Neurochemistry International 53 (2008) 309–316

Contents lists available at ScienceDirect

Neurochemistry International

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

CB_2 cannabinoid receptor antagonist SR144528 decreases mu-opioid receptor expression and activation in mouse brainstem: Role of CB_2 receptor in pain

Eszter Páldy [a], Erika Bereczki [a], Miklós Sántha [a], Tibor Wenger [b], Anna Borsodi [a], Andreas Zimmer [c], Sándor Benyhe [a,*]

[a] Institute of Biochemistry, Biological Research Centre, Hungarian Academy of Sciences, Temesvari krt. 62, H-6726 Szeged, Hungary
[b] Department of Human Morphology and Developmental Biology, Semmelweis University, P.O. Box 95, H-1450 Budapest, Hungary
[c] Institute of Molecular Psychiatry, University of Bonn, Sigmund-Freud-Street 25, 53105 Bonn, Germany

ARTICLE INFO

Article history:
Received 24 August 2008
Accepted 25 August 2008
Available online 29 August 2008

Keywords:
CB_2 cannabinoid receptor
μ-Opioid receptor
[^{35}S]GTPγS binding
Real-time PCR
Radioligand binding
Noladin ether
SR144528
CB_1 receptor knockout mouse
CB_2 receptor knockout mouse
Brainstem

ABSTRACT

Formerly considered as an exclusively peripheral receptor, it is now accepted that CB_2 cannabinoid receptor is also present in limited amounts and distinct locations in the brain of several animal species, including mice. However, the possible roles of CB_2 receptors in the brain need to be clarified. The aim of our work was to study the μ-opioid receptor (MOR) mRNA expression level and functional activity after acute *in vivo* and *in vitro* treatments with the endocannabinoid noladin ether (NE) and with the CB_2 receptor antagonist SR144528 in brainstem of mice deficient in either CB_1 or CB_2 receptors. This study is based on our previous observations that noladin ether (NE) produces decrease in the activity of MOR in forebrain and this attenuation can be antagonized by the CB_2 cannabinoid antagonist SR144528, suggesting a CB_2 receptor mediated effect. We used quantitative real-time PCR to examine the changes of MOR mRNA levels, [^{35}S]GTPγS binding assay to analyze the capability of μ-opioid agonist DAMGO to activate G-proteins and competition binding assays to directly measure the ligand binding to MOR in mice brainstem. After acute NE administration no significant changes were observed on MOR signaling. Nevertheless pretreatment of mice with SR144528 prior to the administration of NE significantly decreased MOR signaling suggesting the involvement of SR144528 in mediating the effect of MOR. mRNA expression of MORs significantly decreased both in CB_1 wild-type and CB_1 knockout mice after a single injection of SR144528 at 0.1 mg/kg when compared to the vehicle treated controls. Consequently, MOR-mediated signaling was attenuated after acute *in vivo* treatment with SR144528 in both CB_1 wild-type and CB_1 knockout mice. *In vitro* addition of 1 μM SR144528 caused a decrease in the maximal stimulation of DAMGO in [^{35}S]GTPγS binding assays in CB_2 wild-type brainstem membranes whereas no significant changes were observed in CB_2 receptor knockouts. Radioligand binding competition studies showed that the noticed effect of SR144528 on MOR signaling is not mediated through MORs. Our data demonstrate that the SR144528 caused pronounced decrease in the activity of MOR is mediated *via* CB_2 cannabinoid receptors.

1. Introduction

The cannabinoid receptor system includes two different receptor subtypes: cannabinoid receptor 1 (CB_1) and cannabinoid receptor 2 (CB_2) (Matsuda et al., 1990; Munro et al., 1993), both – similar to opioid receptors – coupled to G_i/G_o types of GTP-binding proteins (Childers et al., 1992; Howlett, 1995; Reisine et al., 1996; Manzanares et al., 1999). The existence for other cannabinoid sensitive receptor(s) has also been suggested by anatomical and electrophysiological evidence (Hájos et al., 2001). Endogenous ligands for cannabinoid receptors are named endocannabinoids, such as arachidonoyl ethanol amide (anandamide), 2-arachidonyl glyceryl ether (noladin ether, NE) and 2-arachidonoyl glycerol (2-AG) (Hanus, 2007). Impairment of the endocannabinoid system has recently been shown in animal models of Huntington's disease (Bisogno et al., 2008), suggesting novel putative target(s) of cannabinoid medicine. The cannabinoid system constitutes an important biological defense mechanism against acute and pathological pain (Calignano et al., 1998; Rice, 2001). Neuronal effects of cannabinoids are primarily mediated by CB_1 (Rice, 2001;

* Corresponding author at: Institute of Biochemistry, Biological Research Centre, Hungarian Academy of Sciences, P.O. Box 521, H-6726 Szeged, Hungary.
Tel.: +36 62 432 099; fax: +36 62 433 432.
E-mail address: benyhe@brc.hu (S. Benyhe).

0197-0186/$ – see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.neuint.2008.08.005

Mackie, 2006); for instance CB_1 receptor activation inhibits striatal dopamine release (Sidlo et al., 2008) or leads to a decrease in serotonin release in hippocampus (Balazsa et al., 2008). However, the clinical usage of cannabinoids acting at CB_1 receptors is limited because of the central side effects (Iverson, 2003); and tolerance (De Vry et al., 2004; Gonzalez et al., 2005). A molecular basis of the tolerance in spinal neurons might be due to the CB_1 receptor–G-protein-associated sorting protein (GASP1) interaction (Tappe-Theodor et al., 2007). Whereas CB_1 has attracted interest since its discovery, CB_2 has remained almost overlooked. Initial studies revealed that CB_2 receptor was expressed exclusively in peripheral tissues of the immune system (Lynn and Herkenham, 1994). In early 2000s a new opportunity for the development of cannabinoid-based analgetics emerged from data showing that selective CB_2 agonists are antinociceptive in animal pain models (Clayton et al., 2002; Malan et al., 2003; Ibrahim et al., 2003, 2005, 2006). These results are thought to have clear medical implications in treating pain and inflammation without central nervous system (CNS) side effects. However, in contrast to the inability to demonstrate the expression of CB_2 receptors in the normal CNS (Munro et al., 1993; Griffin et al., 1999; Malan et al., 2003) recent studies have confirmed the occurrence of this receptor in other tissues and organs including the brain.

Recently, growing attention is being paid to the investigation of neuronal CB_2 involvement in pain perception and/or modulation (Sagar et al., 2005; Wotherspoon et al., 2005; Onaivi et al., 2008). Nevertheless, the presence of functional CB_2 receptors in the CNS has provoked considerable controversy over the past few years. In our previous study we have shown that the putative endocannabinoid noladin ether inhibits the gene expression and G-protein activation of μ-opioid receptors (MOR) in wild-type and CB_1 knockout mice forebrain. In addition the observed attenuations can be reversed by the CB_2 receptor selective antagonist SR114528. These results demonstrated functional interactions between forebrain CB_2 cannabinoid and MOR and their impact on agonist-mediated signaling (Paldyova et al., 2008). Noladin ether (NE) was chemically synthesized as a stable 2-AG analogue by Mechoulam et al. (1998). Later, the compound was extracted from porcine (Hanus et al., 2001) and rat (Fezza et al., 2002) brain by that means identified as a putative endogenous agonist.

It is known that in some brainstem structures such as the periaqueductal gray (PAG) CB_1 cannabinoid and MOR are codistributed (Mansour et al., 1988; Herkenham et al., 1991; Mailleux and Vanderhaeghen, 1992) and play an important role in antinociception (Lichtman et al., 1996) and in the expression of morphine withdrawal (Maldonado et al., 1992). CB_1 receptors are colocalized with, and has been reported to reciprocally inhibit other members of the GPCR family, e.g., the metabotropic $GABA_B$ receptor (Cinar et al., 2008). It has also been shown that CB_2 receptors are present in the brainstem as well as in the cortex and cerebellum (Nunez et al., 2004; Golech et al., 2004; Van Sickle et al., 2005). In PAG and in substantia nigra, multifocal expressions of CB_2 immunoreactivity in glial and neuronal patterns were observed (Gong et al., 2006) and intense immunoreactive staining was seen for all opioid receptors (Gray et al., 2006).

Along with this background and with our preceding data, first we first examined the possible effects of NE on the MOR system in brainstem. We have analyzed the changes in MOR gene expression and receptor activation by MOR agonist after acute in vivo and *in vitro* NE treatments both in $CB_1^{+/+}$ and $CB_1^{-/-}$ mice (Ledent et al., 1999). Next we analyzed the effect of SR144528 on the MOR system after acute treatment in $CB_1^{+/+}$ and $CB_1^{-/-}$ mice, and *in vitro* in $CB_2^{+/+}$ and $CB_2^{-/-}$ mice (Buckley et al., 2000). Alteration of MOR gene expression was determined by real-time PCR amplification of receptor mRNAs, while agonist-stimulated G-protein activation was measured in [^{35}S]GTPγS binding assay using selective μ-agonist (DAMGO; [D-Ala^2, $_{NMe}$Phe^4, Gly^5-ol] enkephalin) ligand. Direct ligand binding was measured in equilibrium competition assays using single concentration of [^{3}H]DAMGO radioligand at the presence of various concentrations of NE or SR144528. The present study investigated the effects of NE and SR144528 on MOR affinity and activation in mouse brainstem, focusing on the possible interactions between CB_2s and MORs in CB_1 and CB_2 knockout systems.

2. Materials and methods

2.1. Animals

CB_1 wild-type ($CB_1^{+/+}$) and CB_1 cannabinoid receptor knockout mice ($CB_1^{-/-}$) were generated in Dr. Ledent's lab as described (Ledent et al., 1999). The animals were housed in controlled temperature (21 ± 2 °C) and light (on 7 a.m., off 7 p.m.) and were provided with water and food *ad libitum*. Different treatment groups were composed of 7–10 animals in each group. 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.§). CB_2 wild-type ($CB_2^{+/+}$) and CB_2 cannabinoid receptor knockout mice ($CB_2^{-/-}$) were provided by Dr.Zimmer's lab (Buckley et al., 2000).

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. The CB_2 receptor antagonist SR144528 (Rinaldi-Carmona et al., 1998) was provided by SANOFI Research (Montpellier, France). The dose of SR144528 was 0.1 mg/kg dissolved in the same vehicle as noladin ether. Upon acute *in vivo* treatments animals received a single intraperitoneal (i.p.) injection of noladin ether or SR144528. Control mice were injected with DMSO solution. When used in a combined treatment, the CB_2 antagonist compound SR144528 was delivered 30 min prior to the agonist treatment, as suggested by SANOFI Research Laboratory (Rinaldi-Carmona et al., 1998). DAMGO ([D-Ala^2, NMePhe^4, Gly^5-ol]enkephalin) was obtained from Bachem Holding AG, Bubendorf, Switzerland.

2.3. Membrane preparations

Brainstem membrane fractions from $CB_1^{+/+}$, $CB_2^{+/+}$ and $CB_1^{-/-}$, $CB_2^{-/-}$ mice were prepared according to the method previously described (Benyhe et al., 1997). Briefly, mice were decapitated and the brains were quickly removed, separated (forebrain, cerebellum, brainstem) and homogenized on ice in 50 mM Tris–HCl buffer (pH 7.4) using a Teflon-glass homogenizer. The homogenate was centrifuged at 40,000 × *g* for 20 min at 4 °C and the resulting pellet was resuspended in fresh buffer and incubated for 30 min at 37 °C. The 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 −70 °C until use. Before use membranes were thawed, diluted with fresh buffer and centrifuged again to remove sucrose and used immediately in the binding assays.

2.4. Competition binding assay

Aliquots of frozen $CB_1^{+/+}$ and $CB_1^{-/-}$ mice brainstem membranes were centrifuged (40,000 × *g*, 20 min, 4 °C) to remove sucrose and pellets were suspended in 50 mM Tris–HCl buffer (pH 7.4). Membranes were incubated with gentle shaking at 35 °C for 45 min in a final volume of 1 ml with unlabelled DAMGO, noladin ether or SR144528 (10^{-11}–10^{-5} M), and ∼1 nM of [^{3}H]DAMGO. Total binding was measured in the presence of radioligand, non-specific binding was determined in the presence of 10 μM unlabeled naloxone. The reaction was terminated by rapid filtration under vacuum (Brandel M24R Cell Harvester), and washed three times with 5 ml ice-cold 50 mM Tris–HCl (pH 7.4) buffer through Whatman GF/C glass fibers. The radioactivity of the dried filters was detected in UltimaGold™ F scintillation cocktail (Packard) with Packard Tricarb 2300TR liquid scintillation counter. Radioligand binding assays were performed in duplicate and repeated at least three times. Experimental data were analyzed by GraphPad Prism 3.0 to determine the concentration of the drug that displaced 50% of [^{3}H]DAMGO (IC_{50}).

2.5. [^{35}S]GTPγS binding assay

Membrane preparations of both CB_1 and CB_2 mice brainstems were diluted in 50 mM Tris–HCl buffer (pH 7.4) to get appropriate protein content for the assays (∼10 μg of protein/sample). The membrane fractions were incubated at 30 °C for 60 min in Tris–EGTA buffer (pH 7.4) composed of 50 mM Tris–HCl, 1 mM EGTA, 3 mM $MgCl_2$, 100 mM NaCl, containing 20 MBq/0.05 cm^3 [^{35}S]GTPγS (0.05 nM) and