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Intrinsic Relative Activities of Opioid Agonists in Activating Gα proteins and Internalizing Receptor: Differences between Human and Mouse Receptors

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Abstract

Several investigators recently identified biased opioid receptor (KOP receptor) agonists. However, no comprehensive study of the functional selectivity of available KOP receptor agonists at the human and mouse KOP receptors (hKOP receptor and mKOP receptor, respectively) has been published. Here we examined the ability of over 20 KOP receptor agonists to activate G proteins and to internalize the receptor. Clonal neuro-2a mouse neuroblastoma (N2a) cells stably transfected with the hKOP receptor or mKOP receptor were used. We employed agonist-induced [^{35}S]GTPγS binding and KOP receptor internalization as measures of activation of G protein and β-arrestin pathways, respectively. The method of Ehlert and colleagues was used to quantify intrinsic relative activities at G protein activation (RA_{i-G}) and receptor internalization (RA_{i-I}) and the degree of functional selectivity between the two [Log RA_{i-G} − Log RA_{i-I}, RA_{i-G}/RA_{i-I} and bias factor]. The parameter, RA_i, represents a relative estimate of agonist affinity for the active receptor state that elicits a given response. The endogenous ligand dynorphin A (1–17) was designated as the balanced ligand with a bias factor of 1. Interestingly, we found that there were species differences in functional selectivity. The most striking differences were for 12-epi-salvinorin A, U69,593, and ICI-199,441. 12-Epi-salvinorin A was highly internalization-biased at the mKOP receptor, but apparently G protein-biased at hKOP receptor. U69,593 was much more internalization-biased at mKOP receptor than hKOP receptor. ICI199,441 showed internalization-biased at the mKOP receptor and G protein-biased at the hKOP receptor. Possible mechanisms for the observed species differences are discussed.

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CONFLICT OF INTERESTS

There is no conflict of interests.

Keywords

ligand bias; kappa opioid receptor; species difference

1. INTRODUCTION

Functional selectivity, also known as ligand bias or biased signaling, is a relatively recent finding for seven-transmembrane receptors (7TMRs) [reviewed in (Urban et al., 2007; Whalen et al., 2011; Wisler et al., 2014)]. Traditionally, activation of a 7TMR produces signaling through G proteins to regulate second messengers. Repeated agonist exposure causes arrestins-mediated desensitization and internalization of 7TMRs. However, it has recently been shown that arrestins also serve as scaffolds for other second messenger pathways leading to a variety of responses [reviewed in (Lefkowitz and Shenoy, 2005)]. Biased agonists which preferentially activate G protein or arrestin pathways, have been found for many 7TMRs, including μ, δ and opioid receptors (Pradhan et al., 2010; Rivero et al., 2012; Rives et al., 2012; DeWire et al., 2013).

KOP receptor agonists produce analgesic effects (von Voigtlander et al., 1983), anti-scratching behaviors (Togashi et al., 2002; Inan and Cowan, 2004) and water diuretic effects (Slizgi and Ludens, 1982; Leander, 1983) in animal models. KOP receptor agonists produce analgesia without respiratory depression seen with MOPR agonists [reviewed in (Martin, 1983)]. The usefulness of KOP receptor agonists in humans, however, is limited by dysphoria (an unpleasant or aversive state) and psychotomimetic effects these compounds cause (Pfeiffer et al., 1986). The only selective KOP receptor agonist used clinically is nalfurafine, which is used in Japan for the treatment of pruritus in kidney dialysis patients (Kumagai et al., 2010; Kumagai et al., 2012).

Chavkin and colleagues have suggested that antinociception produced by KOP receptor agonists is mediated by the G protein pathway (McLaughlin et al., 2004), whereas dysphoria is mediated by β arrestin-dependent p38 MAP kinase phosphorylation (Bruchas et al., 2007). Therefore, several groups have been actively searching for G protein-biased KOP receptor agonists (Rives et al., 2012; Schmid et al., 2013; Zhou et al., 2013; White et al., 2014) to circumvent the dysphoric effects. However, recently White et al. (2015) showed that the unbiased agonist U69,593 or salvinorin A, or the G protein-biased agonist RB-64 produced similar levels of conditioned place aversion in wildtype and β-arrestin2−/− mice, indicating that either β-arrestin2 is not involved or there are other pathways involved in the aversive effect besides β-arrestin2. In addition, these authors demonstrated while G protein signaling was involved in KOP receptor-mediated antinociception, β arrestin-2 pathway might be associated with motor incoordination. Moreover, RB-64 did not induce sedation or anhedonia-like effects, unlike the unbiased agonists U69,593 and salvinorin A.

Several groups have found some KOP receptor agonists to be G protein-biased at the human KOP receptor (hKOP receptor), including 6'-GNTI (Rives et al., 2012; Schmid et al., 2013; White et al., 2014), triazole and isoquinolinone analogues (Zhou et al., 2013), dynorphin A and its shorter peptides and salvinorin A analogues (White et al., 2014). In addition, balanced agonists and β-arrestin-biased agonists have been reported (Schattauer et al., 2012;

White et al., 2014). Schattauer et al. (2012) compared functional selectivity of four KOP receptor partial agonists and U50,488H between hKOP receptor and rat KOP receptor (rKOP receptor) and found species differences for butorphanol and pentazocine, but not for levorphanol, nalorphine and U50,488.

Drug discovery inevitably involves experimentation on animals, particularly rodents, before proceeding to higher animals. Although comparison between human and rodent receptors is commonly performed in industry, the data are not readily available in the literature. This study sought to quantify the extent of ligand bias for a number of different KOP receptor agonists *in vitro* at both the hKOP receptor and mouse KOP receptor (mKOP receptor) and determine if there are species differences in ligand bias. We used N2a cells transfected with the hKOP receptor or mKOP receptor and performed [^{35}S]GTPγS binding as a measure of G protein activation and the on-cell western (OCW) assay as a measure of β-arrestin-mediated receptor internalization. We then used an approach, originally developed and refined by Ehlert and colleagues (Griffin et al., 2007; Ehlert, 2008; Ehlert et al., 2011b), to estimate the relative affinity constant of an agonist for the active receptor state that elicits the response [intrinsic relative activity (RA_i)]. We used this analysis to detect differences in ligand affinity for the active receptor states that engage G proteins and β-arrestins following receptor activation. We have measured the difference in RA_i for the two pathways to quantify the degree of bias for each ligand. Interestingly, we have found that for several agonists, there are differences in functional selectivity between hKOP receptor and mKOP receptor.

2. MATERIALS AND METHODS

2.1. Materials

[^{35}S]GTPγS (1250 Ci/mmol), [15, 16-^{3}H]diprenorphine (36–50 Ci/mmol), [Phenyl-3, 4-^{3}H]U69,593 (43.6 Ci/mmol) were purchased from PerkinElmer Life Sciences (Boston, MA); EGTA, EDTA, anti-FLAG (M1), polyethyleneimine, formalin, paraformaldehyde (PFA), compound 48/80, Kolliphor EL, leupeptin hydrochloride, bovine serum albumin (BSA), phenylmethylsulfonyl fluoride (PMSF), GDP and GTPγS were purchased from Sigma-Aldrich (St. Louis, MO). HBSS with Ca^{2+} and Mg^{2+} and geneticin (G418) were purchased from Cellgro Mediatech, Inc. (Herndon, VA). Minimal essential medium (MEM), trypsin and penicillin/streptomycin were purchased from Gibco Life Technologies (Grand Island, NY). Dynorphin A (1–17) and dynorphin B were purchased from Phoenix Pharmaceuticals (Belmont, CA). IRDye 800CW goat anti-mouse, Sapphire 700, DRAQ5 and blocking buffer were purchased from LI-COR, Inc. (Lincoln, NE). The following reagents were purchased from the indicated companies: bicinchoninic acid assay (BCA) reagents, Thermo Fisher Scientific, Inc. (Rockford, IL); GF/B glass filters, Brandel, Inc. (Gaithersburg, MD); EcoScint scintillation fluid, National Diagnostics (Atlanta, GA); fetal bovine serum (FBS), Atlanta Biologicals (Atlanta, GA); anti-HA monoclonal antibody HA-11 clone 16B12 MMS-101R, Covance (Princeton, NJ).

The following drugs were generously provided by the indicated companies/institutions: naloxone, U50,488H, ethylketocyclazocine (EKC), butorphanol, ICI-199441, nalbuphine, nalorphine, levorphanol, pentazocine, etorphine HCl and β-funaltrexamine (β-FNA) by the

National Institute on Drug Abuse (Bethsada, MD); bremazocine by Sandoz (Basle, Switzerland); enadoline by Parke-Davis (Cambridge, UK); tifluadom by ICI (Macclesfield, UK); spiradoline (U62,066) and U69,593 from Upjohn Co. (Kalamazoo, MI). 12-epi-Salvinorin A (12epiSalA) was synthesized in the laboratory of Dr. Thomas Prisinzano at the University of Kansas (Lawrence, KS). Ethoxymethyl ester of salvinorin B (EOM-SalB), methoxymethyl ester of salvinorin B (MOM-SalB) and salvinorin A (SalA) were provided by the laboratory of Dr. David Y. Lee at McLean Hospital at Harvard University (Belmont, MA)

2.2. Cell lines and membrane preparation

N2a cells stably transfected with the FLAG-tagged mKOP receptor or 3xHA-tagged hKOP receptor (N2a-FLAG-mKOP receptor and N2a-3HA-hKOP receptor cells, respectively) were established as described previously (Xu et al., 2000; Chen et al., 2011). Cells were cultured in 100-mm culture dishes in MEM supplemented with 10% FBS, 0.2 mg/ml geneticin, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere consisting of 5% CO_2 and 95% air at 37°C.

Membranes were prepared according to a modification of our published procedures (Zhu et al., 1997; Wang et al., 2005). Cells were washed twice and harvested in 1× PBS containing 0.5 mM EDTA and centrifuged at 500 *g* for 3 min. The cell pellet was suspended in lysis buffer (25 mM Tris, pH 7.4, 1 mM EDTA and 0.1 mM PMSF), passed through a 26 3/8-gauge needle 10 times and then centrifuged at 46,000 g for 30 min. The pellet was rinsed twice with lysis buffer and resuspended in 50 mM Tris-HCl buffer / 0.32 M sucrose (pH 7.4), aliquoted and frozen in dry ice/ethanol, and stored at −80°C. Protein concentration was determined by the bicinchoninic acid (BCA) assay. All procedures were performed at 4°C.

2.3. Saturation binding with [^{3}H]diprenorphine

To determine the K_d and B_{max} of the two cell lines being used, we performed saturation binding with 25–40 μg membrane protein per reaction and 11 concentrations of [^{3}H]diprenorphine were used −0.025, 0.05, 0.075, 0.1, 0.2, 0.3, 0.4, 0.5, 1, 1.5, and 2 nM. Binding was performed in 50 mM Tris-HCl buffer containing 1 mM EGTA (pH 7.4) in a final volume of 1 ml. Nonspecific binding was determined in the presence of the opioid antagonist naloxone (10 μM). The reaction mixture was incubated for 1 hr at room temperature and terminated by filtration under reduced pressure with GF/B filters presoaked in 0.2% polyethyleneimine and 0.1 mg/ml BSA. Filters were washed three times with ice-cold 50 mM Tris-HCl buffer containing 0.15 M NaCl (pH 7.4). Radioactivity on filters was determined by liquid scintillation counting. All experiments were performed in duplicate and repeated at least three times.

2.4. Ligand-stimulated [^{35}S]GTPγS binding

[^{35}S]GTPγS binding was performed following a modified protocol of our published method (Zhu et al., 1997; Wang et al., 2005). Briefly, membranes (containing 10 μg protein) were incubated with 20–25 μM GDP and ~0.4 nM [^{35}S]GTPγS in reaction buffer (50 mM HEPES, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, pH 7.4) and various concentrations of a ligand in a final volume of 0.5 ml. Reaction mixtures were incubated for 1 hr at 30°C.

Nonspecific binding was determined in the presence of 10 μM GTPγS. Subsequently, bound and free [^{35}S]GTPγS were separated by filtration with GF/B filters under reduced pressure and the filter was washed with ice-cold buffer containing 50 mM Tris (pH 7.6), 5 mM $MgCl_2$ and 50 mM NaCl. Radioactivity in filters was determined by liquid scintillation counting. All experiments were performed in duplicate and repeated at least three times. [^{35}S]GTPγS binding measurements were normalized relative to the maximal response of U50,488H

2.5. Measurement of receptor internalization with on-cell western assay

The following is a modified protocol from Zhao et al. (2010). Cells were plated at a density of 20,000–30,000 cells/well in 96-well plates and 40 hr later media was aspirated. Cells were incubated with each test drug at various concentrations (0.01 nM – 10 μM) in serum-free MEM for 1 h. Cells were rinsed with cold PBS containing Ca^{2+} and Mg^{2+} and incubated with a mouse monoclonal anti-HA antibody (4 μg/ml) for the hKOP receptor or mouse monoclonal M1 anti-FLAG antibody for the mKOP receptor (6 μg/ml, in 3% BSA + HBSS) on ice for 1 hr at 4°C. They were rinsed and fixed with 4% paraformaldehyde for 10 min. After washing, blocking was performed for 1 hr with LI-COR blocking buffer followed by a 1 hr-incubation in the dark with LICOR 800CW-conjugated goat anti-mouse antibody (1:800 in blocking buffer), which labeled cell-surface receptors in green. Cells were also co-stained with two cellular stains: Sapphire700 and DRAQ5 (1 mM) (1:1000 and 1:2000, respectively), which stained the nuclei and cytoplasm with a red color and was used to normalize for well-to-well variation in cell number. Overlapping cell stain (red) and receptor immunostaining (green) were measured with an Odyssey IR image analysis system (LI-COR Bioscience). Quantitation was performed by taking the ratio of the intensity of the 800 channel (green, receptor) divided by the intensity of the 700 channel (red, cell stain) and comparing to that of the control. A minimum of three independent dose-response experiments were performed in triplicate for each agonist.

2.6. Log RA_i and bias calculation

Intrinsic reactive activity (*RAi*) of each agonist was estimated by global nonlinear regression analysis using the following two equations (Griffin et al., 2007; Ehlert, 2008; Ehlert et al., 2011b):

$$y=\frac{M_{sys}}{1+\left(\frac{1+10^{\log X'+\log K'}}{10^{\log X'+\log R'}}\right)^{m}} \quad 1$$

$$y=\frac{M_{sys}}{1+\left(\frac{1+10^{\log X'+\log K}}{10^{\log X+\log R'+logRA_i}}\right)^{m}} \quad 2$$

The parameters of the standard agonist [dynorphin A (1–17)] are denoted with an apostrophe. In these equations, *y* represents the measured response, M_{sys}, the maximal response of the signaling pathway, *m*, the transducer slope factor, *X*, the concentration of agonist, and *K*, the observed affinity constant of the agonist (inverse concentration units, e.g., M^{-1}). Note that *K* represents the reciprocal of the more commonly used observed

dissociation constant (concentration units, e.g., M). The parameters *R'* and RA_i can be expressed in terms of other parameters as described by the following equations:

$$R' = \tau' K' \quad 3$$

$$RA_i = \frac{\tau K}{\tau' K'} \quad 4$$

In these equations, the parameter τ is proportional to ligand efficacy (fraction of ligand-receptor complexes in the active state) and receptor density and inversely proportional to the sensitivity constant of the signaling pathway (K_E). It has been shown that the τ*K* value of an agonist is proportional to its affinity constant for the active state of the receptor (K_{act}). The proportionality constant is equivalent to the τ value of the unoccupied receptor (τ_{sys}). This latter parameter represents the fraction of the population of unoccupied receptors in the active state (ε_{sys}) divided by the sensitivity constant of the operational model (K_E) ($\tau_{sys} = \varepsilon_{sys}/K_E$). Therefore, RA_i is a relative estimate of the affinity constant of a ligand for the active receptor state (K_{act}) (Tran et al., 2009):

$$RA_i = \frac{K_{\mathrm{act}}}{K'_{\mathrm{act}}} \quad (5)$$

In cases where more than one active receptor state contributes to same response, K_{act} represents a weighted average value.

All of the agonist concentration-response curves for a specific output response (i.e., ligand-induced [^{35}S]GTPγS binding or receptor internalization) and receptor species were analyzed simultaneously using global nonlinear regression analysis with equations 1 and 2. In this analysis, equation 1 was fitted to the data for the standard agonist, and equation 2 was fitted to the data of the various tests agonists. The standard agonist refers to the agonist to which the estimates of RA_i are normalized. Dynorphin A was designated as the standard because it is an endogenous ligand for the KOP receptor. Regression analysis was done sharing the estimates of M_{sys}, *m* and *R'* among the data. Unique estimates of *K'* and *R'* were obtained for the standard agonist and of *K* and RA_i for each test agonist together with their asymptotic standard errors. In all instances it was impossible to obtain reliable estimates of *K* or *K'* for full agonists. Nonetheless, it was always possible to obtain a least squares fit and estimates of *R'* and RA_i as described previously (Griffin et al., 2007). This behavior underscores the independent nature of the microscopic constant, K_{act}, and hence of RA_i, as compared to that of the dependent population parameters, *K* and τ.

The difference between the log RA_i values of an agonist for the [^{35}S]GTPγS-binding (log RA_{i-G}) and receptor-internalization (log RA_{i-I}) assays was estimated as a measure of the log agonist-bias [log (RA_{i-G}/RA_{i-I})]. Estimates of this difference exhibit a *Student*'s t-distribution, and hence, its SEM was estimated from the sum of the variances of the individual estimates. Agonists with positive log bias are G protein-biased; those with negative log values are internalization biased. Finally, for each agonist, the larger of its two RA_i values (RA_{i-G} or RA_{i-I}) was divided by the smaller to estimate the bias factor. For

example, an agonist with a log (RA_{i-G}/log RA_{i-I}) value of 1.0 would exhibit a bias factor denoted as, G, 10, indicating a tenfold selectivity for G protein signaling. Conversely, an agonist with a log (RA_{i-G}/log RA_{i-I}) value of −1.0 would exhibit a bias factor denoted as, I, 10, indicating a tenfold selectivity for the receptor internalization pathway. These bias factors are listed in Tables 2 and 4.

3. RESULTS

3.1. Expression levels of mKOP receptor in N2a-FLAG-mKOP receptor cells and hKOP receptor in N2a-3HA-hKOP receptor cells

Saturation binding of [^{3}H]diprenorphine to mKOP receptor or hKOP receptor in membrane preparations was performed using 11 concentrations of [^{3}H]DIP. The K_d and B_{max} values were calculated. The K_d values of [^{3}H]diprenorphine binding to both FLAG-mKOP receptor and 3HA-KOP receptor were similar at 0.30 ± 0.06 and 0.19 ± 0.004 nM (n=3), respectively. FLAG-mKOP receptor and 3HA-hKOP receptor had B_{max} values of 5.48 ± 0.52 pmol/mg protein and 1.61 ± 0.08 pmol/mg protein (n=3), respectively.

3.2. hKOP receptor

3.2.1. [^{35}S]GTPγS binding and receptor internalization—[^{35}S]GTPγS binding was used as the functional measure of agonist-induced G protein activation. Agonist-promoted receptor internalization was determined by OCW assay. Assays were performed for 21 agonists at the hKOP receptor. Most agonists activated G proteins and internalized receptor in a dose-dependent fashion. Agonists showed varying efficacies in the two end points.

Fig. 1 shows the concentration-response curves of dynorphin A, U50,488H, nalorphine, etorphine and MOM-SalB for inducing [^{35}S]GTPγS binding (A) and receptor internalization (B) at the hKOP receptor. A total of 21 agonists were assayed in this way, and their corresponding EC_{50} and E_{max} values for both responses are shown in Table 1.

For [^{35}S]GTPγS binding, the EC_{50} values ranged from 0.24 nM (ICI199,441) to 333 nM (tifluadom) and the E_{max} values from 20.5% (pentazocine) to 108% (U69,593 and MOM-SalB) of that of U50,488H.

For receptor internalization, the E_{max} values are expressed as a percent of total plasma membrane receptor before agonist treatment. The agonists 12epiSalA, etorphine, and levorphanol did not cause significant internalization. The E_{max} values of the other agonists ranged from 23.9% (nalorphine) to 64.3% (salvinorin A). The EC_{50} values ranged from 1.4 nM (bremazocine) to 92 nM (pentazocine) (Table 1).

3.2.2. Estimation of log RA_i values and log agonist bias values (log RA_{i-G} − log RA_{i-I})—The log RA_i values of the agonists for stimulating [^{3}H]GTPγS binding (log RA_{i-G}) and receptor internalization (log RA_{i-I}) were estimated from the agonist concentration-response curves and summarized in Table 2. The log bias value, which is normalized relative to that of dynorphin A, was estimated for each agonist, and these are also summarized in Table 2. Positive log values indicate a bias for G protein signaling, whereas negative values indicate a bias for receptor internalization. As shown in Table 2,

log bias values ranged from internalization bias (−1.46) for salvinorin A to G protein bias (0.75) for ICI-199441. The antilog of the absolute values of the log bias values yields bias factors.

Only ICI-199, 441 had higher activity for G protein activation with a bias factor of G, 5.7. The following agonists were internalization-biased with the rank order of salvinorin A (I, 29.0) > nalbuphine (I, 27.6) > tifluadom (I, 11.8) >, enadoline (I, 4.2) > EKC (I, 3.1) > U69,593 (I, 2.9).

Pentazocine, butorphanol, nalorphine, U50,488H, β-FNA, bremazocine, EOM-SalB, MOM-SalB, dynorphin B and spiradoline had estimated bias factors; however, the biases did not reach statistical significance. The results indicate that these agonists have similar activities for both responses.

The log bias values could not be calculated for 12epiSalA, etorphine and levorphanol because these ligands lacked an effect on receptor internalization. Although it seems that these agonists should be G protein biased, it is useful to first consider that the standard agonist, dynorphin A, displayed three-fold lower potency in the internalization assay compared to its effect in the [^{35}S]GTPγS assay. The latter results suggest that the [^{35}S]GTPγS assay exhibits greater signal amplification following receptor activation. Thus, even if an agonist lacked bias, it would be difficult to detect an internalization response for it if it behaved as a partial agonist in the [^{35}S]GTPγS assay. It is therefore inappropriate to designate levorphanol as G protein biased given that it behaves as a weak partial agonist in the [^{35}S]GTPγS assay [rationale described in (Griffin et al., 2007)]. However, both 12epiSalA and etorphine behave as full and strong partial agonists, respectively, in the [^{35}S]GTPγS assay, yet they lack significant positive effects in the internalization assay. Consequently, we designate these latter agonists as G protein biased.

3.3. mKOP receptor

3.3.1. [^{35}S]GTPγS binding and receptor internalization—Twelve of the 21 ligands were tested at the mKOP receptor for the following reasons. ICI-199,441, U50,488H, enadoline, salvinorin A EOM-SalB, MOM-SalB, and 12epiSalA were selected because of their high selectivity for the KOP receptor. Levorphanol, nalbuphine and pentazocine were partial agonists at the hKOP receptor, some of which were shown to have ligand bias at the hKOP receptor in a previous study (Schattauer et al., 2012). Etorphine was demonstrated to be a full agonist for [^{35}S]GTPγS binding, but induced low level or no hKOP receptor internalization (Li et al., 2003). Dynorphin A was again designated as the standard agonist.

Fig. 2 shows dose-response curves of enandoline, dynorphin A, MOM-SalB, etorphine and nalbuphine in promoting [^{35}S]GTPγS binding (A) and internalization (B) at the mKOP receptor. Etorphine and nalbuphine did not cause mKOP receptor internalization.

Most agonists activated G proteins and internalized the receptor in a concentration-dependent fashion. Agonists showed varying efficacies for the two end points. The EC_{50} and E_{max} values of the agonists at the mKOP receptor for both endpoints are listed in Table 3.

For [^{35}S]GTPγS binding, the EC_{50} values ranged from 0.060 nM (ICI199,441) to 69 nM (nalbuphine) and the E_{max} values ranged from 46.6% (pentazocine) to 104% (12epiSalA) that of U50,488H.

Etorphine, nalbuphine and pentazocine did not cause significant internalization. For the other agonists, the E_{max} values ranged from 29.7% (levorphanol) to 47.2% (ICI-199,441) of cell surface mKOP receptor being internalized. The EC_{50} values ranged from 0.18 nM (U69,593) to 31.1 nM (MOM-SalB).

3.3.2. Estimation of log RA_i values and agonist bias factors (log RA_{i-G} − log RA_{i-I})—The log RA_i and log bias values were estimated as described above and expressed relative to those of the designated balanced agonist dynorphin A (Table 4). Etorphine, pentazocine and nalbuphine lacked significant effects on receptor internalization. Hence, their log RA_{i-I} values for this response and associated log bias values could not be estimated. The following agonists are internalization-biased with the rank order of U69,593> levorphanol > 12-epi-Salvinorin A > U50,488H > ICI-199441 > EOM-SalB. Although enadoline, salvinorin A and MOM-SalB had mean bias values in the direction of small internalization- or G protein-bias, the values lacked statistical significance.

4. DISCUSSION

We have found that several KOP receptor agonists display biased signaling at both hKOP receptor and mKOP receptor expressed in N2a cells (see Tables 2 and 4), using G protein activation and receptor internalization as two functional end points. Interestingly, there are species differences in functional selectivity of some agonists, most notably 12-epi-salvinorin A, ICI199,441, U69,593, salvinorin A and U50,488H. To the best of our knowledge, this is the first report examining a structurally diverse group of KOP receptor agonists at both mKOP receptor and hKOP receptor and demonstrating species differences in functional selectivity of full KOP receptor agonists. In addition, a salient feature of our study is that we used the same cell line for both functional endpoints to avoid possible confounding effects.

To assay G protein activation, we measured ligand-induced stimulation of [^{35}S]GTPγS binding (0.4 nM). The concentration of guanine nucleotide (i.e., GDP, 0.20 – 0.25 mM) in our assay was much lower than the average concentrations of GTP and GDP measured in a variety of cells (0.47 and 0.15 mM, respectively) (Traut, 1994). When the concentration of guanine nucleotide is low, the barrier to receptor activation is reduced, and the relative efficacy values of partial agonists will increase and approach that of a full agonist. This perturbation in ligand efficacy has no influence on the estimate of RA_i, however, because this constant is a relative estimate of the active state affinity constant (Ehlert et al., 2011a).

4.1. Comparison of relative intrinsic activities of agonists at the hKOP receptor and the mKOP receptor

For convenience, the log bias factors of those agonists with measurable values at both the human and mouse receptors are summarized in Table 5 with dynorphin A as the designated balanced agonist. For the remainder of the discussion, we use the response bias factor to quantify agonist bias. This factor represents the antilog of the absolute value of the log bias

factor. For example, an agonist with a log bias factor (log (RA_{i-G}/RA_{i-I})) of 1.0 would exhibit a *G protein* bias factor of G, 10, whereas an agonist with a log bias factor of −1.0 would exhibit and *internalization* bias factor of I, 10.

Many agonists showed species differences with the greatest differences for 12-epi-salvinorin A, ICI199,441, U69,593, salvinorin A, and U50,488H. The agonist 12-epi-salvinorin A was strongly internalization-biased at the mKOP receptor (I, 208), but did not cause internalization of the hKOP receptor. ICI-199,441 was internalization-biased at the mKOP receptor (I, 10), but G protein-biased at the hKOP receptor (G, 5.7). U69,593 was highly internalization biased at the mKOP receptor (I, 627), but only slightly so at the human receptor (I, 2.9). Salvinorin A displayed internalization-bias at the hKOP receptor, (I, 29), but had similar activity in both pathways at the mKOP receptor (I, 1.8). Conversely, U50,488H displayed internalization-bias at the mKOP receptor (I, 26), but had similar activities for both responses at the hKOP receptor (I, 1.7).

Other compounds also displayed species differences, but not to as great an extent. EOM-SalB was internalization-biased at the mKOP receptor (I, 6.9), but showed balanced activity at the hKOP receptor (I, 1.2). The reverse is true for enadoline, being internalization-biased at the hKOP receptor (I, 4.2), but un-biased at the mKOP receptor. Pentazocine and MOM-SalB did not display significant biases at both hKOP receptor and mKOP receptor.

The following agonists did not cause receptor internalization: at hKOP receptor, 12epiSalA and levorphanol; at the mKOP receptor, nalbuphine and pentazocine; and at both hKOP receptor and mKOP receptor, etorphine. Bias values could not be calculated for these ligands. Nonetheless, we designated 12epiSalA as G protein biased for the rationale described above. Nalbuphine displayed internalization-biased at the hKOP receptor (I, 27.6), whereas levorphanol had strong internalization-biased at the mKOP receptor (I, 244).

Using salvinorin A as the reference unbiased agonist, White et al. (2014, 2015) showed that RB-64 was highly G protein-biased at both hKOP receptor and mKOP receptor and U69,593 was unbiased at both receptors.

Whether the observed species differences in functional selectivity of KOP receptor agonists occur *in vivo* remains to be investigated.

4.2. Possible cellular mechanisms for the species differences

We have recently determined sites of U50,488H-induced phosphorylation in both the hKOP receptor and mKOP receptor by LC-MS/MS (unpublished data). U50,488H (10 μM, 30 min) treatment promotes hKOP receptor phosphorylation at S356, T357, S358 and T363 with T363 having the most hits (Supplemental Materials, Fig. S1). The same treatment enhances phosphorylation of the mKOP receptor at S356, T357, T363 and S369 with S369 having the highest number of hits (Supplemental Materials, Fig. S1). Therefore, there are difference in sites of phosphorylation, namely S358 in the hKOP receptor and S369 in the mKOP receptor. In addition, the highest phosphorylated sites are T363 in the hKOP receptor and S369 in mKOP receptor. Because of these differences, phosphorylated mKOP receptor and

hKOP receptor may recruit downstream effectors differently, including β-arrestins, leading to differences in responses.

4.3. Differences in pharmacological effects of G protein-biased versus unbiased KOP receptor agonists *in vivo*

Whether biased KOP receptor agonists produce different *in vivo* pharmacological profiles from unbiased agonists is in the initial stage of investigation. RB-64, a highly G proteinbiased agonist, caused analgesia and aversion in mice, similar to the unbiased agonists U69,593 and salvinorin A. In contrast, while U69,593 and salvinorin A produced sedation and anhedonia-like actions, RB-64 did not (White et al., 2015).

4.4. Compounds with the same scaffold do not have similar biases

Several arylacetamides were examined in the current study. They did not display similar functional selectivity ranging from being G-biased to arrestin-biased. At the hKOP receptor, the agonists and their bias values are ICI199,441 (G, 5.7), spiradoline (G 1.8), U50,488H (I, 1.7), U69,593 (I, 2.9), and enadoline (I, 4.2). The bias values for spiradoline and U50,488H lacked statistical significance, however. At the mKOP receptor, enadoline, ICI199,441 and U50,488H were all internalization-biased with bias values of I, 3.2 (not significant), I, 10 and I, 26, respectively. White et al. (2014) also reported varying bias values among a group of arylacetamides at the hKOP receptor.

The salvinorin A analogues did not exhibit similar functional selectivity, either. At the hKOP receptor, 12-epi-salvinorin A was G biased. MOM-SalB and EOM-salB lacked bias, but salvinorin A was strongly internalization-biased with the bias value of I, 29. At the mKOP receptor, they also have very different bias values. While MOM-SalB and salvinorin A show no significant bias, EOM-SalB (I, 6.9) and 12-epi-salvinorin A (I, 208) are internalization-biased. These observations are different from those of Zhou et al. (2013) and White et al. (2014). White et al. (2014) found that 2-substituted salvinorin A analogues were all G protein-biased, relative to salvinorin A. Zhou et al. (2013) reported that five triazole analogues and two isoquinolinone analogues were all G protein-biased using U69,593 as the reference agonist.

4.5. Partial agonists

We found that in [^{35}S]GTPγS binding assay, levorphanol, nalbuphine, and pentazocine were partial agonists at both hKOP receptor and mKOP receptor and etorphine was a partial agonist at the hKOP receptor and a full agonist at the mKOP receptor. In contrast, these four agonists did not cause significant KOP receptor internalization in at least one of the two species of opioid receptor. In addition, at the hKOP receptor butorphanol exhibited modest internalization-bias (I, 4.7), but not to a significant extent ($p = 0.064$). The present results with etorphine are similar to those of our previous report (Li et al., 2003). Our results that pentazocine caused significant internalization of the hKOP receptor, but not mKOP receptor, are also consistent with those of Schattauer et al. (2012). These investigators observed that pentazocine was more potent at hKOP receptor compared to the rKOP receptor in activating p38 MAPK, a measure of β-arrestin pathway, but similarly potent in G protein-dependent activation of ERK1/2 via hKOP and rKOP receptors. In contrast, while levorphanol and

U50,488H exhibit differences between the hKOP and mKOP receptors in our study (see Table 5), they did not distinguish between hKOP and rKOP receptors in p38 MAPK activation. It should be noted that the C-tails of the rat and mouse KOP receptors have identical sequences. The discrepancy may be due to differences in the cells (N2a vs. HEK293) and end points (internalization vs. p38 phosphorylation) used. In N2a cells we were not able to detect p38 phosphorylation following U50,488H treatment.

We found that several agonists had higher efficacy in enhancing [^{35}S]GTPγS binding via the mKOP receptor than via hKOP receptor, including etorphine, levorphanol, nalbuphine, pentazocine, dynorphin A (1–17). This disparity may be due to differences in the level of receptors in the cell lines. The mKOP receptor cell line had a higher expression level than the hKOP receptor cell line and higher expression resulted in higher efficacy. Even so, levorphanol, nalbuphine and pentazocine were still partial agonists at the mKOP receptor. Since we used dynorphin A (1–17) as a reference compound for each cell line, differences in receptor level do not affect bias values.

4.6. 12epiSalA and other KOP receptor ligands

We have found that 12epiSalA is a full agonist in [^{35}S]GTPγS binding, but does not cause hKOP receptor internalization. In contrast, Beguin et al. (2012) found 12epiSalA to be a full agonist for β-arrestin recruitment at the hKOP receptor in U2OS cells, and a partial agonist for [^{35}S]GTPγS binding at the hKOP receptor in CHO cells. The differences may be attributed to different cell lines and endpoints used.

White et al. (2014) examined functional selectivity of agonists on the hKOP receptor using salvinorin A as the reference compound. We found that when dynorphin A (1–17) was designated as the balanced agonist, salvinorin A was highly β-arrestin-biased at the hKOP receptor. If we re-calculate our data using salvinorin A as the reference agonist, all the compounds shown in Table 2 will be G protein-biased or balanced. Some will be strongly G protein-biased at the hKOP receptor, including ICI199,441, dynorphin A (1–17), spiradoline, U50,488H, U69,593, MOM-SalB and EOM-SalB. White et al. (2014) found that U62,066 (spiradoline), dynorphin A (1–17) and 2-substituted salvinorin A analogues were G proteinbiased, whereas ICI199,441 and U50,488H are β-arrestin-biased and U69,593 was balanced. Thus, only some of our results are similar to those of White et al. (2014). The reasons for the disparity may include the cell lines and functional endpoints used (see below).

4.7. Designation of a balanced agonist and choice of cells, functional endpoints and assay conditions

It should be noted that ligand bias is defined differently in different laboratories. One important factor is the different reference compounds used. We used dynorphin A (1–17), whereas White et al. (2014, 2015) used salvinorin A and Zhou et al. (2013) used U69,593. In addition, differences in functional end points and cell system are also contributing factors.

We used N2a cells, a neuron-like cell line, transfected with the hKOP receptor or mKOP receptor for both assays to eliminate the potentially confounding effects of variations in cellular machinery and proteins by using different cells. In our study, we chose to measure

receptor internalization, which is more distal than β-arrestin recruitment used by several groups (Rives et al., 2012; Schmid et al., 2013; Zhou et al., 2013; White et al., 2014), but is more proximal than p38 MAPK activation used by Schattauer et al. (2012). We also chose a more upstream readout of G protein activation by measuring [^{35}S]GTPγS binding, which was also used by Schmid et al. (2013) and Zhou et al. (2013). In contrast, Rives et al. (2012) and White et al. (2014) measured cAMP level, while Schattauer et al. (2012) chose a more downstream readout of ERK1/2 activation. Different end points may give different results because of signal amplification at each step that may affect the results. This is why we use the term internalization-biased instead of β-arrestin-biased. In a recent review, Thompson et al. (2014) discussed that It is more desirable to study the cellular events that are closely linked to the system response. However, it is not always known what these events are and the proper tools may not yet exist.

4.8. Conclusion

We have demonstrated that KOP receptor agonists display functional selectivity at both hKOP receptor and mKOP receptor in N2a cells. A new finding in this study is that there are species differences in functional selectivity of several full agonists, such as 12-epiSalA, U69,593 and ICI-199441. It raises the possibility that results obtained from one species with KOP receptor agonists may not be applicable to the other.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

12epiSalA	12-epi-Salvinorin A
7TMR	seven-transmembrane domain receptor
dynorphin A (1–17)	H-Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln-OH
EKC	ethylketocyclazocine
EOM-SalB	ethoxymethyl ester of salvinorin B
FLAG epitope tag	DYKDDDDK
FLAG-mKOP receptor	FLAG-tagged mouse κ opioid receptor
β-FNA	β-funaltrexamine
GTPγS	guanosine-5’-O-(3-thio)triphosphate

hKOP receptor	human κ opioid receptor
KOP receptor	κ opioid receptor
mKOP receptor	mouse κ opioid receptor
MOM-SalB	methoxymethyl ester of salvinorin B
OCW	on-cell western
N2a cells	neuro-2a mouse neuroblastoma cells
N2a-HA-hKOP receptor	N2a cells stably expressing HA-tagged human κ opioid receptor
rKOP receptor	rat κ opioid receptor
N2a-FLAG-mKOP receptor	N2a cells stably expressing FLAG-tagged mouse κ opioid receptor
RA_i	intrinsic relative activity
SalA	salvinorin A
U50,488H	[(−)(trans)-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidiny) cyclohexyl]benzeneacetamide]

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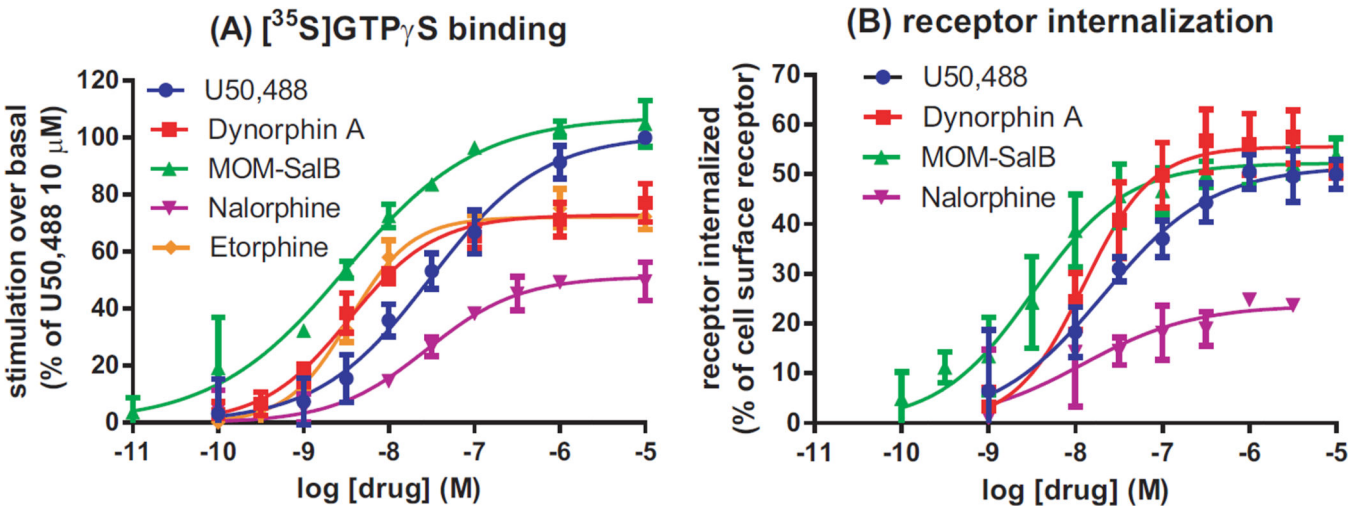

Figure 1. Concentration-response curves of five KOP receptor agonists at the hKOP receptor for [^{35}S]GTPγS binding and receptor internalization in N2a-3HA-hKOP receptorcells

(A) [^{35}S]GTPγS binding was performed on 10 μg membrane protein in the presence of 5 mM $MgCl_2$, 150 mM NaCl and 20–25 μM GDP at 30°C for 60 min as described in the **Materials and Methods**. Each experiment included a concentration-response curve for U50,488H, and the data have been normalized relative to the maximal response to U50,488H at 10 μM since it gave the highest response in preliminary experiments. Each concentration-response curve represents the mean ± SEM of at least three independent experiments performed in duplicate. **(B)** The OCW was performed on live cells and receptor internalization was measured as compared to control. Twenty thousand cells/well were plated in 24-well plates 40 hr before the experiment was performed and the agonists were added in serum-free medium at varying concentrations. The anti-HA monoclonal antibody was added as the primary antibody, cells were fixed and then stained with two cellular stains (Sapphire700 and DRAQ5) with the 800CW goat anti-mouse antibody as described in **Materials and Methods**. Each value represents the mean ± SEM of at least three independent experiments performed in triplicate. The theoretical curves represent the global least squares fit of equations 1 and 2 to the data.

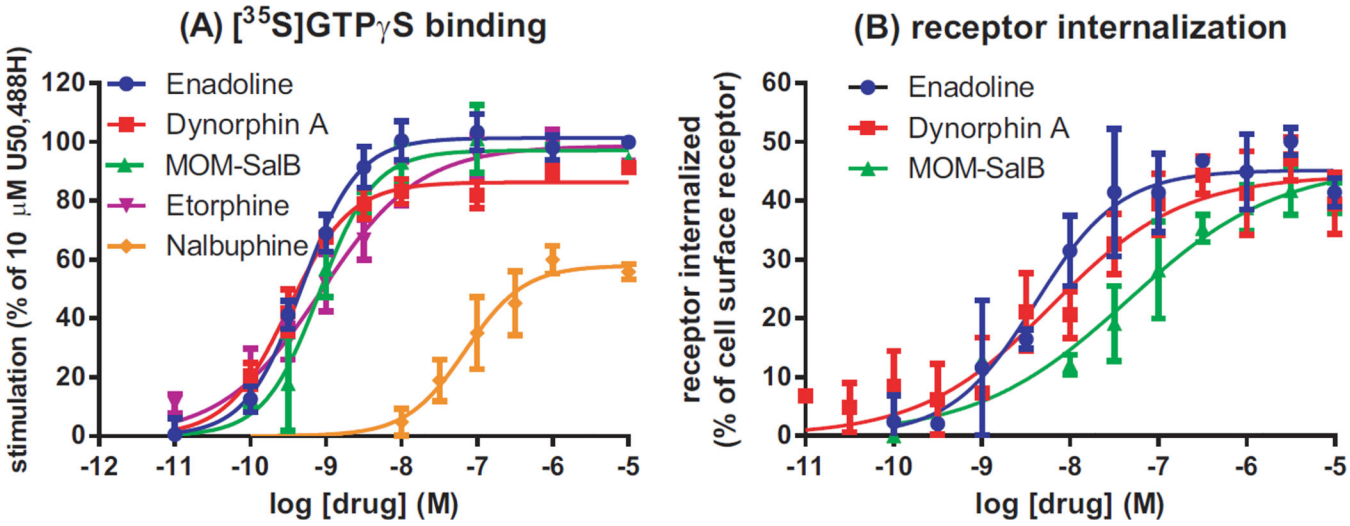

Figure 2. Concentration-response curves of five KOP receptor agonists at the mKOP receptor for [^{35}S]GTPγS binding and receptor internalization in N2a-FLAG-mKOP receptor cells

(A) [^{35}S]GTPγS binding was performed on 10 μg membrane protein in the presence of 5 mM $MgCl_2$, 150 mM NaCl and 20–25 μM GDP at 30°C for 60 min as described in the **Materials and Methods**. Each experiment included a concentration-response curve for enadoline, and the data have been normalized to the maximal response to enadoline at 1 μM since it gave the highest response in preliminary experiments. Each concentration-response curve represents the mean ± SEM of at least three independent experiments performed in duplicate. **(B)** The OCW was performed on live cells and receptor internalization was measured as compared to control. Thirty thousand cells/well were plated in 24-well plates 40 hr before the experiment was performed and the agonists were added in serum-free medium at varying concentrations. The anti-FLAG M1 monoclonal antibody was added as the primary antibody, cells were fixed and then stained with two cellular stains (Sapphire700 and DRAQ5) with the 800CW goat anti-mouse antibody as described in **Materials and Methods**. Each value represents the mean ± SEM of at least three independent experiments performed in triplicate. The theoretical curves represent the global least squares fit of equations 1 and 2 to the data.

Table 1

The human KOP receptor: Summary of EC_{50} and E_{max} values of agonists for receptor internalization as measured by the OCW and G protein activation as measured by ligand-stimulated [^{35}S]GTPγS binding.

	Internalization			[^{35}S]GTPγS binding		
	pEC_{50} (EC_{50}, mean in nM)	**E_{max} (% receptor internalized)**	**n**	**pEC_{50} (EC_{50}, mean in nM)**	**E_{max} (%)**	**N**
Dynorphin A	7.90 ± 0.06 (13)	55.5 ± 1.8	6	8.46 ± 0.07 (3.4)	73.0 ± 2.0	3
12epiSalA	No internalization		3	6.66 ± 0.27 (219)	102 ± 14	3
Etorphine	No internalization		3	8.46 ± 0.05 (3.4)	72.2 +1.9	3
Levorphanol	No internalization		3	7.14 ± 0.21 (72)	38.1 ± 4.8	3
Nalbuphine	8.46 ± 0.19 (3.5)	23.9 ± 1.6	4	7.03 ± 0.46 (92)	39.5 ± 6.8	3
Pentazocine	7.04 ± 1.11 (92)	37.0 ± 13.3	3	7.55 ± 0.85 (28)	20.5 ±6.9	3
Salvinorin A	8.61 ± 0.31 (2.5)	64.3 ± 5.6	6	7.64 ± 0.06 (23)	103 ± 2.9	3
Tifluadom	7.95 ± 0.16 (11)	29.6 ± 2.1	5	6.48 ± 0.21 (333)	97.2 ± 11	3
Butorphanol	8.48 ± 0.24 (3.3)	33.4 ± 3.2	5	8.63 ± 0.14 (2.4)	25.6 ± 1.8	3
Enadoline	8.41 ± 0.14 (3.9)	62.8 ± 2.6	3	8.13 ± 0.08 (7.5)	105 ± 3.3	3
EKC	8.68 ± 0.15 (2.1)	44.8 ± 2.2	5	8.38 ± 0.04 (4.1)	80.6 ± 1.5	3
U69,593	7.37 ± 0.11 (43)	60.0 ± 3.0	3	7.03 ± 0.14 (94)	108 ± 6.6	3
Nalorphine	7.92 ± 0.37 (12)	23.7 ± 4.0	4	7.56 ± 0.09 (28)	51.4 ± 2.3	3
U50,488	7.68 ± 0.10 (21)	51.5 ± 2.2	3	7.52 ± 0.10 (30)	101 ± 4.2	3
β-FNA	8.02 ± 0.47 (9.6)	32.8 ± 5.7	6	8.08 ± 0.09 (8.4)	63.7 ± 2.4	3
Bremazocine	8.87 ± 0.14 (1.4)	39.2 ± 1.8	5	9.02 ± 0.07 (0.95)	68.2 ± 2.0	3
EOM-SalB	8.68 ± 0.35 (2.1)	46.7 ± 4.5	3	8.63 ± 0.13 (2.3)	108 ± 3.4	3
MOM-SalB	8.49 ± 0.08 (3.2)	52.2 ± 1.7	6	8.51 ± 0.09 (3.1)	108 ± 3.3	3
Dynorphin B	7.54 ± 0.17 (29)	51.0 ± 3.6	3	7.83 ± 0.09 (15)	83.7 ± 3.3	3
Spiradoline	7.17 ± 0.50 (68)	54.8 ± 7.9	3	7.94 ± 0.05 (12)	102 ± 2.1	3
ICI-199441	8.62 ± 0.07 (2.4)	54.7 ± 1.0	3	9.61 ± 0.04 (0.24)	98.5 ± 1.4	3

E_{max} values for the OCW are measured as % receptor internalization as compared to control, while E_{max} values for GTPγS are normalized to the response of 10 μM U50,488H. All OCW assays were performed in triplicate and repeated at least 3 times; all [^{35}S]GTPγS assays were performed in duplicate and repeated at least 3 times. The EC_{50} and E_{max} values of etorphine, levorphanol and 12epiSalA could not be estimated because of their weak effect on receptor internalization. The values in the table are expressed as mean ± SEM values.

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Table 2

The human KOP receptor: log RA_{i-G}, log RA_{i-I}, log (RA_{i-G}/RA_{i-I}), bias factor and probability values of agonists.

	log RA_{i-G}	**log RA_{i-I}**	$log\frac{RA_{i-G}}{RA_{i-I}}$	**Bias factor**	**(p value)**
Dynorphin A	0	0	0	1	1
12epiSalA	− 1.47 ± 0.10	ND	ND	G[b], ND	ND
Etorphine	− 0.034 ± 0.11	ND	ND	G, ND	ND
Levorphanol	− 1.84 ± 0.16	ND	ND	ND	ND
Salvinorin A	− 0.47 ± 0.10	0.99 ± 0.12	− 1.46 ± 0.15	I[a], 29.0	3.0×10^{-5}
Nalbuphine	− 1.67 ± 0.19	−0.23 ± 0.25	−1.44 ± 0.32	I, 27.6	0.0061
Pentazocine	− 1.64 ± 0.40	−0.87 ± 0.22	−0.76 ± 0.17	I[c], 5.8	0.17
Tifluadom	− 1.62 ± 0.11	− 0.55 ± 0.19	− 1.07 ± 0.22	I, 11.8	0.0027
Butorphanol	− 0.58 ± 0.23	0.088 ± 0.18	− 0.66 ± 0.30	I[c], 4.7	0.064
Enadoline	0.044 ± 0.10	0.67 ± 0.14	− 0.61 ± 0.17	I, 4.2	0.021
EKC	0.015 ± 0.11	− 0.50 ± 0.14	− 0.48 ± 0.17	I, 3.1	0.033
U69,593	− 0.98 ± 0.11	− 0.51 ± 0.12	− 0.47 ± 0.16	I, 2.9	0.046
Nalorphine	− 1.16 ± 0.14	− 0.76 ± 0.25	− 0.39 ± 0.29	I[c], 2.5	0.22
U50,488	− 0.60 ± 0.10	− 0.37 ± 0.14	− 0.23 ± 0.17	I[c], 1.7	0.25
β-FNA	− 0.48 ± 0.12	− 0.39 ± 0.19	− 0.08 ± 0.22	I[c], 1.2	0.69
Bremazocine	0.50 ± 0.11	0.58 ± 0.16	− 0.08 ± 0.19	I[c], 1.2	0.67
EOM-SalB	0.58 ± 0.10	0.68 ± 0.16	− 0.08 ± 0.19	I[c], 1.2	0.66
MOM-SalB	0.45 ± 0.10	0.47 ± 0.11	− 0.02 ± 0.15	I[c], 1.1	0.89
Dynorphin B	− 0.44 ± 0.11	− 0.51 ± 0.14	0.08 ± 0.18	G[c], 1.2	0.70
Spiradoline	− 0.18 ± 0.10	− 0.43 ± 0.18	0.26 ± 0.21	G[c], 1.8	0.28
ICI-199441	1.46 ± 0.10	0.70 ± 0.15	0.75 ± 0.18	G, 5.7	0.014

The parameters were estimated from the same agonist concentration-response curves that were used to estimate EC_{50} and E_{max} values (Table 1). The endogenous ligand for the KOP receptor, dynorphin A, was designated as the “balanced” or standard reference ligand. The log (RA_{i-G}/RA_{i-I}) values for etorphine, levorphanol and 12epiSalA could not be estimated because of their weak effect on receptor internalization. The bias factor for a given ligand is defined as the ratio of the larger RA_i value (RA_{i-G} or RA_{i-I}) divided by the smaller of the two.

ND: cannot be determined.

[a] Internalization pathway-biased

[b] G protein pathway-biased

[c] Not significantly biased, $P > 0.05$

Table 3

The mouse KOP receptor: Summary of EC_{50} and E_{max} values for receptor internalization as measured by the OCW and G protein activation as measured by ligand-stimulated [^{35}S]GTPγS binding.

	Internalization			[^{35}S]GTPγS binding		
	pEC_{50} (EC_{50}, mean in nM)	**E_{max} (% receptor internalized)**	**n**	**pEC_{50} (EC_{50}, mean in nM)**	**E_{max} (%)**	**N**
Dynorphin A	8.27 ± 0.09 (5.4)	44.5 ± 1.4	7	9.50 ± 0.04 (0.32)	86.3 ± 1.37	4
Etorphine	No internalization		3	9.06 ± 0.08 (0.87)	98.7 ± 3.2	3
Pentazocine	No internalization		3	7.21 ± 0.10 (62)	46.6 ± 2.7	3
Nalbuphine	No internalization		3	7.16 ± 0.11 (69)	58.0 ± 4.1	3
U69,593	9.74 ± 0.16 (0.18)	42.9 ± 2.6	4	7.95 ± 0.05 (11)	99.2 ± 2.4	3
Levorphanol	9.08 ± 0.18 (0.83)	29.7 ± 1.7	2	7.52 ± 0.09 (30)	62.3 ± 2.9	
12epiSalA	8.68 ± 0.18 (2.1)	39.0 ± 2.3	3	7.27 ± 0.28 (54)	104 ± 4.9	3
U50,488	8.86 ± 0.14 (1.4)	41.5 ± 1.7	7	8.39 ± 0.06 (4.1)	94.9 ± 2.8	4
ICI-199441	9.53 ± 0.12 (0.30)	47.2 ± 1.5	7	10.22 ± 0.05 (0.060)	92.4 ± 1.9	4
EOM-SalB	8.63 ± 0.19 (2.4)	46.8 ± 3.8	3	9.28 ± 0.07 (0.53)	101 ± 3.8	3
Enadoline	8.40 ± 0.08 (4.0)	44.8 ± 1.2	7	9.32 ± 0.03 (0.48)	101 ± 1.5	5
Salvinorin A	7.61 ± 0.16 (24.4)	40.1 ± 2.2	5	8.56 ± 0.06 (2.8)	87.7 ± 2.3	3
MOM-SalB	7.50 ± 0.10 (31.3)	40.0 ± 1.6	4	9.09 ± 0.06 (0.81)	97.1 ± 2.9	3

E_{max} values for the OCW are measured as % receptor internalization as compared to control, while E_{max} values for GTPγS are normalized to the response of 1 μM enadoline. The EC_{50} and E_{max} values of etorphine, pentazocine and nalbuphine could not be estimated because of their weak effect on receptor internalization. All OCW assays were performed in triplicate and repeated at least 3 times; all GTPγS assays were performed in duplicate and repeated at least 3 times. The values in the table are expressed as mean ± SEM.

Table 4

The mouse KOP receptor: log RA_{i-G}, log RA_{i-I}, log (RA_{i-G}/RA_{i-I}), bias factor and probability values of agonists.

	log RA_{i-G}	**log RA_{i-I}**	$log\frac{RA_{i-G}}{RA_{i-I}}$	**Bias factor**	**P value**
Dynorphin A	0	0	0	1.0	1.0
Etorphine	− 0.33 ± 0.08	ND	ND	ND	
Nalbuphine	− 2.50 ± 0.11	ND	ND	ND	
Pentazocine	− 2.52 ± 0.13	ND	ND	ND	
U69,593	−1.49 ± 0.08	1.30 ± 0.23	−2.80 ± 0.24	I, 627	0.00033
Levorphanol	− 2.10 ± 0.11	0.29 ± 0.35	−2.39 ± 0.37	I, 244	0.0073
12epiSalA	− 2.17 ± 0.09	0.15 ± 0.23	− 2.32 ± 0.25	I, 208	0.00073
U50,488H	− 1.07 ± 0.08	0.35 ± 0.18	− 1.42 ± 0.20	I, 26	5.0×10^{-5}
ICI-199441	0.75 ± 0.08	1.76 ± 0.21	− 1.01 ± 0.23	I, 10	0.0016
EOM-SalB	− 0.18 ± 0.08	0.66 ± 0.23	− 0.84 ± 0.24	I, 6.9	0.024
Enadoline	− 0.13 ± 0.07	0.37 ± 0.26	− 0.51 ± 0.27	I[c], 3.2	0.086
Salvinorin A	− 0.94 ± 0.09	− 0.70 ± 0.22	− 0.25 ± 0.24	I[c], 1.8	0.34
MOM-SalB	− 0.37 ± 0.08	− 0.71 ± 0.24	0.34 ± 0.25	G[c], 2.2	0.23

The parameters were estimated from the same agonist concentration-response curves that were used to estimate EC_{50} and E_{max} values (Table 3). The endogenous ligand for the KOP receptor, dynorphin A, was designated as the “balanced” or standard reference ligand. The log (RA_{i-G}/RA_{i-I}) values for etorphine nalbuphine and pentazocine could not be estimated because of their weak effect on receptor internalization. The bias factor for a given ligand is defined as the ratio of the larger RA_i value (RA_{i-G} or RA_{i-I}) divided by the smaller of the two.

ND: not determined

[a] Internalization pathway-biased

[b] G protein pathway-biased

[c] Not significantly biased, P > 0.05

Table 5

Comparison of bias factors of agonists between human and mouse KOP receptors expressed in N2a cells.

	hKOP receptor	mKOP receptor
12epiSalA	G[a]	I[b], 208
ICI-199441	G, 5.7	I, 10
U69,593	I, 2.9	I, 627
Salvinorin A	I, 29	I[c], 1.8
U50,488	I[c], 1.7	I, 26
Nalbuphine	I, 27.6	ND
Levorphanol	ND	I, 244
EOM-SalB	I[c], 1.2	I, 6.9
Enadoline	I, 4.2	I[c], 3.2
Pentazocine	I[c], 5.8	ND
MOM-SalB	I[c], 1.1	G[c], 2.2

Data are compiled from Table 2 and Table 4. Dynorphin A(1–17) is the designated balanced agonist.

[a] G protein pathway-biased

[b] Internalization pathway-biased

[c] Not significantly biased, $P > 0.05$

ND: cannot be determined.