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Receptor reserve reflects differential intrinsic efficacy associated with opioid diastereomers

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ABSTRACT

Structure–activity relationships built around receptor binding or cell-based assays are designed to reveal physiochemical differences between ligands. We hypothesized that agonist receptor reserve may provide a unique approach to distinguish structurally-related agonists exhibiting similar functional characteristics. An intracellular calcium activation assay in Chinese Hamster Ovary (CHO) cells expressing cloned human μ-opioid receptors was developed. We examined two isomers exhibiting indistinguishable receptor binding and *in vitro* potency profiles. Oxymorphone, a clinically-available congener of codeine has at least two active diastereomeric metabolites (6α- and 6β-oxymorphols) found to be similar for μ-opioid receptor binding affinity (K_d = 15 versus 14 nM) and calcium activation (EC_{50} = 22 versus 14 nM). Calcium activation was then inhibited in CHO cells in a concentration-dependent manner using the irreversible μ-opioid receptor antagonist, β-funaltrexamine (β-FNA). Under these conditions, ~10-fold greater receptor reserve was found for 6α-oxymorphol compared to 6β-oxymorphol. This difference between the oxymorphols corresponded to a rank order of intrinsic efficacy (Emax): DAMGO>oxymorphone = 6α-oxymorphol = oxycodone>6β-oxymorphol. In addition, 6α-oxymorphol exhibited greater relative potency than the 6β-oxymorphol in mouse tail-flick, hot-plate and phenylquinone writhing antinociceptive assays, regardless of route of administration. Thus the β-FNA/calcium model provides a novel, cell-based approach to distinguish structurally related μ-opioid agonists, and in the specific case of the oxymorphols, receptor reserve differences provided a means to bridge functional *in vitro* and *in vivo* models.

1. Introduction

Congeners of codeine, oxycodone and oxymorphone are clinically useful drugs for the treatment of pain (Kaplan et al., 1998; Marco et al., 2005). Oxymorphone is an O-demethylated metabolite of oxycodone with a higher *in vitro* affinity for the μ-opioid receptor (Chen et al., 1991; Thompson et al., 2004; Peckham and Traynor, 2005). Differences in μ-opioid receptor efficacy between oxycodone and oxymorphone are due to the O-demethylation of the C_3 position of the molecule. Further ketone reduction at the C_6 position of 6-hydroxyoxymorphone, a metabolite of oxymorphone, results in two diastereomers: 6α-oxymorphol and 6β-oxymorphol (Cone et al., 1983), differing by the (R)- or the (S)-configuration of the hydroxylated carbon at the sixth position of the molecule (structures are illustrated in Fig. 1).

Differences in binding, intrinsic efficacy and receptor reserve for oxycodone and oxymorphone have been described (Prather et al., 1994; Selley et al., 1998; Thompson et al., 2004). However, while the α and β oxymorphols are indistinguishable for μ-opioid receptor binding affinity (Carliss et al., 2006) differences in receptor reserve have not been demonstrated. Following the lead of studies using μ-opioid blockade to reveal receptor reserve (Zernig et al., 1995; Selley et al., 1998; Peckham et al., 2005) we hypothesized that calcium signaling mediated by agonist binding in the presence of an irreversible antagonist would progressively diminish as greater fractions of opioid receptors were blocked. That is, as suggested by Kenakin (1993) the degree of agonist intrinsic efficacy in the presence of continuous receptor blockade would predict the receptor reserve for that agonist.

To measure intrinsic agonist efficacy we developed a novel calcium stimulation assay in CHO cells using the μ-opioid receptor antagonist β-funaltrexamine (β-FNA) to block human μ-opioid receptor-mediated intracellular calcium activity. β-FNA is reported to form highly selective, irreversible covalent bonds within the receptor pharmacophore (Liu-Chen et al., 1990). In addition, the kinetic rate constants generated for β-FNA binding in CHO cells expressing μ-opioid receptors result in smooth time- and concentration-dependent receptor fractional occupancy curves suggestive of uniform conformational states (Spivak and Belgan, 2004). Finally, since agonist binding to μ-opioid receptors

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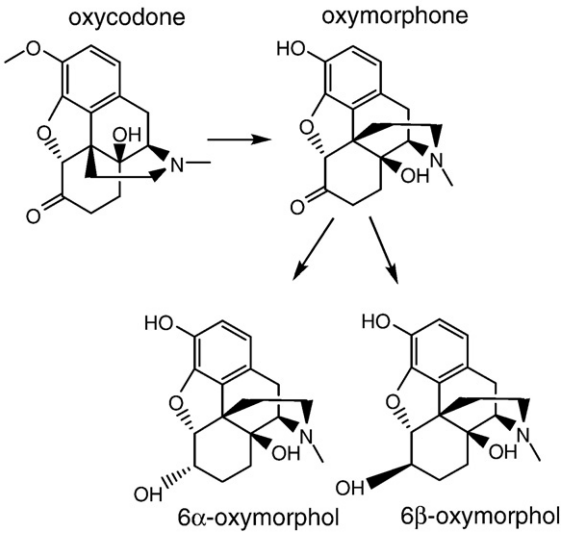

Fig. 1. Chemical structures are illustrated for the parent oxycodone, its primary metabolite oxymorphone and in turn, its 6-hydroxyoxymorphone metabolite diastereomers, 6α-oxymorphol and 6β-oxymorphol. Oxycodone and oxymorphone have a ketone group at carbon 6, while oxycodone is methylated at carbon 3. The diastereomers differ by an α or β hydroxyl group at carbon 6.

activate $G_{i/o}$ coupled inhibitory signaling proteins, cells were primed with ATP in order to enhance the calcium signal, probably through activation of extracellular P2X receptors (Law et al., 2000; Samways and Henderson, 2006). Thus if a functional distinction between the receptor reserves for structurally similar oxymorphol diastereomers could be established, a pharmacological rationale for the clinical development of one agonist over the other could be provided.

2. Methods and materials

2.1. Chemicals and drugs

Morphine sulfate, naloxone, β-FNA and [D-Ala^2, *N*-MePhe^4, Gly^5-ol]-enkephalin (DAMGO) were obtained from Sigma-Aldrich Corp (St. Louis, MO). Oxycodone and oxymorphone (HCl) were obtained from the National Institute on Drug Abuse (NIDA; Bethesda, MD). [^{3}H]-diprenorphine was obtained from Amersham (Arlington Heights, IL). Diastereomeric metabolites of oxymorphone (syn:6a-hydroxy oxymorphone, 14-hydroxy-7,8-dihydoisomorphine, hydromorphinol) 6α-oxymorphol (morphinan-3,6,14-triol, 4,5-epoxy-17-methyl-, (5α,6α)-(9Cl)) and 6β-oxymorphol (morphinan-3,6,14-triol, 4,5-epoxy-17-methyl-, (5α,6β)-(9Cl)) as HCl salts were obtained from Cerilliant (Austin, Tx). All compound concentrations were calculated in free base. For the antinociception studies the drugs were prepared fresh daily in distilled water vehicle and either injected subcutaneously (s.c.) or delivered orally (p.o.) at 0.1 cm^3/10 g body weight. Phenylquinone was dissolved in 10% trappsol and sterile water. In mg/kg, doses of either 6α- or 6β-oxymorphol used in the tail-flick and hot-plate tests were delivered s.c. at 0.1, 0.3, 0.5, and 0.8 for oxymorphone; 0.5, 1, 2, 3, 5, 7, 10, and 20 for 6α-oxymorphol and 1, 2, 5, 10 and 20 for 6β-oxymorphol, and 1, 3, 5, 7, and 10 for morphine. Doses of either 6α- or 6β-oxymorphol used in the phenylquinone writhing test were delivered p.o. at 0.5, 1.5, 2.5, 3.5, 5.0, 10 and 15 mg/kg, respectively. All antinociceptive testing was done within a post administration one hour time interval.

2.2. Antinociception assays

Relative differences in the potency of agonists were assessed in CD1 mice. Three standard antinociceptive assays were used: tail-flick, hot-plate and phenylquinone writhing. Validation of the antinociceptive assays was done with morphine, a predominately μ-opioid receptor ligand. In the phenylquinone writhing assay the racemate 6-hydroxyoxymorphone was used as a positive control for diastereomeric activity.

2.2.1. Tail-flick, hot-plate and phenylquinone writhing tests

The tail-flick and hot-plate assays were conducted at Virginia Commonwealth University in accordance with guidelines from the Institutional Animal Care and Use Committee. These studies were conducted using male CD1 mice weighing 20–30 g obtained from Harlan Laboratories. The mice were housed six per cage in plastic cages, and maintained on a fixed 12:12 light/dark cycle at an ambient temperature of 22 ± 2 °C. Water and food (Harlan Mouse Chow) were provided ad libitum. Mice were tested for antinociception using a modification of the tail-flick procedure established by D'Amour and Smith (1941). The test employs a lamp beam focused on a mouse's tail. Mice were freely able to remove their tails in reaction to the heat source. In the absence of a reaction a total of 10 s was used as a cutoff time in order to prevent tissue damage to the tail. Testing was performed at 20 min following drug pretreatment. Six mice were used for each dose of agonist and tested only once. At least 4–5 doses of each drug were used to generate dose–response curves. For hot-plate nociception mice were exposed to a footsource of radiant heat while standing on a ceramic plate. The animals could freely escape from the heat by either a footlick or by jumping. Baseline (pre-drug) time to escape was 4–10 s. A cut-off of 30 s at a temperature of 56 °C was set to prevent tissue damage. Reaction times of 2 to 4 s were employed for the control baseline. Six mice were used per dose of agonist and tested only once.

The phenylquinone writhing experiments were performed at Psychogenics, Inc. (Tarrytown, NY) and done in accordance with AAALAC guidelines. CD1 mice were used in the experiments at a weight of 35–44 g. The animals were housed six per cage in plastic cages and maintained on a fixed 12:12 light/dark cycle at an ambient temperature of 22 ± 2 °C. Water and food (Harlan Mouse Chow) were provided ad libitum. Five doses of each of the opioids were used. Ten mice were used per dose of each drug and each animal was tested only once. Including water–trappsol vehicle, single doses of morphine and the racemate composed of a 1:1 mixture of 6α-oxymorphol and 6β-oxymorphol, were included in the phenylquinone writhing test as reference controls. Opioids were administered orally 40 min before phenylquinone administration. An oral route of administration was used to anticipate a possible clinical route of administration and to assess continuity of potency compared to the s.c. route. Phenylquinone at 2.5 mg/kg was given 5 min before counting the number of twitches produced within a 10-minute period.

2.3. Receptor binding assay

DAMGO was used as a full agonist reference standard for the human μ-opioid receptor-CHO calcium assay. Membranes from CHO cells transfected with cDNA for the human μ-opioid receptor were obtained from Perkin Elmer Life Sciences (Boston, MA) and thawed for use on the day of experiment. The membranes were rapidly thawed, diluted to 10 units/ml with assay buffer (50 mM Tris, 5 mM $MgCl_2$, pH 7.4) and incubated with or without 5 nM β-FNA for 2 h at 37 °C. The concentration of 5 nM β-FNA used in these studies was taken from the IC_{50} for irreversible β-FNA binding (Liu-Chen et al., 1990) that had previously been found to reduce specific binding by about 54% (from 14 to 6.5 fmol protein). The membrane preparations were pelleted at 20,000 g for 10 min at 4 °C and washed in 20 ml of assay buffer. The pellets were then resuspended in 140 ml of assay buffer (1.43 units/ml or 70 μg/ml). Assay components were then added to 700 μl aliquots of tissue membrane preparation (control or 5 nM β-FNA-pretreated) as follows: 100 μl 4% dimethylsulfoxide ± different concentrations of agonist or naloxone, 100 μl 3.7 nM [^{3}H]-diprenorphine, 100 μl assay buffer with or without 1 M NaCl. Concentration–response curves were generated with 8

concentrations of [^{3}H]-diprenorphine where total and nonspecific determinations were done in triplicate. The opioid agonists (oxycodone, oxymorphone, 6α-oxymorphol and 6β-oxymorphol) or naloxone were prepared as 10× concentrations with 4% dimethylsulfoxide. Each of the compounds was tested at 12 concentrations in duplicate. The concentration range used for these experiments was determined previously (data not shown). Following an incubation time of 150 min at room temperature (to attain equilibrium) the assay contents were then rapidly filtered across Whatman GF/B filters that had been presoaked with 0.3% polyethyleneimine. The filters were washed 5 times with 1 ml of ice-cold 50 mM NaCl. Radioactivity remaining on the filters was determined by liquid scintillation counting. Nonspecific binding was defined as radioactivity remaining in the presence of 5 μM naloxone.

2.4. Ca^{2+} fluorescence assay

Chinese hamster ovary cells expressing the stably transfected recombinant human MOR1 gene (Wang et al., 1994) were obtained from Dr. George Uhl at NIDA. A calcium-sensitive fluorescent dye (FLIPR Calcium Assay Kit; Molecular Devices) was used to measure changes in intracellular Ca^{2+} stimulation via a Flexstation fluorometer (Molecular Devices) with range settings at 485 nm for an excitation wavelength and 525 nm for an emission wavelength.

To estimate receptor reserve, the intrinsic efficacy and relative potency of the agonists were assessed by comparing the effects of saturating concentrations of agonists at different concentrations of β-FNA. The methods used in the present calcium stimulation assay were modified from methods previously developed for both *in vitro* and *in vivo* assays in which irreversible or insurmountable antagonists have been used to block a portion of receptors, but where agonist binding to the unblocked receptor pool could produce a full or maximal antinociceptive response (Zernig et al., 1995; Selley et al., 1998; Peckham and Traynor, 2005; Peckham et al., 2005). Similarly, in our studies fluorescence from intracellular calcium stimulation was measured using increasing concentrations of β-FNA in the presence of different doses of the agonists DAMGO, oxycodone, oxymorphone, 6α-oxymorphol, 6β-oxymorphol and morphine. The concentrations of each of the drugs were taken to full solubility as indicated by clarity.

CHO cells expressing the human μ-opioid receptor were maintained in Ham's F-12 medium supplemented with FBS 10%, 400 μg/ml antibiotic G418 at 37 °C with 5% CO_2 humidified atmosphere. Subcultures were obtained by removing medium from monolayer rinsing with fresh 0.25% trypsin–0.03% EDTA solution for 1 to 2 min at room temperature before removing the trypsin. Fresh medium was added, the cells aspirated and dispensed into new flasks. The subcultures were split 1:10 every 3–4 days. The day before the experiment, the cells were again trypsinized, cell counts performed and the cell number adjusted to ~5×10^5 cells/ml in fresh media. Approximately 5×10^4 cells/well were aliquoted to 100 μl of media and incubated overnight to obtain about 80–100% well confluency.

On the day of the experiment, the cells were pretreated with 0.1–1000 nM of β-FNA for 2 h under the incubation conditions. One hour before the experiment the cells were washed twice with Hank's Basic Salt Solution (HBSS) and then incubated for at least 1 h at 37 °C in 5% CO_2 and then resuspended in 80 μl of HBSS to load dye. The HBSS contained 118 mM NaCl, 4.6 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose and 20 mM HEPES sodium salt adjusted to pH 7.4 into which 80 μl of the calcium-specific fluorescent dye in HBSS was added. Cells were then removed from the incubator and incubated again for at least 5 min with 20 μl of 1 mM ATP in HBSS in order to prime the calcium response (Law, 2000). The cells were then allowed to equilibrate for 30 min at room temperature in a Flexstation fluorometer after which different opioids (DAMGO, oxymorphone, 6α-oxymorphol, 6β-oxymorphol, oxycodone, and morphine) were added to the wells as 10× concentrates in 1% dimethylsulfoxide HBSS. Finally 20 μl calcium ionophore A23187 (10 μM final concentration) was added to each well as a positive control to insure calcium channel patency. DAMGO was included as a full agonist and positive control, where its efficacy for induction of the calcium response was set at 100% against which the calcium responses for the other agonists were normalized. Morphine was also included as a μ-opioid receptor binding reference opioid. After addition of each of the opioids, fluorometric measurements of intracellular calcium stimulation at 485 nm excitation/525 nm were taken every 2 s for at least 60 s. The peak calcium response typically occurred within 15 s after addition of the agonist.

2.5. Data analysis

2.5.1. Antinociception assays

Central measurements of ED_{50} values and their 95% confidence limits were calculated for all dose–response curves using unweighted least-squares linear regression for the ED_{50} as described by Bliss (1967). GraphPad Prism for Windows '98 (GraphPad Version 3.0 Software, San Diego, CA) was used to plot the data.

Data from the tail-flick and hot-plate assays were quantified as the percent maximum possible effect (MPE) according to:

$$\%\text{MPE} = 100 \times [(\text{test} - \text{control}) / (10 - \text{control})].$$

The MPE represents a response latency time in s up to a maximum cut-off time of 10 s. Since there are baseline control values that are not used for writhing in the phenylquinone writhing test, data from this assay were quantified as the percent maximal effect (ME) according to:

$$\%\text{ME} = 100 \times [(\#\text{writhes with drug}) / (\#\text{writhes with vehicle})].$$

All dose–response curves, ANOVAs and post hoc statistical comparisons were generated using GraphPad Prism Version 3.0. A one-way ANOVA followed by Wilcoxon's signed rank nonparametric test was used to evaluate statistical differences between ED_{50}s for the tail-flick, hot-plate and phenylquinone writhing tests. For the phenylquinone writhing test a one-way ANOVA was used to assess differences between vehicle and morphine or vehicle and 6-hydroxyoxymorphone followed by Tukey's post hoc testing of differences between groups.

2.5.2. Binding assays

Binding assay results were analyzed using 1- and 2-site competition models from GraphPad Prism Version 3.0. Fittings for the plots used the sigmoidal algorithm with variable slope models for curves that were a better fit by the 1-site model. The models incorporated 0 and 100 as constants for the bottom and top of the curves, respectively. The affinity constants (K_i values) were derived from a 1-site model. When the 2-site model was preferred values from both 1- and 2-site K_i values were presented. Paired *t*-tests were used to assess statistically-significant differences between agonist-generated K_is.

2.5.3. Calcium stimulation

Fluorometry was monitored by SoftMax Pro, 4.1 from Molecular Devices (Sunnyvale, CA). All concentration curves and ED_{50} and IC_{50} parameter estimates for calcium response data were fitted and generated by GraphPad Prism Version 3.0 with statistical differences evaluated by ANOVA and Tukey's Multiple Comparison test (q). Since all agonists in these assays acted as partial agonists when compared to DAMGO, the intrinsic efficacy of an agonist was reduced to:

$$\%E_{max} = 100 \times (\text{maximal agonist response}) / (\text{maximal DAMGO response})$$

as defined theoretically by Ehlert (1985) and as adapted by Selley et al. (1998). ANOVA followed by the non-parametric Dunnett's *t*-test was used for comparing families of concentration–response curves.

Table 1
ED_{50} and mean ± S.E.M. values for drugs or vehicle used in mouse antinociceptive assays.

	Tail-flick	Hot-plate	Phenylquinone writhing	
Drug	ED_{50} (mg/kg-s.c.)	ED_{50} (mg/kg-s.c.)	ED_{50} (mg/kg-p.o.)	Mean ± S.E.M. (number/10 min)
Oxymorphone	0.35 (0.22–0.56)	0.31 (0.27–0.39)		
6α-Oxymorphol	1.4 (0.89–2.1)	1.0 (0.11–8.86)[a]	1.7 (0.92–2.5)	
6β-Oxymorphol	5.1 (3.0–8.6)	8.8 (6.2–12.4)[a]	4.3 (1.93–6.66)	
Morphine	4.2 (2.5–7.0)	5.5 (3.8–7.85)[a]		10.2 ± 3.18[b]
Racemate (6α and 6β oxymorphols; 1:1)				0.60 ± 0.34[b]
Vehicle				29.4 ± 22.49

The data were expressed as %MPE (%MPE = [(drug − control) / (control)] × 100) for the tail-flick and hot-plate assays. The control was the baseline time without drug. For the phenylquinone writhing assay (%ME = [(#writhes with drug) / (#writhes with vehicle)] × 100) the vehicle represented the mean for the number of writhes over a 10 min interval without drug. Standard errors of the mean (S.E.M.) were calculated for each point. ED_{50}s were derived from least square curve-fitting. Confidence intervals (95%) for the ED_{50}s are given in parentheses. A mean ± S.E.M. was calculated where a single-dose was used for vehicle, morphine or for 6-hydroxyoxmorphone in the phenylquinone writhing test. Drug was administered subcutaneously (s.c.) 20 min prior to tail-flick or hot-plate testing, or orally (p.o.) 40 min prior to phenylquinone writhing testing. Each animal was tested one time per agonist dose after a baseline control (non-drug) value was set. There were 4–5 doses used per dose curve. Values for tail-flick or hot-plate were based on $N = 6$ mice/dose; phenylquinone writhing on $N = 10$ mice/dose.

[a] Compared to oxymorphone: Wilcoxon's rank sum test, $P < 0.05$. The ED_{50}s were generated from full efficacy curves.

[b] Compared to vehicle: Tukey's Multiple Comparisons, $P < 0.05$. The means were generated from single-dose experiments.

Estimation of fractional receptor occupancy in the presence of β-FNA was done using the formula $B / B_{max} = L / (K_d + L)$, as described in Lodish et al. (2000).

3. Results

3.1. Antinociceptive potency

The diastereomers exhibited consistent 6α-oxymorphol > 6β-oxymorphol *in vivo* potency for the three antinociceptive assays, tail-flick, hot-plate and phenylquinone writhing (Table 1). ED_{50}s were calculated from agonist dose–response curves using least-squares regression analyses to approximate curve fits (GraphPad Prism, version 3.0). ED_{50} values were consistent with doses previously reported for oxycodone, oxymorphone and morphine (Eddy and Lee, 1959). The Wilcoxon's nonparametric signed rank test was used to assess differences between ED_{50}s with comparisons made against oxymorphone, the more potent agonist. Use of the signed rank test was due to the derivation of nonparametric values from the percent maximal possible effect which does not lend itself to the Gaussian scaling restrictions assumed in a probit analysis. The relative potency differences between oxymorphone and the other opioids were statistically significant by Wilcoxon's signed rank test for the tail-flick (ED_{50} oxymorphone = 0.35, $P < 0.001$, 2-tailed) and hot-plate (ED_{50} oxymorphone = 0.31, $P < 0.001$, 2-tailed). ED_{50}s for the 6α and 6β oxymorphols in the phenylquinone writhing test did not reach statistical significance ($P > 0.05$, one-tailed) although the rank order of the oxymorphols for suppressing writhing was in the same direction as the other assays. A single dose of 10 mg/kg-p.o. morphine, vehicle and the racemate (6α and 6β oxymorphols; 1:1) delivered at 5 mg/kg-p.o. were used to generate mean values in the phenylquinone writhing test (Table 1). The racemate and morphine were both statistically different from vehicle by ANOVA ($F = 11.96$, df = 29, $P < 0.001$; Tukey's $P < 0.01$). Overall, these results including the single-dose racemate phenylquinone assay experiment, revealed a 6α-oxymorphol > 6β-oxymorphol *in vivo* rank order potency.

Equivalent bioavailability for the diastereomers was established from a separate pharmacokinetic study (Zhongping et al., 2004) where an almost identical plasma profile was found after administration of 5 mg/kg of either drug over the first hour of sampling. In these studies, plasma concentrations for both diastereomers peaked at 0.25 h following oral administration with equivalent concentrations continuing for at least 1 h. Following administration of 5 mg/kg of either 6α- or 6β-oxymorphol, plasma concentrations (ng/ml) at sampling times 0.25, 0.5 and 1 h were 69 ± 16, 50 ± 15 and 32.3 ± 1.25 for 6α-oxymorphol and 68 ± 12, 44 ± 12 and 35 ± 12 for 6β-oxymorphol (mean ± SD). The timing of these measurements was inclusive of the antinociceptive testing interval. Over the total sampling period of 26 h the kinetic parameters were area under the curve (AUC):AUC = 127, AUC = 161 mg h/ml; maximum concentration (Cmax)C_{max} = 69, 68 ng/ml; time to maximum concentration (Tmax):T_{max} = 0.25, 0.25 h, for 6α and 6β. Thus it is not likely that the pharmacokinetics of these drugs would account for the observed differences in relative potency.

3.2. Effect of β-FNA and NaCl on agonist receptor binding

The change in μ-opioid receptor binding in CHO membranes by competitive displacement of [^{3}H]-diprenorphine ($K_i = 0.2$ nM) in the presence of different agonists following pretreatment with the irreversible antagonist, β-FNA was evaluated. A baseline was established with naloxone binding that was unaffected by 5 nM β-FNA pretreatment either with or without 100 mM NaCl (data not shown).

Table 2
Summary of human μ-opioid receptor agonist binding affinities (K_i/nM).

	Oxycodone	Oxymorphone	6α-Oxymorphol	6β-Oxymorphol
−β-FNA	340 ± 10	4.4 ± 13	15 ± 13	14 ± 9
+β-FNA	200 ± 12	4.7 ± 11	8.4 ± 15	14 ± 10
−β-FNA + NaCl	2900 ± 9	36 ± 9	106 ± 9	86 ± 9
+β-FNA + NaCl	300 ± 11*	29 ± 11	40 ± 10*	27 ± 11*

Total [^{3}H]-diprenorphine binding to human μ-opioid receptor-CHO membranes was done with or without (+/−, respectively) 5 nM β-FNA pretreatment and with or without (+/−) 1 M NaCl. Data represent K_is for [^{3}H]-diprenorphine ± standard errors (nM) derived from 12 concentrations of agonist used to generate competition curves. Each agonist was tested in duplicate. Data were fitted using both 1- and 2-site binding models depending on best fit, using GraphPad Prism Version 3.0. Specific binding was determined in the presence and absence of 5 μM naloxone. In the presence of NaCl, β-FNA pretreatment produced statistically-lower K_i values for oxycodone, 6α-oxymorphol and 6β-oxymorphol (paired *t*-test; *$P < 0.05$; β-FNA with versus without NaCl).

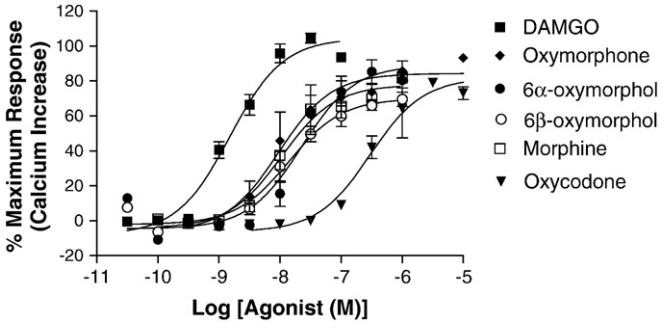

Fig. 2. The effect of DAMGO (■), oxymorphone (♦), morphine (□), 6α-oxymorphol (●), 6β-oxymorphol (○) and oxycodone (▼) on μ-opioid receptor-mediated calcium stimulation is shown in human μ-opioid receptor-CHO cells. The concentration curves were plotted by normalizing all agonist response to DAMGO. Each value represents the peak response for one of 8–10 concentrations. Each data point represents two runs per concentration.

Table 3
EC_{50} and relative efficacy for receptor-activated changes in intracellular Ca^{2+}.

Drug	EC_{50} (nM)	95% CI (nM)	$E_{max} \pm$ S.E.M. (%)
DAMGO	1.5	0.7–3	100
Oxymorphone	9.5	4.6–20	82 ± 5.7
Morphine	11	5.5–20	78 ± 0.2
6α-Oxymorphol	22	8.3–61	82 ± 4.5
6β-Oxymorphol	14	7.5–26	67 ± 4.0
Oxycodone	350	150–590	76 ± 1.8

The EC_{50}s and 95% confidence intervals (CI) were calculated using values from the concentration-dependent fluorescence curves measured for each agonist. Efficacy ($\%E_{max}$) is expressed as a percent ratio using the maximal response of agonist relative to the maximal response for DAMGO for each concentration of agonist. DAMGO: [D-Ala^2, *N*-MePhe^4, Gly^5-ol]enkephalin. A statistically significant difference was not found between the EC_{50}s of the oxymorphols, whereas statistical differences were found between the EC_{50} for oxycodone and EC_{50}s representing all other ligands ($F = 97.6$; Tukey's Multiple Comparison test, df = 5, $P > 0.001$). Each EC_{50} was based on 8–10 concentrations of agonist with 2–8 replications (see Fig. 2).

Alternatively, as indicated by the agonist affinity constants (K_i) in Table 2 binding of the agonists was significantly decreased in the presence of 1 M NaCl. NaCl was less effective after β-FNA pretreatment. These results indicate that receptor conformational changes were consistent for each of the agonists under our experimental conditions. Statistically-significant differences between the K_i values occurred ($F = 1.14$, df = 15, $P > 0.05$) where the NaCl effect was diminished after pretreatment with β-FNA for oxycodone ($t = 4.38$, df = 11, $P < 0.0011$), 6α-oxymorphol ($t = 4.53$, df = 11, $P < 0.0009$) and 6β-oxymorphol ($t = 2.48$, df = 11, $P < 0.031$). The K_i for oxymorphone was consistent with the effect of NaCl to lower the K_i for the other agonists before and after β-FNA pretreatment, but did not become low enough to achieve a statistical difference. Since there was no difference in the variance between the assays, it is suggested that the K_i for oxymorphone may have reached a maximum due to the relatively higher binding of this particular agonist.

3.3. Ca^{2+} measurement—μ-opioid receptor agonist assay

Concentration–response curves for receptor-activated changes in intracellular Ca^{2+} are shown in Fig. 2. The curves were normalized to 100% of the curve for DAMGO. As indicated by the EC_{50} estimates DAMGO was the most potent agonist (Table 3). The EC_{50} for oxycodone was about 37 times greater than for oxymorphone. This number is in range (~27×) with the ratio of values for these same ligands from Thompson et al. (2004) using a [^{35}S]GTPγS activation assay. Statistical differences were not found between potencies (EC_{50}) for the 6α and 6β diastereomers ($EC_{50}\alpha = 22$; $EC_{50}\beta = 14$ nM; two-tailed unpaired *t*-test, df = 18, $P > 0.025$). In this analysis statistical significance only occurred for potencies between oxycodone and the other ligands (df = 5, $P < 0.0001$). Estimates of relative efficacy ($\%E_{max}$) are expressed as the percent ratio of the maximal agonist response to the maximal DAMGO response. The calcium activation efficacy of 6α-oxymorphol was about 20% greater than that for 6β-oxymorphol. Oxycodone, oxymorphone and morphine exhibited relative efficacies of about 80% compared to DAMGO (Table 3). Thompson et al. (2004) also report partial agonism for oxycodone and oxymorphone in the [^{35}S]GTPγS activation assay.

Different concentrations of β-FNA were used to block calcium activity induced by DAMGO, 6α-oxymorphol, 6β-oxymorphol, oxycodone and oxymorphone. Each curve in Figs. 3 and 4 is an agonist concentration–response curve obtained with pretreatment of 7–8 concentrations of β-FNA. Thus as more receptors were blocked with increasing concentrations of β-FNA the curves shifted to the right and downward. The 6α and 6β diastereomers occupied different receptor fractions as indicated from the blockade of agonist binding with β-FNA, resulting in differential calcium activity (Fig. 3). Receptor reserves between the diastereomers reflected differences due to higher concentrations of β-FNA required to produce depression of the agonist concentration–response curves. When each of the concentration curves for β-FNA generated in the presence of agonist were compared to the parent curve representing 0 nM β-FNA, concentrations of 100 and 300 nM β-FNA were required before effectively depressing the curve for 6α-oxymorphol ($F = 3.81$, $P < 0.01$; Dunnett's *t*, $P < 0.05$). Alternatively, lower concentrations of 10 and 30 and 100 nM β-FNA were required to depress the 6β-oxymorphol curve ($F = 4.24$, $P < 0.01$; Dunnett's *t*, $P < 0.01$). Thus 6α-oxymorphol required a 10-fold higher amount of β-FNA than 6β-oxymorphol to suppress the calcium response. Concentration curves for DAMGO, oxymorphone and oxycodone illustrated in Fig. 4 demonstrate controls for rank order of potency between the agonists under the same experimental conditions.

To verify the results obtained for the diastereomers, a replication of the experiment shown in Fig. 3 was conducted. The data from the original experiment and the replication were combined and plotted such that the data for an agonist at a given β-FNA concentration (i.e., data from different 6α- and 6β oxymorphol concentrations (10^{-9}, 10^{-8}, 10^{-7} M, etc.)) were averaged to get an overall effect at a specific β-FNA concentration as shown in Fig. 5. This allowed us to evaluate the variation between both experiments by collapsing each agonist concentration curve at a single concentration of β-FNA. Thus statistically significant differences were found for the 6α agonist response at 10, 30 and 100 nM of β-FNA compared to the β-FNA baseline ($F = 5.39$, $P < 0.05$, Dunnett's multiple comparison test). For the 6β agonist, differences between 0 concentration of β-FNA and 1, 3, 10, 30 and 100 nM β-FNA were found ($F = 11.77$, $P < 0.05$; Dunnett's multiple comparison test). That is, higher concentrations of the 6α agonist were needed to suppress the calcium response from baseline than was needed for the 6β agonist. These results used an additional data set along with a different assessment and still

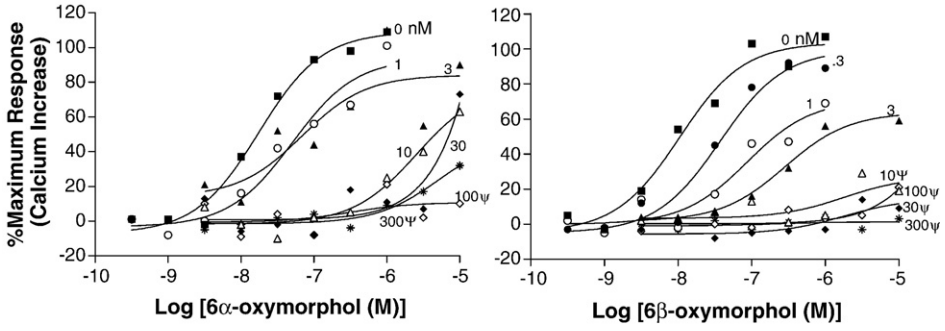

Fig. 3. The concentration-dependent effect of 6α-oxymorphol and 6β-oxymorphol binding to human μ-opioid receptor-CHO cells in the presence of different concentrations of β-FNA (in nM; 0: ■, 0.3: ●, 1.0: ○, 3.0: ▲, 10: △, 30: ♦, 100: ◇, 300: ∗). The percent maximum response represents intracellular calcium stimulation as a function of agonist concentration. For 6α-oxymorphol there were significant differences between the curve with 0 concentration of β-FNA and the curves representing 100 and 300 nM of β-FNA ($F = 3.81$, $P < 0.05$, indicated by Ψ; Dunnett's multiple comparison test) and for 6β-oxymorphol between the curve with 0 concentration of β-FNA and the curves representing 10, 30 and 100 nM β-FNA ($F = 4.24$, $P < 0.01$; indicated by Ψ; Dunnett's multiple comparison test). This corresponds to the 13% free receptor fraction for 6α and the 23% free receptor fraction for 6β-oxymorphol estimated from IC_{50}s for β-FNA.

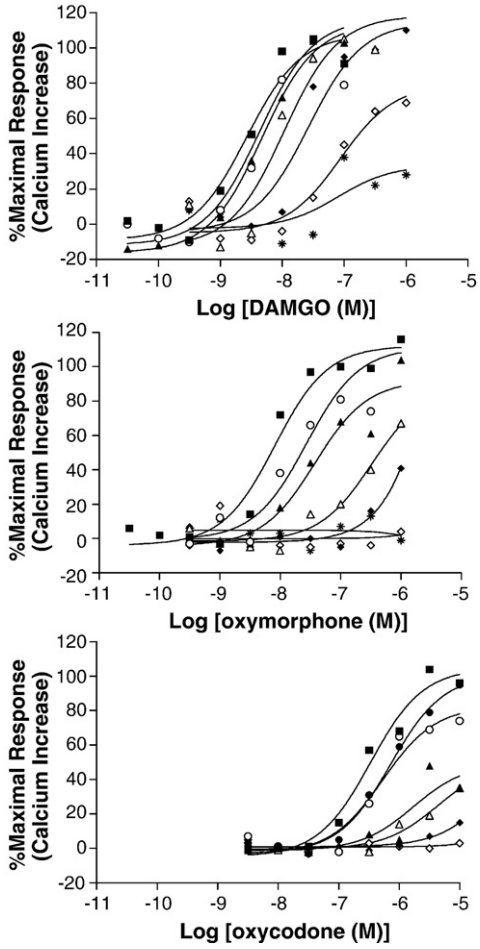

Fig. 4. The concentration-dependent effect of DAMGO, oxymorphone and oxycodone activation of human μ-opioid receptor-CHO cells in the presence of different concentrations of β-FNA (in nM; 0: ■, 0.3: ●, 1.0: ○, 3.0: ▲, 10: △, 30: ♦, 100: ◇, 300: ∗). The percent maximum response represents intracellular calcium stimulation as a function of agonist concentration. Cells were plated into 96-well plates at a density of 50,000 cells per well and cultured overnight then pretreated with 0.1–1000 nM of β-FNA for 2 h, washed three times and loaded with calcium-sensitive dye. ATP was added in order to prime the calcium response before adding different concentrations of agonists. Values represent the means of the % response of DAMGO for each plotted data point.

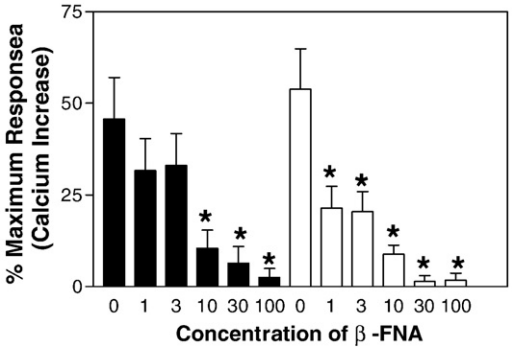

Fig. 5. Increases in intracellular calcium in human μ-opioid receptor-CHO cells are produced with either the 6α-oxymorphol (filled) or 6β-oxymorphol (open) diastereomer. The percent maximum response represents calcium stimulation as a function of the values for 6–8 concentrations of agonist in the presence of different concentrations of β-FNAn (*x*-axis). To generate these data, the experiment done to obtain the data in Fig. 3 was repeated and the new results added. For 6α-oxymorphol there were significant differences between 0 concentration of β-FNA and concentrations of 10, 30 and 100 nM of β-FNA ($F=5.39$, $P<0.05$, Dunnett's multiple comparison test) and for 6β-oxymorphol between 0 concentration of β-FNA and 1, 3, 10, 30 and 100 nM β-FNA ($F=11.77$, $P<0.05$; Dunnett's multiple comparison test).

Table 4
The number of transfected human μ-opioid receptors calculated to be available for binding and activation after treatment of cells with β-FNA[a].

β-FNA (nM)	Receptors remaining per cell (calculated[b])	Receptors remaining (%)
0.001	84,000	100
0.01	83,600	100
0.1	80,000	95
1	56,000	67
10	14,000	17
100	1650	2
1000	168	0.2

[a] β-FNA = β-funaltrexamine.
[b] Calculations used $B/B_{max}=L/(K_d+L)$ where K_d is 2 nM for β-FNA and the ligand concentration (L) is the β-FNA concentration as shown. Total receptor number remaining per cell is based on $84{,}000\times[1-(L/(K_d+L)]$.

reflected the results found in the first experiment (Fig. 3) consistently demonstrating a greater receptor reserve for the 6α-oxymorphol agonist.

Fractional receptor occupancy would be expected to mediate the level of the cell-signaling response as a function of agonist binding. Estimates of the number of human μ-opioid receptors available for binding and activation as a function of β-FNA blockade were made (Table 4). The calculations were based on previous estimates (data not shown) using 0.7 pmol receptor/mg protein and 5 million cells/mg protein that yielded 84,000 receptors/cell or, 0.7×10^{-12} mol $\times 6.02\times10^{23}$ molecules/mol/5×10^{6} cells.

The IC_{50}/K_i for β-FNA in the calcium assay was determined to be 0.77/0.11 nM for short-term competitive interaction and 10/1.4 nM for long-term noncompetitive versus 10 nM DAMGO. Thus the K_i we found at about 1.4 nM, was consistent with the reported value for μ opioid receptor β-FNA binding (Tam and Liu-Chen, 1986). Therefore 2 nM was considered to be an accurate approximation for the K_d parameter in the one site binding equation $B/B_{max}=L/(L+K_d)$ (GraphPad Prism, Version 3.0). These parameters were then used to calculate receptor number remaining after β-FNA pretreatment. Thus at 10 nM β-FNA 83% of the original receptor pool would have been blocked. When the IC_{50}s for the agonists obtained in the presence of β-FNA, a rank ordering of calcium activation was obtained. IC_{50} calculations for DAMGO, oxymorphone, 6α-oxymorphol, 6β-oxymorphol and oxycodone were 200, 15, 26, 3 and 4 nM β-FNA, respectively. Based on the estimates from Table 4, the IC_{50} for 6α-oxymorphol corresponds to ~13% of the remaining free receptor fraction, while the IC_{50} for 6β-oxymorphol corresponds to ~23% of the free receptor fraction. This analysis confirms that a relatively higher concentration of β-FNA was required to suppress calcium activity induced with 6α-oxymorphol at equivalent concentrations of the diastereomers.

4. Discussion

Agonist receptor reserve was used to differentiate structurally-related opioid diastereomers that exhibit similar biological functionality. Agonist-mediated calcium response characteristics of CHO cells transfected with the human μ-opioid produced results comparable to the stimulation of [^{35}S]GTPγS activation found for oxymorphone and oxycodone (Thompson et al., 2004). The hydroxyoxymorphone metabolites 6α and 6β oxymorphols, were shown to have identical μ-opioid receptor binding affinities and to exhibit similar intrinsic efficacy ($\%E_{max}$) and potency (EC_{50}). Differential *in vivo* μ-opioid receptor reserve has been demonstrated to mediate different actions of morphine including locomotion, reward and dependence (Sora et al., 2001). The present data add to this by demonstrating that antinociception is also mediated by functional μ-opioid receptor reserves. Further, insurmountable or long-lasting antagonists can reveal receptor reserve by antagonizing a variety of agonist-mediated effects including [^{35}S]GTPγS activation in CHO cells and thalamic tissue (Selley et al., 1998), and rodent antinociception, (Zernig et al., 1995; Peckham et al., 2005). In

these studies differential μ-opioid receptor fractions blocked with β-FNA, an irreversible μ-opioid receptor antagonist, were activated with the agonists 6α and 6β oxymorphols. That is, since about a 10-fold higher concentration of β-FNA was required to depress the same magnitude of calcium activity induced by 6α-oxymorphol as that induced by 6β-oxymorphol, a greater receptor reserve for the 6α is indicated. In addition, the IC_{50} for β-FNA in the presence of 6α-oxymorphol indicated that a relatively larger fraction of receptors were blocked. Receptor conformational changes were consistent for each of the agonists under our experimental conditions. Reduction of agonist binding affinity in opioid receptors (Selley et al., 2000) and increases in binding affinity with β-FNA in the presence of NaCl (Chen et al., 1995) are well documented. Taken together, these data provide a demonstration that the oxymorphols are associated with differential receptor reserves. The order of intrinsic efficacy of the oxymorphols was reflected across different *in vivo* assays of antinociception where the 6α exhibited greater relative potency than 6β oxymorphol.

To underscore the differences in fractional occupancy between the diastereomers, we estimated the range of receptor reserves based on concentration-dependent β-FNA blockade. Receptor blockade at 10 nM β-FNA corresponded to an available pool of 14,000 receptors per cell, or 17% unblocked receptors. From our estimates 56,000 unblocked receptors per cell were remaining at 1 nM and 1650 receptors were remaining at 100 nM β-FNA. This would indicate that an insufficient fraction of receptors were blocked at 1 nM β-FNA, necessary to resolve a difference in calcium activation between the diastereomers, whereas at 100 nM β-FNA a larger fraction of receptors were blocked that inhibited calcium activity for both of the diastereomers. Thus differences in receptor-mediated calcium activation between the diastereomers could be resolved at 10 nM β-FNA. Such a range of functional receptors is consistent with the results of Fehman et al. (1998). These authors created several clones of another G-protein-coupled receptor, the GLP-1 (Glucagon-like peptide I). Between 1800 and 5600 receptors per cell was insufficient for production of an agonist-mediated cAMP response whereas cells that expressed between 13,000 and 380,000 receptors could produce the response.

We have also assessed the relationship between endogenous levels of receptor and the level of expression of the cloned human μ-opioid receptor in our system. CHO cell human μ-opioid receptor levels are approximately 700 fmol/mg protein (data not shown). This is about 5–10 times the endogenous expression of the μ-opioid receptor reported (as fmol/mg protein) in SH-SY5Y cells (77), mouse brain (130), mouse spinal cord (124), rat brain (150) and rat spinal cord (145), respectively (Zhao et al., 2003). At 10 nM β-FNA, where we can differentiate receptor reserves for the diastereomers, we estimate the level of functional μ-opioid receptor to be about 11 to 21% or about 70–100 fmol/mg protein, within the expression level estimated by Zhao et al. (2003).

Different opioid agonists generally induce formation of receptor conformations resulting in different signaling complexes. For example, morphine and its synthetic congener etorphine stimulate response patterns of extracellular and intracellular calcium activation differently, suggesting functionally distinct binding site conformations (Quillan et al., 2002). In the case of the μ-opioid receptor there is evidence supporting the hypothesis that different agonists preferentially induce desensitization by different mechanisms (reviewed in Kelly et al., 2008). In addition, some β2AR agonists selectively activate β-arrestin mediated signaling rather than Gs-mediated signaling (Shenoy and Lefkowitz, 2005; DeWire et al., 2007; Shukla et al., 2008). The different intrinsic efficacy of the oxymorphols may likewise to some extent result from different agonist-receptor conformations. However the present results provide a clear role for fractional occupancy in mediating differences in intrinsic efficacy reflected by different agonist receptor reserves. In this case, it is not likely that β-FNA produced changes in ligand receptor affinity that would have been linked to agonist intrinsic efficacy. First of all the addition of β-FNA did not change the agonist K_i values for [^{3}H]-diprenorphine binding, indicating that receptor binding affinity was not substantially altered by pretreatment with β-FNA. This is consistent with the effects of NaCl and β-FNA linear binding kinetics as demonstrated by Chen et al. (1995). Secondly, it has been shown that a reduction in [^{3}H]-diprenorphine binding sites to about 23% of control levels can be produced with 10 nM β-FNA with no change in receptor affinity (Alt et al., 2001). Finally, evaluations of kinetic rate constants for μ-opioid receptor expressing membrane fragments and μ-opioid receptor expressing CHO cells have been reported to be in close agreement (Liu-Chen et al., 1990; Spivak and Belgan, 2004). Therefore it is not likely that agonist-mediated calcium stimulation would be due to changes in μ-opioid receptor affinity state as a consequence of pretreatment with β-FNA.

The antinociceptive potency of the agonists exhibited a general rank order of: oxymorphone > 6α-oxymorphol > 6β-oxymorphol = morphine as demonstrated in the tail-flick, hot plate and writhing assays. The relative potency of the 6α diastereomer was greater than the 6β. Oxymorphols administered either s.c. or p.o., have a similar potency order of 6α > 6β. Plasma pharmacokinetics is also similar for the oxymorphols up to the 1 h time interval during antinociceptive testing. Thus we attribute the action of the oxymorphols to receptor activation rather than to metabolic differences. The present overall potency values were in range of ED_{50} values in the rat warm-water tail-withdrawal assay previously reported for the codones and morphine (Peckham and Traynor, 2005). Oxymorphone (Metzger et al., 2001) and the 6α and 6β oxymorphols (data not shown) are both selective for human μ, versus kappa and delta opioid-receptor clones. Alternatively, oxycodone has been reported to have significantly more activity at the kappa-opioid receptor subtype (Ross et al., 2000) but exhibits an ED_{50} of 3.24 mg/kg-s.c. in mouse tail-flick (Duttaroy and Yoburn, 1995) similar to our *in vivo* doses for the oxymorphols.

In drug discovery structure–activity relationships are usually built around receptor binding affinity profiles that separate ligands often associated with complex stereochemistry. However, the correspondence of chemical structure to biological efficacy may not be immediately evident without extensive *in vivo* testing. The present results highlight the importance of different receptor reserve in bridging *in vivo* and *in vitro* results and in providing for a unique approach in the prediction of efficacy and potency in the search for new analgesic drugs.

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