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Research Report

A cannabinoid receptor 1 mutation proximal to the DRY motif results in constitutive activity and reveals intramolecular interactions involved in receptor activation

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ABSTRACT

Activation of a G-protein-coupled receptor involves changes in specific microdomain interactions within the transmembrane region of the receptor. Here, we have focused on the role of L207, proximal to the DRY motif of the human cannabinoid receptor 1 in the interconversion of the receptor resting and active states. Ligand binding analysis of the mutant receptor L207A revealed an enhanced affinity for agonists (three- to six-fold) and a diminished affinity for inverse agonists (19- to 35-fold) compared to the wild-type receptor, properties characteristic of constitutive activity. To further examine whether this mutant adopts a ligand-independent, active form, treatment with GTPγS was used to inhibit G protein coupling. Under these conditions, the L207A receptor exhibited a 10-fold increase in affinity for the inverse agonist SR141716A, consistent with a shift away from an enhanced precoupled state. Analysis of the cellular activity of the L207A receptor showed elevated basal cyclic AMP accumulation relative to the wild type that is inhibited by SR141716A, consistent with receptor-mediated Gs precoupling. Using toxins to selectively abrogate Gs or Gi coupling, we found that CP55940 nonetheless induced only a Gi response suggesting a strong preference of this ligand-bound form for Gi in this system. Molecular dynamics simulations reveal that the single residue change of L207A impacts the association of TM3 and TM6 in the receptor by altering hydrophobic interactions involving L207, the salt bridge involving the Arg of the DRY motif, and the helical structure of TM6, consistent with events leading to activation. The structural alterations parallel those observed in models of a mutant CB_1 receptor T210I, with established constitutive activity (D'Antona, A.M., Ahn, K.H. and Kendall, D.A., 2006. Mutations of CB1 T210 produce active and inactive receptor forms: correlations with ligand affinity, receptor stability, and cellular localization. Biochemistry, 45, 5606–5617).

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1. Introduction

The human cannabinoid receptor 1 (CB_1) is a member of the G-protein-coupled receptor (GPCR) superfamily and the family A rhodopsin-like receptors. The CB_1 receptor was originally isolated from a human brain stem cDNA library (Gerard et al., 1991) and is primarily found in the central nervous system. It selectively binds the major psychoactive constituent of *Cannabis sativa*, Δ^9-tetrahydrocannabinol, and endogenous cannabinoids such as anandamide and 2-arachidonylglycerol (for a review, see Howlett et al., 2002). The cannabinoid receptor pathway is believed to be involved with synaptic transmission and the depression of neurotransmitter release in particular (Wilson and Nicoll, 2002). Like other GPCRs, the CB_1 receptor has seven α-helical transmembrane segments connected by three intracellular and three extracellular loops and is oriented with an extracellular amino terminus and an intracellular carboxyl terminus. Upon ligand binding and receptor activation, the intracellular face of the receptor transduces the signal to a heterotrimeric guanine nucleotide binding protein (G protein). Studies have shown that the CB_1 receptor is primarily coupled to pertussis toxin (PTX)-sensitive Gi/o type G proteins (Howlett and Fleming, 1984), which in turn interact with adenylate cyclase to inhibit its activity. However, in striatal neurons, the CB_1 receptor has also been shown to couple with Gs though to a lesser extent (Glass and Felder, 1997). Other events following CB_1 receptor activation can include increased conductance of K^+ channels (Felder et al., 1995), decreased Ca^+ channel conductance (Mackie and Hille, 1992), as well as the release of arachidonic acid (Burstein et al., 1994).

The 2.2 Å crystal structure of rhodopsin (Okada et al., 2004) provides insight into the conformation of GPCRs and possible changes that may occur during receptor activation. However, the derived structure is of the resting state, and a detailed model of the activated state based on structural data does not exist. Studies focusing on the conformation changes of rhodopsin and the β_2-adrenergic receptor during receptor activation have suggested a rearrangement of the transmembrane (TM) segments in which TM6 plays a primary role (Gether, 2000; Gouldson et al., 2004). In the resting state, the bend at a conserved CWXP motif within TM6 seems to be preserved through interactions with TM3. Located on TM3 is the highly conserved DRY motif in which the Arg 3.50 (Ballesteros and Weinstein, 1995) has 94% identity among all family A GPCRs. The Arg 3.50 is believed to form a salt bridge with Asp 3.49 on TM3 and Glu/Asp 6.30 on TM6 (Palczewski et al., 2000). Receptor activation is hypothesized to involve protonation of Asp 3.49, thereby disrupting the salt bridge and thus the stability of the inactive form of the receptor (Ballesteros et al., 2001; Scheer et al., 1996). These changes are accompanied by a straightening of TM6 that allows closer association with TM5.

Even with recent advances in X-ray structure determination of GPCRs, many questions remain unanswered regarding the interactions involved in stabilization of the resting state, the specific helical rearrangements in the progression through intermediate states, and the role of specific ligands in promoting these states and the activated form. Using an in situ reconstitution system, Glass and Northup (1999) showed that the efficacy of different cannabinoids for activating different CB_1–G protein complexes varies widely. These observations and others (Howlett, 2004) suggest that the cannabinoid receptor, like other GPCRs, may be induced to form ligand-specific activated forms. Evidence that distinct ligand-directed activated forms promote coupling to different G proteins comes from studies of several GPCRs. Agonists of the α_{1B}-adrenergic receptor (Perez et al., 1996) and the 5-hydroxytryptamine-2 receptor (Berg et al., 1998) differentially activate PTX-sensitive versus -insensitive G proteins. Furthermore, the α_2-adrenergic receptor displays agonist-specific Go versus Gi coupling (Yang and Lanier, 1999). Studies of the CB_1 receptor using rat brain membrane preparations (Houston and Howlett, 1998) and CHAPS extracts from N18TG2 neuroblastoma cell membranes (Mukhopadhyay and Howlett, 2005) also showed that different agonists promote specific receptor–Gαi subtype complexes. The chemically distinct ligands tested are thought to provide different "microconformational" changes within the receptor binding pocket. It is these distinct changes within the protein that dictate specific G protein coupling and therefore signaling to different cellular pathways.

In this study, we have focused on the role of L207, proximal to the DRY motif of the CB_1 receptor, in the interconversion of the receptor resting and active states. Characterization of the mutant receptor L207A revealed enhanced agonist and diminished inverse agonist affinity relative to the wild type, a hallmark of constitutive activity as predicted by the extended Ternary Complex Model (Samama et al., 1993). Furthermore, the change in SR141716A affinity was GTPγS sensitive, as expected for a mutation-induced shift toward the active state. Interestingly, examination of the consequences of the mutation on cyclic AMP levels reveals a deviation in G protein subtype coupling for ligand-dependent versus -independent coupling.

2. Results

2.1. *Rationale for the L207A mutation*

In this study, we characterized the mutant CB_1 receptor L207A in which a Leu to Ala substitution was generated in the transmembrane region adjacent to the DRY sequence of TM3 (Fig. 1A). This location was particularly interesting because Leu 207 is predicted to be on the same face as the Arg 3.50 and because the Leu is conserved (Fig. 1B) among family A receptors (71%) and among all human GPCRs (62%). This Leu 3.43 is the most invariant residue of TM3 aside from the Arg of the DRY motif in this region of the helix (Horn et al., 2003). Moreover, Leu 207 is also found on the same face of TM3 as a constitutively active CB_1 mutant, T210I (D'Antona et al., 2006), which is only one turn away from Arg 3.50 (Fig. 1C).

2.2. *The L207A receptor exhibits shifts in agonist and inverse agonist preferences*

The L207A receptor expressed in HEK 293 cells displayed a marked change in its ligand-binding pattern as compared to