellular cAMP production was measured in the presence of 1 μM naloxone, a 340±50% increase in the adenylyl cyclase activity was observed as compared to that measured in cells not treated with DAMGO (***Figure 5A***). In HEK293 cells expressing the MORS196L mutant receptor, similar treatment only resulted in 160±9.2% increase in the adenylyl cyclase activity. This could be due to the partial agonistic properties of naloxone observed with the S196L mutant. However, this might not be only reason, since 1 μM nalorphine treatment could induce similar level of increase in the adenylyl cyclase activity (330±30%) in HEK293 cells expressing the wild-type receptor. Since the magnitude of adenylyl cyclase activity increase is dependent on the receptor density (Law et al., 1994), the ~ 2-fold difference in the

receptor density between wild-type and MORS196L (***Table 1***) could contribute to the difference in the amount of cAMP produced. Nevertheless, with HEK293 cells expressing similar levels of MORS196L, MORS196LT327A, and MORS196LC330S, chronic treatment of these cells with DAMGO resulted in the different intracellular cAMP levels in the presence of naloxone. In particular, displacing DAMGO with naloxone from the MORS196LC330S or MORS196LCSTA receptor did not result in an increase of adenylyl cyclase activity after chronic agonist treatment. These results are in contrast with those observed with the wild-type MOR and MORS196L receptors. Thus, naloxone had the activities of a full agonist in the MORS196L mutant receptors when the Cys^{330} and Thr^{327} residues are mutated to the corresponding residues of DOR.

The observed differences in the magnitude of adenylyl cyclase superactivation could be due to differences in level of receptor desensitization. However, as previously reported with DOR (Law et al., 1983), there did not appear to be a correlation between receptor desensitization and superactivation of adenylyl cyclase in chronic DAMGO treatment of cells expressing MOR. Maximal increase in the superactivation of adenylyl cyclase activity was achieved without complete loss of DAMGO-mediated inhibition. 1 μM DAMGO pretreatment produced a slow rate of desensitization of MOR mediated inhibition of adenylyl cyclase activity (Law et al., 2000a). After 4 hours of 1μM DAMGO pretreatment of HEK293 cells expressing wild-type MOR, 73.6±1.4% of DAMGO maximal inhibition remained (***Figure 5B***). Similar DAMGO treatment resulted in complete loss of DAMGO response in cells expressing MORS196L mutant receptor, or 25.9±9.2% of maximal inhibition remained in cells

expressing MORS196LT327A mutant receptor. Even with greater degree of desensitization in these two mutant MOR and the other MORS196L mutant receptors, the magnitude of adenylyl cyclase superactivation in cells expressing the mutant receptors was much lower than that observed with wild-type receptor in the presence of naloxone. These results again suggested the naloxone efficacies in these receptors, not the degree of desensitization, determined the magnitude of superactivation observed.

Antagonist activation of GIRK1 with $\mu\delta_2$S196L, $\mu\delta_2\mu_{67}$S196L and $\mu\delta_2\mu_7$S196L

As it was possible that the observed effect was specific to the adenylyl cyclase effector system or unique to stably expressing clonal cell lines, the magnitude of the agonistic effects of the antagonist naloxone at the G protein-coupled inwardly rectifying potassium channel (GIRK1) channel in oocytes transiently co-expressing $\mu\delta_2$S196L, $\mu\delta_2\mu_{67}$S196L or $\mu\delta_2\mu_7$S196L with GIRK1 were examined. Previously, we described the activation of GIRK1 by antagonists in *Xenopus laevis* oocytes co-expressing the $\mu\delta_2$S196L opioid receptor and GIRK1 channel (Claude et al., 1996). GIRK1 channel activity was assayed electrophysiologically using whole-cell two-electrode voltage clamp in *Xenopus laevis* oocytes. Perfusion of opioid ligands in the presence of a high K^+ buffer, produced an inward current when the GIRK1 channel was activated ***(Figure 6A)***. To confirm that the chimeric receptors were coupled to the GIRK1 channel via pertussis-toxin sensitive G-proteins, oocytes were injected with either the activated A protomer of pertussis toxin or vehicle. In oocytes co-expressing

$\mu\delta_2$S196L with the GIRK1 channel, injection with pertussis toxin inhibited 98 $\pm$ 2% (n=10) of the current induced by 1 μM DPDPE in vehicle injected oocytes (n=10) (data not shown). As it has been suggested that G-proteins activate the GIRK1 channel via $\beta\gamma$-subunits (Wickman et al., 1994; Huang et al., 1995), the chimeric receptors appeared to be coupled to the GIRK1 channel via $\beta\gamma$-subunits liberated from pertussis-toxin sensitive G-proteins, G_i/G_o.

In oocytes co-expressing the $\mu\delta_2$S196L opioid receptor with the GIRK1 channel, perfusion of naloxone or DPDPE produced dose-dependent inward potassium currents with similar maximal currents ***(Figure 6B)***. In oocytes co-expressing the $\mu\delta_2\mu_{67}$S196L or $\mu\delta_2\mu_7$S196L opioid receptor with the GIRK1 channel, however, the maximal current induced by naloxone was only about 40% of the maximal current produced by DPDPE ***(Figure 6C and 6D)***. The magnitude of the maximal current induced by DPDPE varied between experiments (388 $\pm$ 102 nA, 269 $\pm$ 43 nA and 244 $\pm$ 61 nA, for $\mu\delta_2$S196L, $\mu\delta_2\mu_{67}$S196L and $\mu\delta_2\mu_7$S196L, respectively) without affecting either the potency of the opioid ligands to induce the potassium current or the relative magnitude of the current induced by naloxone. Potassium currents were not induced when non-injected (n=3), GIRK1 only injected (n=3) or $\mu\delta_2$S196L only injected (n=3) oocytes were perfused with either 1 μM DPDPE or 10 μM naloxone (data not shown). Taken together, these results suggested that, again, the antagonist naloxone induces the same level of receptor activation at the $\mu\delta_2$S196L opioid receptor as compared to the agonist DPDPE, but not at the $\mu\delta_2\mu_{67}$S196L and $\mu\delta_2\mu_7$S196L opioid receptors coupled to the GIRK1 channel.

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DISCUSSION

Previously we found that antagonists had full agonist activity at the chimeric opioid receptor $\mu\delta_2$S196L (Claude et al., 1996). Originally, we hypothesized that substitution of the hydrogen bonding serine residue with the non-hydrogen bonding leucine residue in this receptor chimera released some of the intra- or inter-transmembrane interactions, thereby producing a less restricted binding pocket (Claude et al., 1996). However, when the analogous mutation was created in the μ-opioid receptor (MORS196L), antagonists had only partial agonist activity. Similar naloxone partial agonistic properties were observed in knock-in mice with the S196A mutation in the MOR gene (Wang et al., 2003). The differences between naloxone efficacy in the μδ2S196L receptor chimera and MORS196L suggest other intracellular molecular interaction within the receptor must participate in determining antagonist efficacy. Resolution of the rhodopsin crystal structure and additional mutational analysis of opioid receptor indicate that receptor activation requires the disruption of the salt bridge and hydrogen bonding formed between the conserved D/ERY motif within the 2nd intracellular loop and the $X_1BBX_2X_3B$ motif within the 3rd intracellular loop that stabilizes the inactive conformation of the receptor (Palczewski et al.,, 2000; Huang et al., 2001; Li et al., 2001). Receptor activation also requires the disruption of the van der Waals interaction between TM3 and TM6 that stabilizes the receptor inactive form (Teller et al., 2001). In addition, the structural domain formed between TM6 and the conserved NPXXY motif in TM7 holding the receptor in its inactive state

must be overcome in receptor activation (Teller et al., 2001). Thus it is not surprising to predict from molecular modeling that opioid receptor activation involves movement of TM7 and TM6 upon agonist binding (Strahs and Weinstein, 1997). Disruption of intra-helical interactions among the transmembrane domains that facilitates the TM6-TM7 movement could affect the ligand efficacies. Since the distinctive difference between the μδ2S196L receptor chimera and MORS196L is the composition of TM. In μδ2S196L, TM1 sequence is that of MOR while TM6 and TM7 sequences correspond to that of DOR. The disruption of probable hydrogen bonds and van der Waal interactions among amino acid side chains between TM1 and TM7 within this receptor chimera could contribute to the full agonistic properties of opioid antagonists observed.

In our current studies, in chimeric receptors that have compatible TM domains, such as $\mu\delta_2\mu_{67}$S196L and $\mu\delta_2\mu_7$S196L, antagonists naloxone exhibited lower efficacy than in chimeric receptor that do not have compatible TM, $\mu\delta_2$S196L. In three functional assays of opioid receptor activation (inhibition of adenylyl cyclase activity, activation of the GIRK1 channel and down-regulation of the opioid receptor after chronic drug exposure) the opioid antagonists naloxone, naltriben and TIPPψ were full agonists at the $\mu\delta_2$S196L receptor but only partial agonists at the $\mu\delta_2\mu_{67}$S196L or $\mu\delta_2\mu_7$S196L receptor. One could argue that the observed differences in the antagonist activities among these chimeric receptors were caused by sequence divergence in the intracellular or extracellular loops between MOR and DOR. However, this does not appear to be the case because mutation of the TM7 sequence of MORS196L to the corresponding DOR could reproduce the chimeric receptor

results. The combination mutation of Thr^{327} and Cys^{330} in MORS196L to Ala and Ser respectively resulted in an increase in the efficacy of naloxone and that of the partial agonist, nalorphine. Since these two amino acid residues are adjacent to the conserved NPXXY motif that could be involved in stabilizing the inactive receptor conformation (Teller et al., 2001), such mutation could affect the TM7 stacking and destabilizing the interactions between TM7 and TM6. However, this probably is not the case since full agonistic properties were observed with naloxone in the chimeric receptor, $\mu\delta_2$S196L where TM6 and TM7 are of the same receptor sequence, DOR. Thus, the tight interaction between TM1 and TM7 most likely ddetermines the ligand efficacies.

The results of this study are unique in that they are the first to demonstrate that alterations to the receptor may result in alterations to ligand efficacy without appreciably changing ligand affinity or potency. In our studies, neither the presence of the Ser^{196} to Leu point mutation nor the replacement of DOR TM7 or TM6 with the analogous regions of MOR appeared to alter ligand interaction. Binding of the non-selective opioid receptor partial agonist, [^{3}H]-diprenorphine, was similar for each of the chimeric receptor constructs. A site-directed mutagenesis study demonstrated that the mutation of a TM6 His^{267} residue in MOR resulted in receptor activation by alkaloid opioid antagonists (Spivak et al., 1997). However, none of the antagonists tested in that study induced full agonist activity and there was a dramatic reduction in ligand affinity. Such studies would suggest that there are possibly many mutations within the transmembrane regions of GPCRs that allow for activation by antagonists. The magnitude of activation by antagonist or partial agonist will depend on the

disruption of intra- or inter-helical interactions that stabilize the inactive receptor conformation.

The combination of Ser^{196} to Leu mutation with those in the TM7 region allows the feasibility to engineer a receptor that could be fully activated by opiate antagonists. Such receptor could be the future for pain treatment paradigm. One of the major side effects of opioid analgesics is tolerance development. Our *in vivo* studies with the MORS196A knockin-in mice indicated that analgesic responses were observed with naloxone and nalorphine, while tolerance was not developed in mice treated chronically with these two opiate antagonist and partial agonist (Wang et al., 2003). Such observations were in accord with reports in which tolerance was not observed in mice either lacking DOR (Zhu et al., 1999) or in animals when DOR activity was blocked by receptor specific antagonist (Abdelhamid et al., 1991; Hepburn et al., 1997). The chronic treatment of the MORS196A knock-in mice with naloxone or nalorphine will inactive the endogenous opioid receptors, including DOR, while the mutant receptor was activated. Thus, by optimizing the method to deliver such engineered receptor at the sites of nociceptive responses, such as the dorsal horn area of the spinal cord, analgesia could be elicited without the tolerance development, or any side effects of the drug, which is the ultimate goal for the current studies.

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FIGURE LEGENDS

Figure 1. Schematic representation of the μδ$_2$S196L, μδ$_2$μ$_{67}$S196L and μδ$_2$μ$_7$S196L chimeric receptors. View from the extracellular surface of the putative packing arrangement of the chimeric opioid receptors and schematic of the seven-transmembrane spanning domains (upper right). Solid circles represent regions from the μ-opioid receptor. Open circles represent regions from the δ-opioid receptor. Shaded circle indicates the position of the fourth transmembrane region serine to leucine (S196L) mutation. (A) μδ$_2$S196L (a.a. MOR1-120/DOR103-372) (B) μδ$_2$μ$_7$S196L (a.a. MOR1-120/DOR103-305/MOR324-398) (C) μδ$_2$μ$_{67}$S196L (a.a. MOR1-120/DOR103-261/MOR280-398).

Figure 2. Maximal inhibition of adenylyl cyclase activity with DPDPE, naloxone, TIPPy and naltriben in CHO cells stably expressing the μδ$_2$S196L, μδ$_2$, μδ$_2$μ$_{67}$S196L or μδ$_2$μ$_7$S196L chimeric opioid receptors. Maximal inhibition of 10 μM forskolin-stimulated adenylyl cyclase activity in CHO cells stably expressing (A) μδ$_2$; (B) μδ$_2$S196L; (C) μδ$_2$μ$_{67}$S196L; and (D) μδ$_2$μ$_7$S196L chimeric receptors was determined using 1 μM of each of the ligands assayed (DPDPE (□), naloxone (■), naltriben (▤) and TIPPψ(▨)). Graph represents mean ± SEM from two to five independent experiments conducted in triplicate. The inserts represent DPDPE (○) and NTB (●) dose-response curves for the respective chimera receptors. * denotes

p<0.01 using ANOVA and Fisher PLSD post-hoc for antagonist treatment as compared to DPDPE-mediated inhibition in each respective clonal cell line.

Figure 3. Maximal inhibition of adenylyl cyclase activity with DAMGO, nalorphin and naloxone in HEK293 cells expressing wild type MOR and various mutants of MORS196L. Maximal inhibition level of 10 μM forskolin-stimulated intracellular cAMP production by DAMGO (□), nalorphine (■) and naloxone (▤) in HEK293 cells expressing MOR, MORS196L, and various mutants of MORS196L were determined by the non-linear regression analysis of the dose response curves. The values represent the averages from three separate experiments and * denotes statistical significance with the non-paired t-test analysis, with p <0.05. The insert represents the dose response curves of DAMGO (○,●) and naloxone (Δ,▲) in HEK293 cells expressing the wild type MOR (———) and MORS196LCSTA (--------) respectively.

Figure 4. Downregulation of chimeric opioid receptors by chronic treatment with DPDPE, naloxone or TIPPy in CHO cells stably expressing the μδ$_2$, μδ$_2$S196L, μδ$_2$μ$_{67}$S196L or μδ$_2$μ$_7$S196L chimeric opioid receptors. Cells were treated with 1 μM opioid ligand for 24 hours prior to binding assay. The graph represents results obtained from two or three independent experiments performed in triplicate (average ± SEM). Statistical significance has been determined using ANOVA and Fisher PLSD post-hoc (*p <0.05, + p<0.01) for antagonist treatment as compared to untreated control in each respective clonal cell line. Control binding levels were as follows: μδ$_2$

140 ± 25 fmole/mg-protein; μδ$_2$S196L, 235 ± 25 fmole/mg-protein; μδ$_2$μ$_{67}$S196L, 120 ± 14 fmole/mg-protein; μδ$_2$μ$_7$S196L, 479 ± 47 fmole/mg-protein.

Figure 5. Increase in adenylyl cyclase activity after chronic DAMGO treatment in HEK293 cells stably expressing the various mutants of MORS196L. HEK293 cells expressing the wild type MOR, MORS196L, MORS196LT327A , MORS196LC330S, or MORS196LCSTA were treated with 1 μM DAMGO for 4 hours. Then ability of 10 μM forskolin to stimulate the production of intracellular cAMP was measured in the presence of (A) 1 μM DAMGO and (B) 1 μM naloxone or nalorphine as described in Methods. In (A), the amount of forskolin-stimulated intracellular cAMP production in cells treated with 1 μM DAMGO for 4 hours was compared to that in control cells. The bars (□) represent the amount of cAMP produced when assayed in the presence of 1 μM naloxone. The bars (■) represent the amount of cAMP produced in the presence of 1 μM nalorphine. In (B), the amount forskolin-stimulated cAMP production in the presence of DAMGO was compared to that observed with forskolin alone in control HEK293 cells (□) or in cells treated with 4 hours of 1 μM DAMGO (■). The values represent the average ± s.d. of 3 separate experiments.

Figure 6. Dose response relationship of DPDPE and naloxone activation of the GIRK1 channel in *Xenopus* oocytes co-expressing the μδ$_2$S196L, μδ$_2$μ$_{67}$S196L or μδ$_2$μ$_7$S196L chimeric opioid receptors with the GIRK1 channel. Each point on the dose response curve represents an average +_SEM value from a total of 8-10 oocytes. The data from the two experiments were normalized by comparing the currents

produced by naloxone to the maximum currents produced by DPDPE. Each oocyte was exposed to only one concentration of drug. (* $p \leq 0.05$, using Student's t-test for naloxone treatment as compared to DPDPE treatment). (A) Electrophysiological record of potassium current induced by 1 μM DPDPE or 1 μM naloxone in hK^+ buffer at the $\mu\delta_2$S196L chimeric opioid receptor. (B) DPDPE and naloxone dose-response curves for $\mu\delta_2$S196L, (C) $\mu\delta_2\mu_{67}$S196L and (D) $\mu\delta_2\mu_7$S196L. Open circles represent DPDPE treatment, closed circles represent naloxone treatment.

Table 1. Characterization of clonal cells stably expressing the chimeric opioid receptors or the TM7 mutants of MORS196L.

	[^{3}H] Diprenorphine Binding	
	K_D (nM)	B_{max} (pmole/mg-protein)
$\mu\delta_2$*	1.1 ± 0.4	0.7 ± 0.1
$\mu\delta_2$S196L*	0.9 ± 0.2	0.6 ± 0.1
$\mu\delta_2\mu_{67}$S196L*	1.9 ± 0.6	0.9 ± 0.2
$\mu\delta_2\mu_7$S196L*	1.4 ± 0.7	1.5 ± 1.0
MOR†	0.42±0.10	2.1±0.13
MORS196L†	1.5±0.084	1.2±0.04
MORS196LT327A†	1.3±0.44	1.2±0.30
MORS196LC330S†	0.96±0.36	0.87±0.14
MORS196LCSTA†	1.8±0.13	2.3±0.073

The affinity (K_D) and B_{max} of [^{3}H]diprenorphine binding in CHO and HEK293 cells stably expressing various receptors was determined by saturation binding analysis as described in the Methods section. Concentrations of [^{3}H]diprenorphine ranged from 0.01 to 10 nM. * denotes mutant receptors expressed in CHO cells, and † denotes mutant receptors expressed in HEK293 cells.

Table 2. Affinity of opioid ligands at $\mu\delta_2$S196L, $\mu\delta_2\mu_{67}$S196L, $\mu\delta_2\mu_7$S196L and the $\mu\delta_2$ chimeric opioid receptors.

	K_i (nM)			
	$\mu\delta_2$	$\mu\delta_2$S196L	$\mu\delta_2\mu_{67}$S196L	$\mu\delta_2\mu_7$S196L
DPDPE	1.5 ± 1.2	5.4 ± 1.1	19 ± 1	16 ± 3
DAMGO	710 ± 280	245 ± 57	>1000	>1000
Naloxone	54 ± 11	26 ± 5	34 ± 4	38 ± 3
NTB	0.38 ± 0.06	0.10 ± 0.08	1.7 ± 0.1	1.2 ± 0.1
TIPPψ	5.9 ± 0.9	3.2 ± 0.4	4.8 ± 1.7	8.1 ± 1.3
CTOP	>1000	>1000	>1000	>1000

The affinity of various ligands was determined by their ability to compete for 1 nM [^{3}H] diprenorphine binding as described in the Methods section.

Table 3. Potency of opioid ligands to inhibit forskolin-stimulated adenylyl cyclase activity in CHO cells stably expressing $\mu\delta_2$S196L, $\mu\delta_2\mu_{67}$S196L, $\mu\delta_2\mu_7$S196L or $\mu\delta_2$ chimeric opioid receptors.

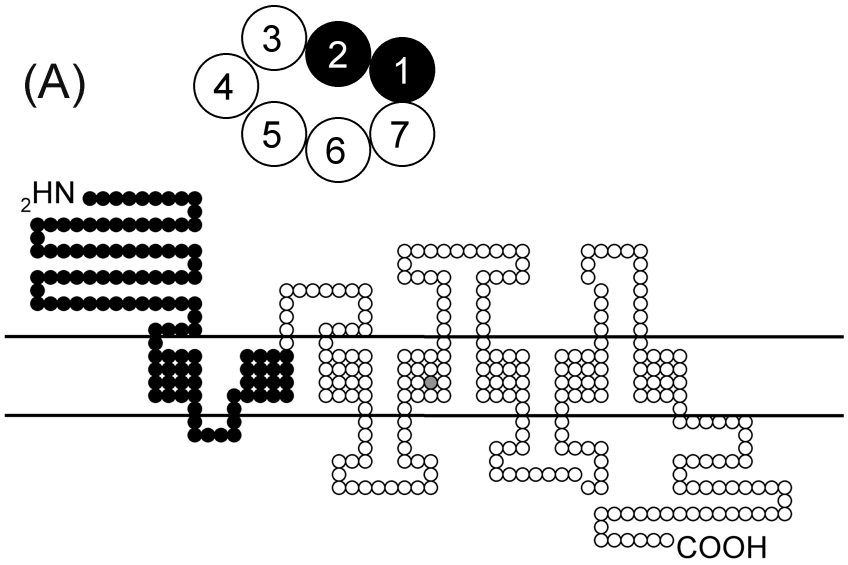
	EC_{50} (nM)			
	$\mu\delta_2$	$\mu\delta_2$S196L	$\mu\delta_2\mu_{67}$S196L	$\mu\delta_2\mu_7$S196L
DPDPE	2.9 ± 0.4	0.7 ± 0.2	5.4 ± 1.4	5.8 ± 0.1
Naloxone	NI	53 ± 4	38 + 14	38 ± 3
NTB	NI	1.0 ± 0.01	2.9 ± 0.5	1.7 ± 0.1
TIPPψ	NI	1.4 ± 0.9	1.2 ± 0.2	4.8 ± 1.7

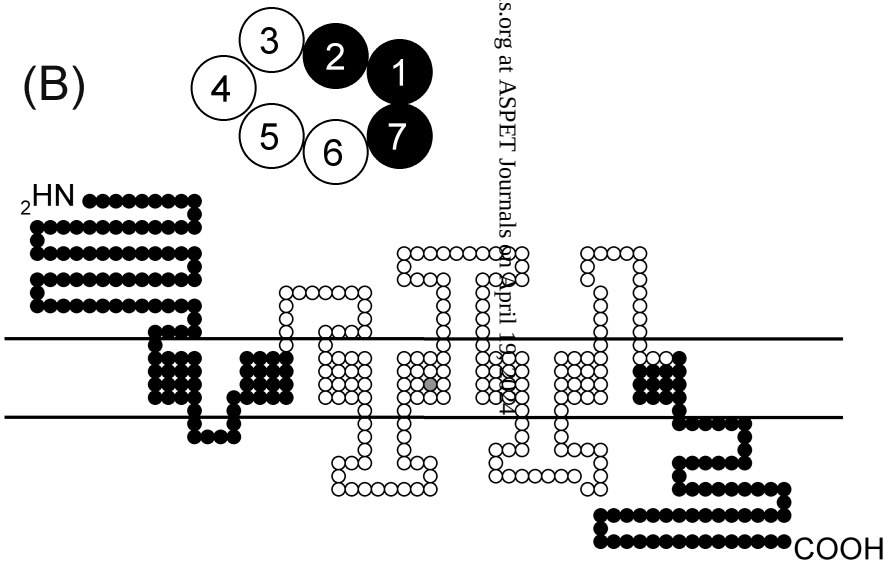
The potency of opioid ligands to inhibit 10 μM forskolin-stimulated adenylyl cyclase activity was determined as described in the Methods section. Values represent average ± SEM of at least three experiments. NI= no inhibition

Table 4. The ability of DAMGO and naloxone to inhibit forskolin-stimulated production of intracellular cAMP in HEK293 cells stably expressing various MORS196L mutant receptors.

	EC50, nM		
	DAMGO	**Naloxone**	**Nalorphine**
MOR	2.6±0.56	NI	10±3.1
MORS196L	0.53±0.04	0.64_0.11	1.7±0.18
MORS196LT327A	0.72±0.20	0.48±0.09	0.74±0.25
MORS196LC330S	0.097±0.014	0.51±0.13	0.19±0.04
MORS196LCSTA	0.43±0.057	7.8±3.4	0.74±0.03

The values in parentheses represent the maximal inhibition levels calculated from the dose response curves. NI = no inhibition





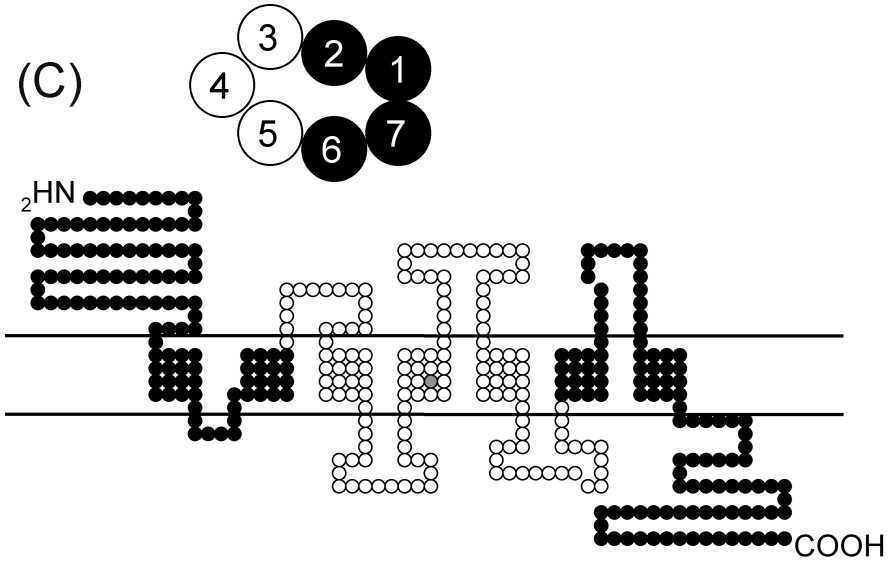


Figure 1

Figure 2

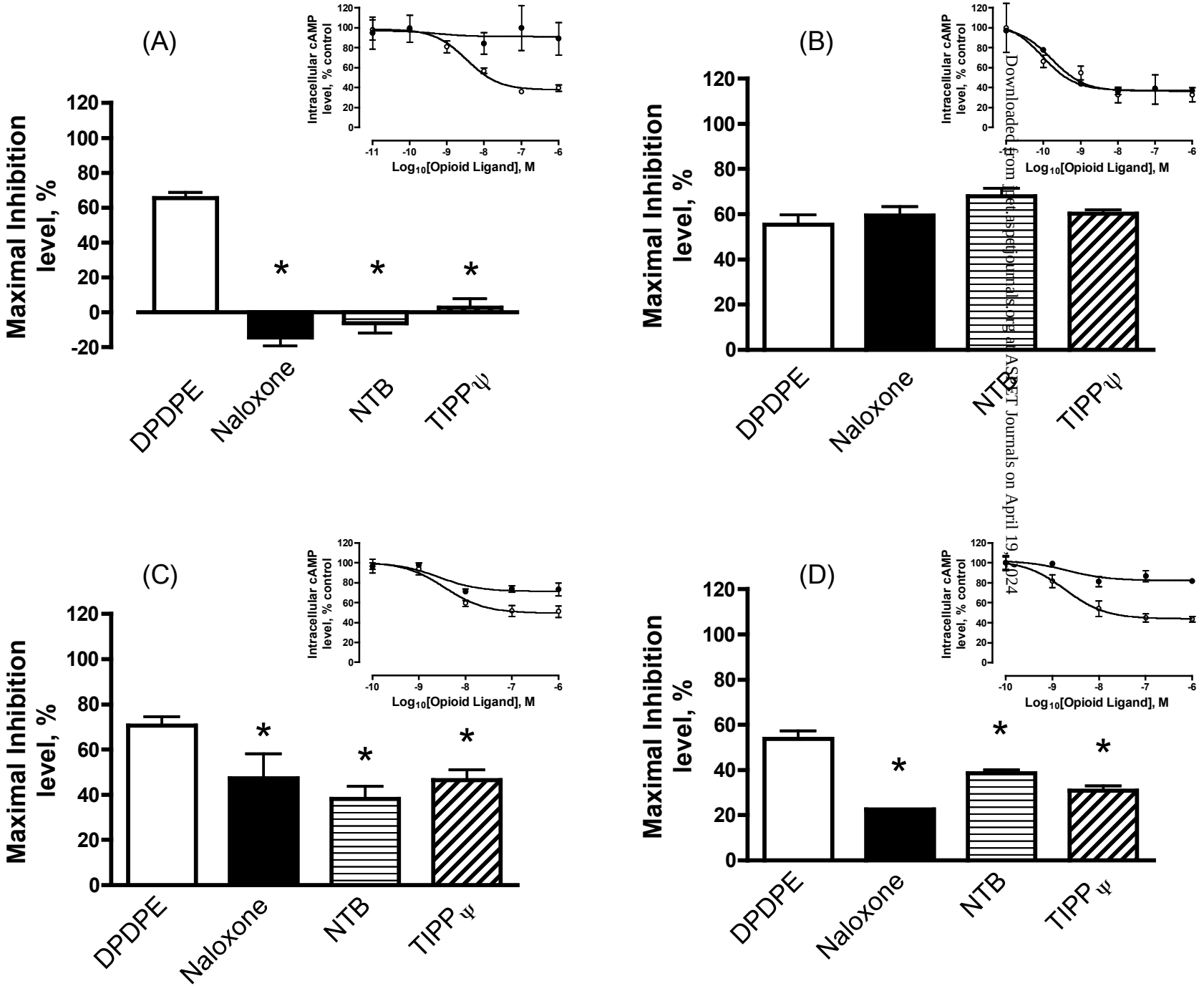


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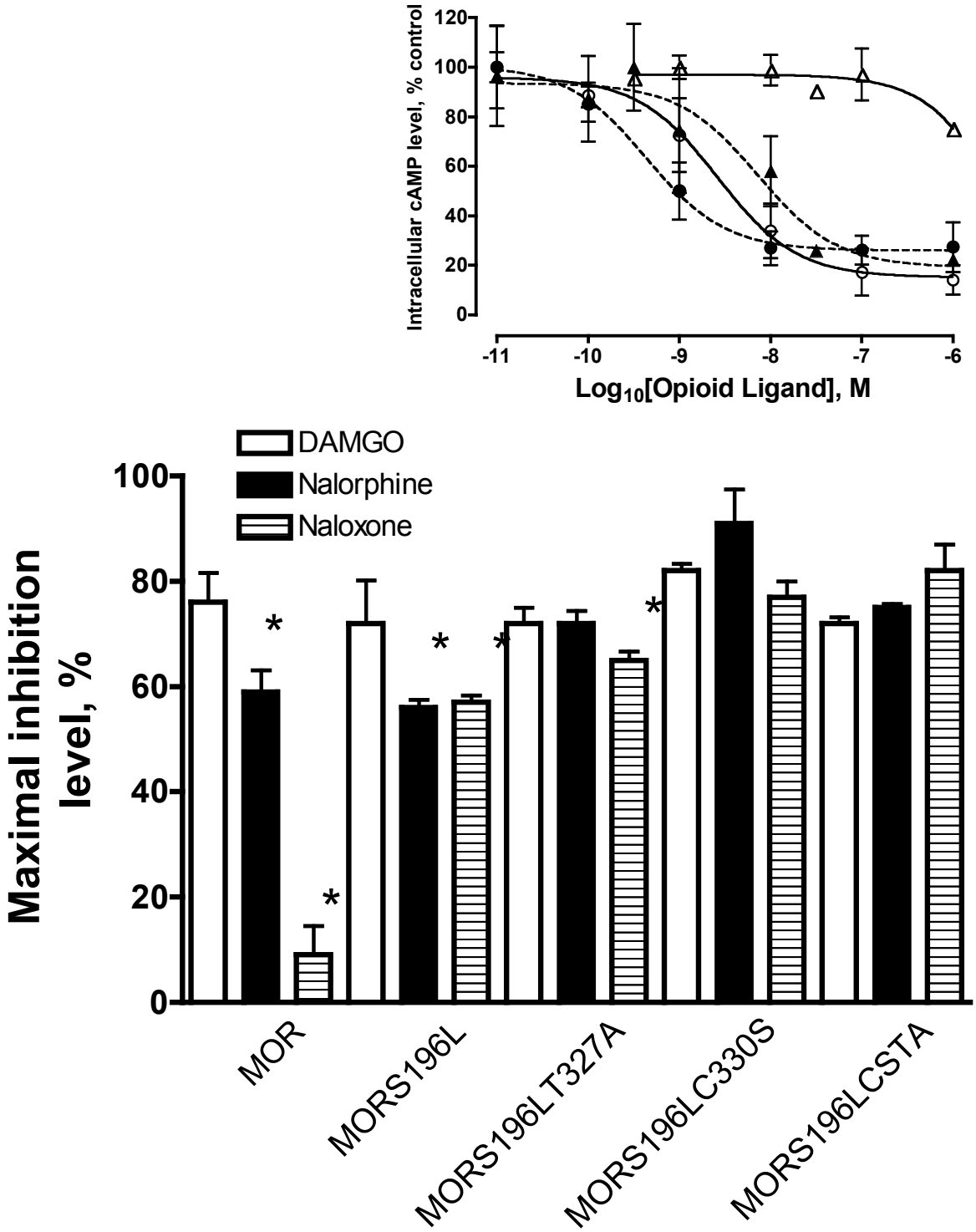
Figure 3



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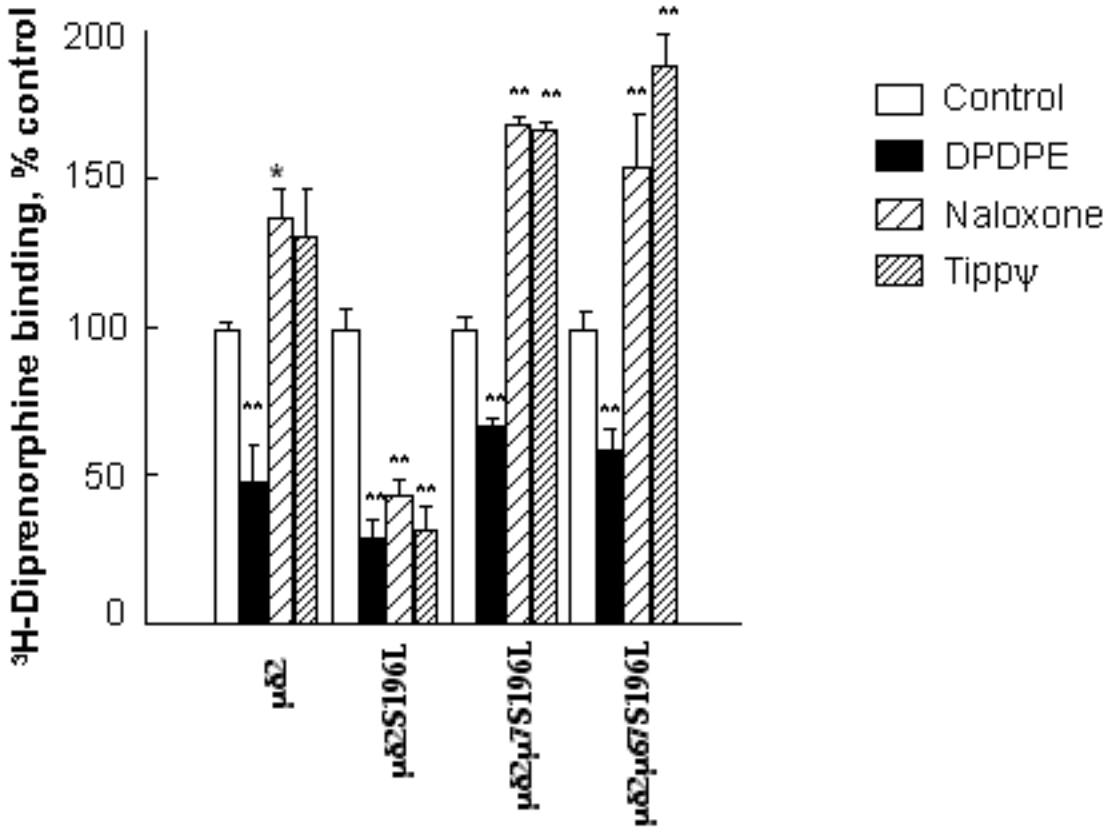

Figure 4

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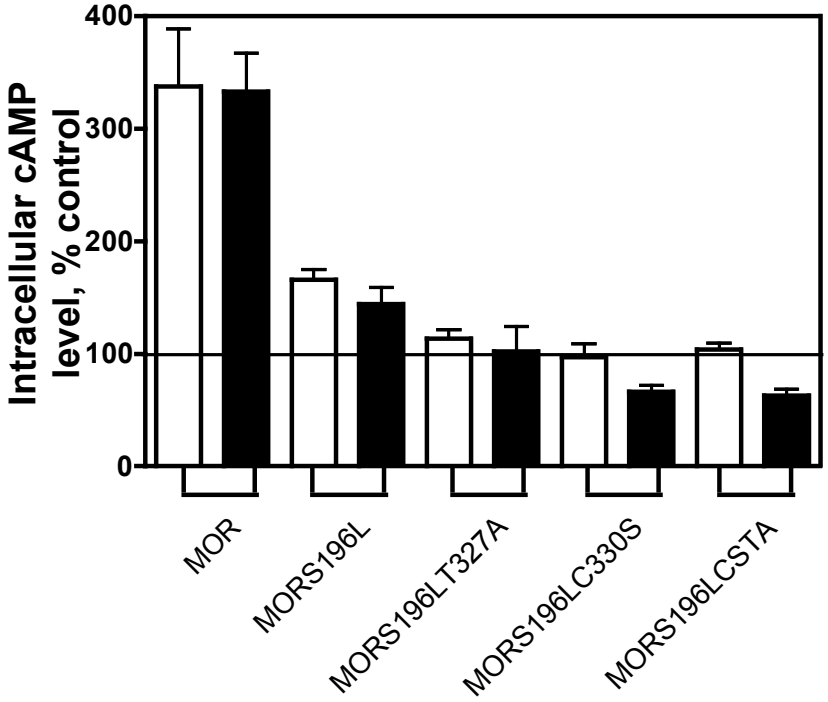
Figure 5

(A)



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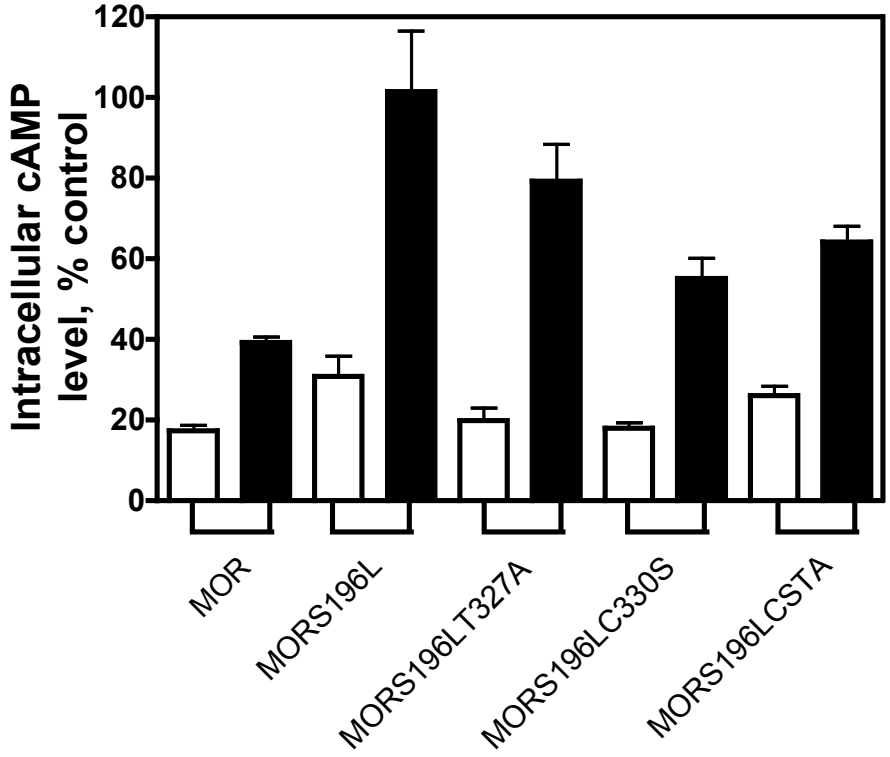

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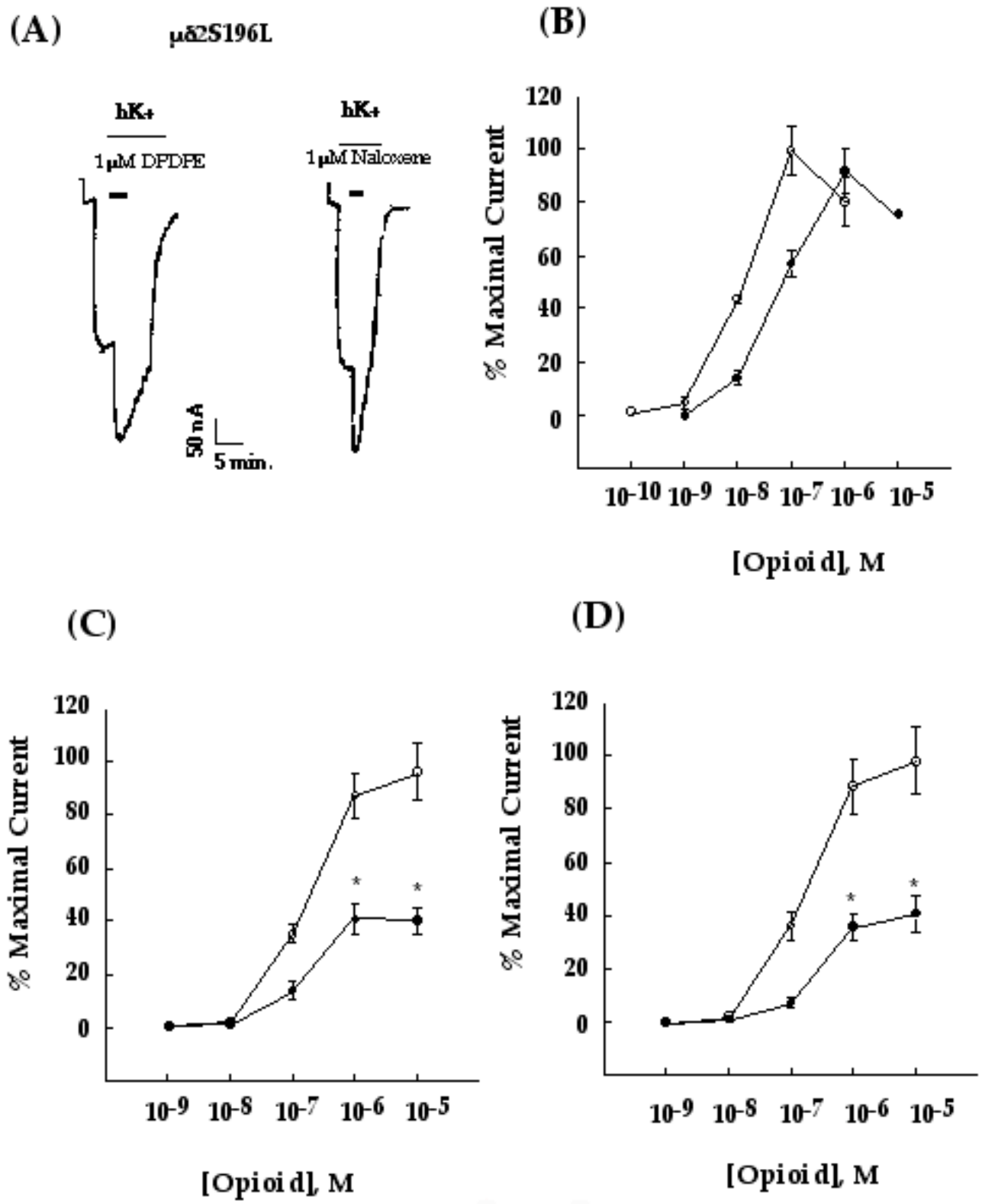

Figure 6