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Differential β-arrestin2 requirements for constitutive and agonist-induced internalization of the CB_1 cannabinoid receptor

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ABSTRACT

CB_1 cannabinoid receptor (CB_1R) undergoes both constitutive and agonist-induced internalization, but the underlying mechanisms of these processes and the role of β-arrestins in the regulation of CB_1R function are not completely understood. In this study, we followed CB_1R internalization using confocal microscopy and bioluminescence resonance energy transfer measurements in HeLa and Neuro-2a cells. We found that upon activation CB_1R binds β-arrestin2 (β-arr2), but not β-arrestin1. Furthermore, both the expression of dominant-negative β-arr2 (β-arr2-V54D) and siRNA-mediated knock-down of β-arr2 impaired the agonist-induced internalization of CB_1R. In contrast, neither β-arr2-V54D nor β-arr2-specific siRNA had a significant effect on the constitutive internalization of CB_1R. However, both constitutive and agonist-induced internalization of CB_1R were impaired by siRNA-mediated depletion of clathrin heavy chain. We conclude that although clathrin is required for both constitutive and agonist-stimulated internalization of CB_1R, β-arr2 binding is only required for agonist-induced internalization of the receptor suggesting that the molecular mechanisms underlying constitutive and agonist-induced internalization of CB_1R are different.

1. Introduction

Internalization of G protein-coupled receptors (GPCRs) is an important process in the regulation of receptor function. Although its main function is the modulation of receptor number on the cell surface, thereby adjusting the sensitivity of the cell to external stimuli, it also plays role in the resensitization and signaling of GPCRs (Ferguson, 2001; Hunyady and Catt, 2006; Shenoy and Lefkowitz, 2011). At the molecular level, β-arrestins are key regulatory proteins of receptor internalization, as they can bind to activated GPCRs, as well as to clathrin and the adaptor protein AP-2, thus directing the receptor towards clathrin-mediated endocytosis (Shenoy and Lefkowitz, 2011). β-Arrestins also mediate receptor desensitization, as their binding to the activated GPCRs causes the uncoupling of the receptor from its cognate G protein (Shenoy and Lefkowitz, 2011). Furthermore, they play important roles in the activation of G protein-independent signal transduction pathways, e.g. the activation of MAP kinases, phosphatidylinositol 3 kinase, Akt or the small GTP-ase RhoA (DeWire et al., 2007; Wei et al., 2003).

β-Arrestin1 (β-arr1) and β-arrestin2 (β-arr2) are two ubiquitously expressed isoforms of β-arrestins (Ferguson, 2001). Although β-arrestin binding is a general property of most activated GPCRs, the selectivity and stability of these binding shows receptor specific differences. Namely, class A receptors (e.g. the β_2 adrenergic receptor) bind β-arr2 with a higher affinity than β-arr1, and this binding is transient, i.e. it can only be detected at or near the plasma membrane. In contrast, class B GPCRs, such as the AT_1 angiotensin receptor, bind both β-arr1 and β-arr2 with relatively high affinity and form stable complexes with β-arrestins, so that β-arrestins remain bound to the receptor after internalization and can be detected on intracellular vesicles (Oakley et al., 2000).

The CB_1 cannabinoid receptor (CB_1R) belongs to the superfamily of G protein-coupled receptors (GPCRs). The receptor plays role in many important physiological processes, such as learning, thinking, nociception or the regulation of food-intake (Pacher et al., 2006). Activation of presynaptic CB_1Rs by postsynaptic endocannabinoid release, which mediates retrograde transmission, is a key regulatory mechanism in the central nervous system, but paracrine activation of CB_1Rs with a similar mechanism can also occur in extraneural tissues (Freund et al., 2003; Gyombolai et al., 2012; Sanchez et al., 2001; Turu et al., 2009; Szekeres et al., 2012). The cellular signaling events following CB_1R activation are mainly associated with the activation of heterotrimeric $G_{i/o}$-proteins and include inhibition of adenylyl cyclases, activation of K_{ir} channels, inhibition of Ca_v channels and phosphorylation and activation of different subtypes of mitogen-activated protein kinases (MAP kinases) (Turu and Hunyady,

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2010). G protein-independent signaling events following CB_1R stimulation have also been reported (Sanchez et al., 2001).

Similar to most GPCRs, CB_1R internalizes upon agonist stimulation. This has been demonstrated in many cell lines, including CHO (Rinaldi-Carmona et al., 1998), AtT20 (Hsieh et al., 1999; Jin et al., 1999; Roche et al., 1999), F11 (Coutts et al., 2001), neuroblastoma N18TG2 (Keren and Sarne, 2003) and HEK293 (Keren and Sarne, 2003; Leterrier et al., 2004) cells, as well as in hippocampal neurons, which naturally express CB_1R (Coutts et al., 2001; Leterrier et al., 2006). According to different studies, this agonist-induced CB_1R endocytosis occurs via clathrin- and/or caveolin-mediated pathways in different cell types (Bari et al., 2008; Hsieh et al., 1999; Keren and Sarne, 2003; Wu et al., 2008).

Numerous studies suggest that β-arr2 is involved in the regulation of CB_1R. Co-expression of both GRK3 and β-arr2 was needed for the proper desensitization of the receptor in Xenopus oocyte (Jin et al., 1999), and dominant negative GRK2 and β-arrestin constructs reduced CB_1R desensitization in hippocampal neurons (Kouznetsova et al., 2002). In β-arr2 knockout mice desensitization and downregulation of CB_1R were impaired in certain regions of the central nervous system (Nguyen et al., 2012). Recruitment of β-arr2 to the activated CB_1R has also been demonstrated (Daigle et al., 2008a). Mutation of amino acids S426 and S430 was shown to inhibit receptor desensitization as well as late phase receptor endocytosis, but not β-arrestin binding (Daigle et al., 2008b; Jin et al., 1999). It has been demonstrated that serine and threonine residues at the C-terminus of CB_1R are involved in its β-arr2 binding and agonist-induced endocytosis (Daigle et al., 2008b; Hsieh et al., 1999). In contrast to the various studies that clearly point to an interaction between CB_1R and β-arr2 upon agonist stimulation, no direct data have been hitherto presented concerning the β-arr1 binding of CB_1R. In some structural studies, the association of β-arr1 with a synthesized CB_1R C-terminus has been shown (Bakshi et al., 2007; Singh et al., 2011), however, such binding has not been demonstrated with the intact CB_1R in living cells.

Constitutive internalization of CB_1R (i.e. spontaneous internalization in the absence of CB_1R agonists) has also been detected in hippocampal neurons, as well as in CHO and HEK cells (Leterrier et al., 2004, 2006; McDonald et al., 2007a; Turu et al., 2007). It has been suggested that constitutive CB_1R internalization is the consequence of its basal activity, since inverse agonist treatment or inhibition of basal activity with a DAG lipase inhibitor (e.g. tetrahydrolipstatin) interfered with this process (Leterrier et al., 2004, 2006; Rinaldi-Carmona et al., 1998; Turu et al., 2007). However, other studies have concluded that constitutive internalization occurs independently of receptor activity (McDonald et al., 2007a, 2007b; Kleyer et al., 2012). The latter statement raises the possibility that constitutive and agonist-induced internalization of CB_1R may occur via distinct endocytic mechanisms (McDonald et al., 2007a). However, no evidence has been hitherto presented showing that these two processes are truly different in that they require distinct endocytic machinery to take place.

Our main goal was, considering the important consequences of β-arrestin recruitment in the regulation of cell function, to characterize the β-arrestin binding properties of CB_1R and to reveal possible differences in constitutive and agonist-driven CB_1R endocytosis by investigating the role of β-arrestins in these processes.

2. Materials and methods

2.1. Materials

The cDNAs of the rat vascular CB_1R and CB_1R-eYFP were provided by Zsolt Lenkei (Centre National de la Recherche Scientificue, Paris). β-Arrestin1, β-arrestin2 and β-arrestin2-eGFP cDNAs were kindly provided by Dr. Marc G. Caron (Duke University, Durham, NC). Molecular biology enzymes were obtained from Fermentas (Vilnius, Lithuania) and Stratagene (La Jolla, CA). pcDNA3.1 vector, coelenterazine h, fetal bovine serum (FBS), OptiMEM, Lipofectamine 2000, and PBS-EDTA were from Invitrogen (Carlsbad, CA). WIN55,212-2 and AM251 were from Tocris (Bristol, UK). Cell culture dishes and plates for BRET measurements were from Greiner (Kremsmunster, Austria). HaloTag® Alexa Fluor® 488 Ligand was from Promega (Madison, WI). Control siRNA, human β-arrestin2-specific siRNA, mouse β-arrestin2-specific siRNA and clathrin heavy chain-specific siRNA (with sequences 5′-UUCUCCGAACGU GUCACGU-3′, 5′-GGACCGCAAAGUGUUUGUG-3′, 5′-ACGUCCAUG UCACCAACAA-3′ and 5′-GAAAGAAUCUGUAGAGAAA-3′, respectively) were from Eurofins MWG Operon (Ebersberg, Germany). HeLa and Neuro-2a cells were from ATCC (American Type Culture Collection, Manassas, VA). Anti-β-arrestin2 and HRP-conjugated anti-rabbit and anti-mouse antibodies were from Cell Signaling Technology Inc. (Beverly, MA). Anti-clathrin heavy chain antibody was from Transduction Laboratories (Lexington, KY). Unless otherwise stated, all other chemicals and reagents were from Sigma (St. Louis, MO).

2.2. Plasmid constructs and site-directed mutagenesis

The mVenus-tagged rat AT_{1a} receptor (AT_1R-mVenus), human β_2 adrenergic receptor (β_2AR-mVenus) and rat CB_1R (CB_1R-mVenus) were created by exchanging the sequence of fluorescent proteins in AT_1R-YFP (Turu et al., 2006), β_2AR-Sluc (Toth et al., 2012) or CB_1R-eYFP, respectively, to the sequence of mVenus using AgeI and NotI restriction enzymes. The mCherry-tagged AT_1R (AT_1R-mCherry) and Cerulean-tagged β_2AR (β_2AR-Cerulean) were created similarly. β-Arrestin2-Rluc was constructed as described previously (Turu et al., 2006). β-Arrestin1-Rluc and β-arrestin1-eGFP were generated from β-arrestin2-Rluc and β-arrestin2-eGFP, respectively, by replacing the cDNA of β-arrestin2 with that of β-arrestin1. CB_1R-mCherry and β-arrestin2-RFP were constructed by subcloning the cDNAs of CB_1R or β-arrestin2 into mCherry or RFP containing vectors (provided by Dr.R. Tsien, University of California, San Diego, CA), respectively. For the construction of Halo-CB_1R, the cDNA of HaloTag was first amplified from the HaloTag® pHT2 vector (Promega, Madison, WI) by PCR with the sense primer containing a cleavable signal sequence of influenza hemagglutinin (MKTIIALSYIFCLVFA) to achieve proper plasma membrane localization of the final construct (Guan et al., 1992). This product was inserted into a pEGFP-C1 vector (Clontech, Palo Alto, CA) in the place of eGFP sequence (pHalo-C1 vector). CB_1R cDNA was then inserted into pHalo-C1 after the HaloTag cDNA to yield Halo-CB_1R. CB_1R-Sluc was generated from CB_1R-eYFP by replacing the eYFP coding sequence with the cDNA of super Renilla luciferase (Woo and von Arnim, 2008). EYFP-tagged ICAM-1 (ICAM-YFP) was constructed as described previously (Varnai and Balla, 2007). V54D mutation was inserted into the appropriate β-arrestin2 constructs by the QuikChange® site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to manufacturer's suggestions. Sequences of all constructs were verified using automated DNA sequencing.

2.3. Cell cultures and transfection

HeLa and Neuro-2a cells were maintained in DMEM supplemented with 10% FBS, (Invitrogen, Carlsbad, CA), 100 μg/ml streptomycin, and 100 IU/ml penicillin in 5% CO_2 at 37 °C. For confocal microscopy experiments, HeLa and Neuro-2a cells were grown on glass coverslips (coated with poly-L-lysine in the case of Neuro-2a cells) in 6-well plates and transfected with the indicated constructs using 1 μg/well of receptor constructs, 0.5 μg/well of β-arrestin constructs (as indicated) and/or 25 pmol/well of siRNA