Neurochemistry International 61 (2012) 378–388

Contents lists available at SciVerse ScienceDirect

Neurochemistry International

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

Inhibition of forebrain μ-opioid receptor signaling by low concentrations of rimonabant does not require cannabinoid receptors and directly involves μ-opioid receptors

Ferenc Zádor [a], Ferenc Ötvös [a], Sándor Benyhe [a,*], Andreas Zimmer [b], Eszter Páldy [a,1]

[a] Institute of Biochemistry, Biological Research Centre, Hungarian Academy of Sciences, Temesvari krt. 62, H-6726 Szeged, Hungary
[b] Institute of Molecular Psychiatry, University of Bonn, Sigmund-Freud-Street 25, 53105 Bonn, Germany

ARTICLE INFO

Article history:
Received 21 December 2011
Received in revised form 12 April 2012
Accepted 10 May 2012
Available online 18 May 2012

Keywords:
Rimonabant
CB_1 cannabinoid receptor
μ-Opioid receptor
[^{35}S]GTPγS binding
Radioligand binding
Docking
CB_1 receptor knockout mouse
CB_1/CB_2 receptor double knockout mouse
Forebrain

ABSTRACT

Increasing number of publications shows that cannabinoid receptor 1 (CB_1) specific compounds might act in a CB_1 independent manner, including rimonabant, a potent CB_1 receptor antagonist. Opioids, cannabinoids and their receptors are well known for their overlapping pharmacological properties. We have previously reported a prominent decrease in μ-opioid receptor (MOR) activity when animals were acutely treated with the putative endocannabinoid noladin ether (NE). In this study, we clarified whether the decreased MOR activation caused by NE could be reversed by rimonabant in CB_1 receptor deficient mice. In functional [^{35}S]GTPγS binding assays, we have elucidated that 0.1 mg/kg of intraperitoneal (i.p.) rimonabant treatment prior to that of NE treatment caused further attenuation on the maximal stimulation of Tyr-D-Ala-Gly-(NMe)Phe-Gly-ol (DAMGO), which is a highly specific MOR agonist. Similar inhibitory effects were observed when rimonabant was injected i.p. alone and when it was directly applied to forebrain membranes. These findings are cannabinoid receptor independent as rimonabant caused inhibition in both CB_1 single knockout and CB_1/CB_2 double knockout mice. In radioligand competition binding assays we highlighted that rimonabant fails to displace effectively [^{3}H]DAMGO from MOR in low concentrations and is highly unspecific on the receptor at high concentrations in CB_1 knockout forebrain and in their wild-type controls. Surprisingly, docking computational studies showed a favorable binding position of rimonabant to the inactive conformational state of MOR, indicating that rimonabant might behave as an antagonist at MOR. These findings were confirmed by radioligand competition binding assays in Chinese hamster ovary cells stably transfected with MOR, where a higher affinity binding site was measured in the displacement of the tritiated opioid receptor antagonist naloxone. However, based on our *in vivo* data we suggest that other, yet unidentified mechanisms are additionally involved in the observed effects.

1. Introduction

Cannabinoids mediate their effects via activating at least two types of cannabinoid receptors, CB_1 and CB_2 both G protein-coupled (for review, see Howlett, 1998). CB_1 cannabinoid receptor is the most abundant G-protein coupled receptor (GPCR) type in the brain with 10 times higher expression levels than other GPCRs. In the central nervous system, the distribution of CB_1 receptors greatly varies between different parts of the brain and in different neuronal cell types. They are widely expressed in several forebrain regions including the olfactory bulbs (Herkenham et al., 1991), all regions of the cerebral neocortex (Egertová and Elphick, 2000; Glass et al., 1997; Herkenham et al., 1991; Matsuda et al., 1990), the hippocampal formations (Herkenham et al., 1991; Jansen et al., 1992), the subcortical regions (Breivogel et al., 1997; Herkenham et al., 1991; Julian et al., 2003; Matsuda et al., 1990; Robbe et al., 2001), among others. CB_2 receptors are predominantly expressed in immune and hematopoietic cells. However, there are many recent publications showing that CB_2 receptors are also present in some central and peripheral neurons (Beltramo et al., 2006; Ross et al., 2001; Skaper et al., 1996; Van Sickle et al., 2005), however, the role of the neuronal CB_2 receptors has still to be established.

Rimonabant, which was the first selective and orally active CB_1 antagonist (Rinaldi-Carmona et al., 1994), together with many other CB_1 and CB_2 antagonists behave as an inverse agonist rather than as a neutral antagonist (for review see Pertwee, 2005)

* Corresponding author. Address: Institute of Biochemistry, Biological Research Centre, Hungarian Academy of Sciences, P.O. Box 521, H-6701 Szeged, Hungary. Tel.: +36 62 432 099; fax: +36 62 433 432.
E-mail address: benyhe@brc.hu (S. Benyhe).
[1] Present address: Pharmacology Institute, University of Heidelberg, Im Neuenheimer Feld 366, 69120 Heidelberg, Germany.

0197-0186/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.neuint.2012.05.015

indicating that CB_1 and CB_2 receptors can exist in a constitutively active stage. It was the first CB_1 antagonist to be approved for the treatment of obesity (Padwal and Majumdar, 2007), but was withdrawn from the market in 2008 as it was found to cause strong psychiatric disorders. Before, as well as after entering rimonabant to the market there were several publications indicating its non-CB_1 receptor related actions (Breivogel et al., 2001; Hough et al., 2009) and its dose related side effects (Beyer et al., 2010; Christensen et al., 2007; Mitchell and Morris, 2007), suggesting rather unspecific behavior at higher concentrations (reviewed in Raffa and Ward, 2011).

It is well known that cannabinoid receptor system shares several features with the μ-opioid receptor (MOR) system. Both receptor types are GPCR, mainly coupled to the inhibitory $G_{i/o}$ proteins (Burford et al., 2000; Demuth and Molleman, 2006). At this level they might even functionally interact (Canals and Milligan, 2008; Rios et al., 2006). The expression patterns of CB_1 and MOR overlaps in several parts of the CNS. In certain forebrain regions, such as caudate putamen, dorsal hippocampus, substantia nigra and nucleus accumbens, the MOR and CB_1 receptors are not only co-localized, but also co-expressed in the same neurons (Pickel et al., 2004; Rodriguez et al., 2001; Salio et al., 2001). It has also been shown that these two receptor subtypes can be cross-regulated (Schoffelmeer et al., 2006) via a direct (Rios et al., 2006) or indirect interactions (Hur and Kim, 2002). When studied in behavioral aspects rimonabant reduced opiate self-administration and reward (Braida et al., 2001; Fattore et al., 2005; Navarro et al., 2001) and suppress morphine-induced feeding (Verty et al., 2003).

Previously we have shown that the putative endocannabinoid noladin ether (NE; Hanus et al., 2001) is capable of attenuating the functional activity of MOR in mouse forebrain and this effect can be partially reversed by a CB_2 antagonist (Páldyová et al., 2008). Now we clarified whether the decreased MOR activity caused by NE, which is rather acting at CB_1 receptors than at CB_2, could be reversed by rimonabant as well as we addressed to investigate the effect of rimonabant on the MOR G protein-activation alone, without NE. Recently, it is believed that rimonabant applied at high concentrations acts on a CB_1 receptor independent manner involving MORs (Cinar and Szücs, 2009), among others (Begg et al., 2005; Gibson et al., 2008; Pertwee et al., 2010; Savinainen et al., 2003). We designed our [^{35}S]GTPγS binding experiments in a way to use low concentrations of rimonabant that we either injected intraperitoneally (alone or in combination with NE) or we directly added to CB_1 wild type ($CB_1^{+/+}$) and CB_1 knockout ($CB_1^{-/-}$) mice forebrain membranes. We tested CB_1/CB_2 double knockout mice as well ($CB_1^{-/-}/CB_2^{-/-}$) to elucidate the role of the CB_2 receptors. We investigated the direct binding properties of rimonabant to MOR in receptor binding assay experiments using CB_1 knockout forebrain tissues and Chinese hamster ovary cells stably transfected with MOR (CHO-MOR). Next, we carried out docking calculations by docking rimonabant to a homology modeled MOR of its active and inactive states, to gather more information about the interaction between MOR and rimonabant.

Unspecific actions of high concentrations of rimonabant at various non-CB_1 receptors are well known. This study aims to clarify the *in vivo* and *in vitro* effects of rimonabant at MOR and MOR mediated signaling when administered in low doses, highlights the preferred orientations of rimonabant to MOR via *in silico* computational simulation and tests its direct binding ability to MOR.

2. Materials and methods

2.1. Animals

CB_1 receptor knockout ($CB_1^{-/-}$) mice and their controls ($CB_1^{+/+}$) were generated on CD1 background in Dr. Ledent's lab as described in Ledent et al., 1999. $CB_1^{-/-}/CB_2^{-/-}$ double knockout mice were provided by Dr. Zimmer's lab (Járai et al., 1999) and C57BL/6J mice were used as appropriate controls ($CB_1^{+/+}/CB_2^{+/+}$). All the animals were housed at 21–24 °C under a 12:12 light:dark cycle and were provided with water and food *ad libitum*. Different treatment groups were composed of 7–10 animals, each. All housing and experiences were conducted in accordance with the European Communities Council Directives (86/609/ECC) and the Hungarian Act for the Protection of Animals in Research (XXVIII.tv. 32.§).

2.2. Drugs and treatments

2-Arachidonyl glyceryl ether (noladin ether, NE) was purchased from Tocris and injected at the dose of 1 mg/kg in DMSO solution. SR141716 (rimonabant) was provided by SANOFI Research Laboratory (Montpellier, France) and was injected at the dose of 0.1 mg/kg in DMSO solution. Upon acute *in vivo* treatments animals received a single intraperitoneal (i.p.) injection of NE or rimonabant. Control mice were injected with DMSO solution. When used in a combined treatment, rimonabant was delivered 30 minutes prior to the NE treatment as suggested by SANOFI Research Laboratory (Rinaldi-Carmona et al., 1994). Mice were decapitated 24 h after they received the last injection. The enkephalin analog Tyr-D-Ala-Gly-(NMe)Phe-Gly-ol (DAMGO) was obtained from Bachem Holding AG, Bubendorf, Switzerland. [^{3}H]DAMGO (41 Ci/mmol) and [^{3}H]naloxone (31 Ci/mmol) was radiolabeled in the Isotope Laboratory of BRC, Szeged, Hungary.

2.3. Forebrain membrane preparations

Forebrain membrane fractions from $CB_1^{-/-}$ and $CB_1^{-/-}/CB_2^{-/-}$ mice and their controls ($CB_1^{+/+}$ and $CB_1^{+/+}/CB_2^{+/+}$, respectively) were prepared according to the method previously described (Benyhe et al., 1997). Briefly, mice were decapitated and the brain was quickly removed. The forebrain part was collected and homogenized on ice in 50 mM Tris–HCl buffer (pH 7.4) with a Teflon-glass homogenizer. The homogenate was centrifuged at 40,000*g* for 20 min at 4 °C and the pellet was resuspended in fresh buffer and incubated for 30 min at 37 °C. This centrifugation step was repeated, and the final pellet was resuspended in 50 mM Tris–HCl buffer (pH 7.4) containing 0.32 M sucrose and stored at −80 °C until use.

2.4. Cell culture and cell membrane preparations

Chinese hamster ovary cells stably transfected with MORs (MOR-CHO) were kindly provided by Dr. Zvi Vogel (Rehovot, Israel). MOR-CHO cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco) and in α-minimum essential medium (αMEM, Gibco), respectively. Both media were supplemented with 10% fetal calf serum, 2 mM glutamine, 100 IU/ml penicillin, 100 mg/ml streptomycin, 25 mg/ml fungizone and 0.5 mg/ml geneticin. Cells were kept in culture at 37 °C in a humidified atmosphere consisting of 5% CO_2 and 95% air.

Membranes were prepared from subconfluent cultures. Cells were rinsed three times with 10 ml PBS and removed with 50 mM Tris–HCl pH 7.4, 1 mM EGTA, 1 mM EDTA and 0.1 mM PMSF buffer and homogenized for 15 s with a polytron homogenizer in an ice-bath. Homogenates were centrifuged two times at 18,000 g for 20 min. The final pellet was resuspended in the above buffer and stored in aliquots at −80 °C until use.

2.5. Functional [^{35}S]GTPγS binding experiments

Membrane preparations of $CB_1^{-/-}$ and $CB_1^{-/-}/CB_2^{-/-}$ forebrains and their proper controls were diluted in 50 mM Tris–HCl buffer