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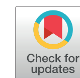
Rimonabant, a potent CB1 cannabinoid receptor antagonist, is a $G\alpha_{i/o}$ protein inhibitor



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ABSTRACT

Rimonabant is a potent and selective cannabinoid CB1 receptor antagonist widely used in animal and clinical studies. Besides its antagonistic properties, numerous studies have shown that, at micromolar concentrations rimonabant behaves as an inverse agonist at CB1 receptors. The mechanism underpinning this activity is unclear. Here we show that micromolar concentrations of rimonabant inhibited $G\alpha_{i/o}$-type G proteins, resulting in a receptor-independent block of G protein signaling. Accordingly, rimonabant decreased basal and agonist stimulated $[^{35}S]GTP\gamma S$ binding to cortical membranes of CB1- and $GABA_B$-receptor KO mice and Chinese Hamster Ovary (CHO) cell membranes stably transfected with $GABA_B$ or D2 dopamine receptors. The structural analog of rimonabant, AM251, decreased basal and baclofen-stimulated $GTP\gamma S$ binding to rat cortical and CHO cell membranes expressing $GABA_B$ receptors. Rimonabant prevented G protein-mediated $GABA_B$ and D2 dopamine receptor signaling to adenylyl cyclase in Human Embryonic Kidney 293 cells and to G protein-coupled inwardly rectifying K^+ channels (GIRK) in midbrain dopamine neurons of CB1 KO mice. Rimonabant suppressed GIRK gating induced by $GTP\gamma S$ in CHO cells transfected with GIRK, consistent with a receptor-independent action. Bioluminescent resonance energy transfer (BRET) measurements in living CHO cells showed that, in presence or absence of co-expressed $GABA_B$ receptors, rimonabant stabilized the heterotrimeric $G\alpha i/o$-protein complex and prevented conformational rearrangements induced by $GABA_B$ receptor activation. Rimonabant failed to inhibit $G\alpha s$-mediated signaling, supporting its specificity for $G\alpha_{i/o}$-type G proteins. The inhibition of $G\alpha_{i/o}$ protein provides a new site of rimonabant action that may help to understand its pharmacological and toxicological effects occurring at high concentrations.

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1. Introduction

Heterotrimeric guanine nucleotide binding proteins (G proteins) are primarily activated by G protein coupled receptors (GPCRs) that transduce extracellular stimuli from the cell surface to intracellular signaling cascades (Milligan and Kostenis, 2006; Denis et al., 2012; Syrovatkina et al., 2016). G protein signaling is regulated by “the regulator of G-protein-signaling” (RGS) proteins that activate the GTPase activity of the $G\alpha$ subunits (Sato et al., 2006; Roman, 2009; Sjögren et al., 2010, Sjögren, 2011). RGS proteins are, therefore, potential targets for therapeutic agents intended to prolong and enhance receptor-induced G protein signaling. There is also evidence that $G\alpha$ or $G\beta\gamma$ subunits of the heterotrimeric G protein can be directly targeted by small molecules with potential therapeutic action (see Smrcka, 2013 for a review). For example, the anti-helminthic drug suramin and some of its analogues were found to inhibit the exchange of GDP for GTP at $G\alpha$ subunit (Smrcka, 2013). Likewise, the cyclic depsipeptide YM-254890 stabilizing

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Abbreviations

BRET	Bioluminescence resonance energy transfer
CAMYEL	cAMP sensor using YFP-EPAC-Rluc
CB1	Cannabinoid receptor type 1
CB1-KO	CB1-knock out
CNS	Central nervous system
CHO	Chinese Hamster Ovary
GDP	Guanosine 5′-diphosphate
GIRK	G-protein-coupled inwardly rectifying K^+ channels
GPCR	G-protein coupled receptor
GTPγS	guanosine 5′-O-(3-thiotriphospate)
HEK-293	Human Embryonic Kidney 293

the GDP bound form of $G\alpha_q$ subunit (Freissmuth et al., 1996; Takasaki et al., 2004). This compound delineates a target site for the development of inhibitors of G protein signaling, which would be of therapeutic benefit in ovarian cancer treatment (Nishimura et al., 2010; Kan et al., 2010). Moreover, fluorescein analogues that bind with micromolar affinity to Gβγ subunit have been identified; one of these potentiated morphine analgesia in rats (Bonacci et al., 2006). Recently, Ayoub et al. (2009) have identified two imidazopyrazine derivatives (BIM-46174 and BIM-46187) that prevented the GPCR-induced activation of all the G protein families tested. The compound BIM-46174 displayed anti-proliferative activity while the compound BIM-46187, which binds to the $G\alpha_{i2}$ subunit, induced pain relief (Prévost et al., 2006; Favre-Guilmard et al., 2008).

Rimonabant, (SR, 141716A (*N*-(Piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide hydrochloride) is a highly potent and selective cannabinoid receptor (CB1) antagonist (Ki = 1.98 nM) (Rinaldi-Carmona et al., 1994) that displays a plethora of pharmacological effects under several pathophysiological conditions, including obesity-related disorders (Van Gaal et al., 2005; Scheen et al., 2006; Pi-Sunyer et al., 2006; Padwal and Majumdar, 2007; Xie et al., 2007), drug addiction (i.e., alcohol, nicotine) (Le Foll et al., 2008; Soyka et al., 2008; Beardsley et al., 2009; Cahill and Ussher, 2011) and anticancer effects *in vitro* (Flygare et al., 2005; Bifulco et al., 2007; Malfitano et al., 2007; Ciaglia et al., 2015). Rimonabant was approved as an anti-obesity treatment (Acomplia, European Public Assessment Report 2007) in more than 50 countries worldwide. Rimonabant counteracts an increased tone of endogenous cannabinoids responsible for excessive appetite and metabolic alterations associated with obesity. While the efficacy of rimonabant in weight reduction was demonstrated in a series of major reports, the meta-analysis of clinical studies has revealed that its beneficial effect on obesity was associated with adverse psychiatric events, including anxiety and depression, increased risk of suicidal ideation and suicide attempts (Christensen et al., 2007; Rucker et al., 2007; Moreira et al., 2009). Due to these adverse effects rimonabant was withdrawn from the market in 2009.

Besides being a potent and selective CB1 receptor antagonist, rimonabant at nanomolar concentrations exhibits CB1-dependent inverse agonistic effects, including the inhibition of basal GTPγS binding (Landsman et al., 1997; MacLennan et al., 1998; Pertwee, 2005; Howlett et al., 2011). In addition, numerous studies indicated that rimonabant at micromolar concentrations reliably inhibits GTPγS binding also in the presence or absence of CB1 receptors, both, in rodent and human brain tissues as well as in heterologous system (Sim-Selley et al., 2001; Breivogel et al., 2001; Savinainen et al., 2003; Cinar and Szücs, 2009; Erdozain et al., 2012; Pertwee, 2005). Several explanations have been proposed for the CB1-independent inverse agonist activity of rimonabant, but the mechanism is still highly debated (Pertwee, 2005; Howlett et al., 2011; Raffa and Ward, 2012).

Here we demonstrate that at micromolar concentrations, higher than those commonly used to block the CB1 receptor, rimonabant inhibited activation of heterotrimeric G protein by acting at the $G\alpha_{i/o}$ subunit. Accordingly, rimonabant produced a receptor-independent reduction of basal activity of $G\alpha_{i/o}$, prevented GPCR-mediated activation of $G\alpha_{i/o}$ proteins and signaling to their effectors. BRET experiments support that rimonabant induced conformational changes in heterotrimeric G protein. Our study identifies a new site of action of rimonabant thereby indicating that micromolar concentrations inhibit $G_{\alpha i/o}$-type G proteins.

2. Material and methods

2.1. Animals

All procedures and experiments were carried out according to Italian (D.L. 26/2014) and European Council directives (63/2010) and in compliance with the approved animal policies by the Ethical Committee for Animal Experiments (CESA, University of Cagliari) and the Italian Department of Health. All possible efforts were made to minimize animal pain and discomfort and to reduce the number of experimental subjects. Male Sprague Dawley rats and DBA mice (Harlan Nossan, San Pietro al Natisone, Italy), weighing 200 to 250 and 17–20 g, respectively, were used. $GABA_{B1}$ knockout ($GABA_{B1}$-KO) and CB1-KO mice were obtained and genotyped as previously described (Schuler et al., 2001; Marsicano et al., 2002). Rats and mice were housed 4 and 6 per cage, respectively, in a temperature- and light-controlled room. Light was on a 12-h cycle, and food and water were available ad libitum.

2.2. Drugs and plasmid constructs

Guanosine 5′-diphosphate (GDP) and guanosine 5′-O-(3-thiotriphosphate) (GTPγS) were purchased from Sigma/RBI (St.Louis, MO, USA). $[^{35}S]$GTPγS (125 Ci/mM) and $[^{3}H]$CGP54626 (85 Ci/mM) were obtained from PerkinElmer and American Radiolabeled Chemicals Inc. (St. Louis, MO, USA), respectively. Luciferase substrate, Coelenterazine, was purchased from NanoLight Technologies (US), Lipofectamine 2000 transfection reagents were obtained from Thermo Fisher Scientific (Waltham, MA). CGP54626, (R)-baclofen, quinpirole and A68293 were purchased from Tocris Bioscience (Ellisville, MO, USA). Rimonabant was a generous gift from G. Le Fur (Sanofi-Aventis Recherche, France), while the structural analog AM251 *N*-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide was purchased from Tocris Bioscience (Ellisville, MO, USA). Drugs were dissolved in 100% DMSO and then diluted in an assay buffer. The concentration of DMSO used in the different assays never exceeded 0.1% (v/v) and had no effects on $[^{3}H]$CGP54626 and $[^{35}S]$GTPγS binding assay, electrophysiological recordings and BRET measurements. Specifically, rimonabant or other drugs were dissolved at a concentration of 0.1 M in 100% DMSO (stock solution) and diluted in buffer to obtain the working solution, i.e. dilution 1/1000 to obtain the final concentration of 0.0001 M of drug in buffer containing 0.1% of DMSO.

The plasmid pcDNA3L-His-(cAMP sensor using YFP-Epac-RLuc) (CAMYEL, MBA-277) (Jiang et al., 2007) was purchased from ATCC.

$G\alpha_o$-Rluc, $G\gamma_2$-Venus, $G\beta_1$-Flag, Gγ2-HA were a kind gift from Jean Philippe Pin (Institute of Functional Genomics, Montpellier,

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