European Journal of Pharmacology 761 (2015) 235–244

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Molecular and cellular pharmacology

Intrinsic relative activities of κ opioid agonists in activating Gα proteins and internalizing receptor: Differences between human and mouse receptors

Kelly M. DiMattio [a], Frederick J. Ehlert [b], Lee-Yuan Liu-Chen [a,*]

[a] Center for Substance Abuse Research and Department of Pharmacology, Temple University School of Medicine, 3500 N. Broad Street, Philadelphia, PA 19140, USA

[b] Department of Pharmacology, School of Medicine, University of California, Irvine, CA 92697, USA

ARTICLE INFO

Article history:
Received 11 February 2015
Received in revised form
17 April 2015
Accepted 18 May 2015
Available online 6 June 2015

Keywords:
Ligand bias
Kappa opioid receptor
Species difference

ABSTRACT

Several investigators recently identified biased κ 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 ($RA_{i\text{-}G}$) and receptor internalization ($RA_{i\text{-}I}$) and the degree of functional selectivity between the two [Log $RA_{i\text{-}G}$ – log $RA_{i\text{-}I}$, $RA_{i\text{-}G}/RA_{i\text{-}I}$ 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.

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 κ 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

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 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 ether 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; RAi, intrinsic relative activity; SalA, salvinorin A; U50,488H., (−)(trans)-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidiny) cyclohexyl]benzeneacetamide

* Corresponding author. Fax: +1 215 707 6661.
E-mail addresses: tuc21238@temple.edu (K.M. DiMattio), fjehlert@uci.edu (F.J. Ehlert), lliuche@temple.edu (L.-Y. Liu-Chen).

http://dx.doi.org/10.1016/j.ejphar.2015.05.054
0014-2999/

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, 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 analogs (Zhou et al., 2013), dynorphin A and its shorter peptides and salvinorin A analogs (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, Sapphire700, 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