FOR THE RECORD

Cannabinoid receptor-G protein interactions: $G_{\alpha i1}$ -bound structures of IC3 and a mutant with altered G protein specificity

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Abstract

The structure of the C-terminal region of the third cytoplasmic loop (IC3) of the cannabinoid receptor one (CB1) bound to $G_{\alpha i1}$ has been determined using transferred nuclear Overhauser effects (NOEs). The wild-type IC3 sequence is helical when associated with $G_{\alpha i1}$. In contrast, a peptide containing the amino-acid inversion, Ala³⁴¹-Leu³⁴² adopts a single turn. These findings correlate with the attenuated G_i association of CB1 with the Ala³⁴¹-Leu³⁴² mutation previously observed in vivo and the diminished stimulation of $G_{\alpha i1}$ GTPase activity by the corresponding peptide demonstrated in vitro here. These results, the first to report the structure of a GPCR domain while associated with G protein, imply the C-terminus of CB1 IC3, a region with high-sequence conservation among G-protein coupled receptors, must be helical for efficient coupling and activation of the G_i protein.

Keywords: Cannabinoid receptor (CB1); G-protein coupled receptors; G-protein association; constitutively active receptors; third cytoplasmic loop (IC3); transferred NOEs

Two subtypes of G-protein coupled cannabinoid receptors, CB1 and CB2, have been identified and cloned. The CB1 receptors are found predominantly in the central nervous system (Gerard et al. 1991) while CB2 receptors are found

mainly in association with immune cells, including the marginal zone of the spleen and in macrophages (Munro et al. 1993). The receptors have been implicated in mediation of cannabinoid-induced analgesia, antiinflammation, and immunosuppression. Recently, cannabinoid receptor agonism has been shown to control spasticity in a multiple sclerosis mouse model (Baker et al. 2000) and may regulate food intake as part of the neural circuitry regulated by leptin (Di Marzo et al. 2001).

CB1 and CB2 heterologously expressed in cultured cells have been shown to inhibit the accumulation of cAMP in a ligand-dependent manner (Felder et al. 1995; Slipetz et al. 1995), consistent with the notion that coupling to inhibitory G proteins (G_i) is a major signaling pathway for CB receptors (Howlett et al. 1986). CB1, but not CB2, can also couple to G_s but the functional significance of this association is not yet known (Glass and Felder 1997).

The identification of the regions of the receptors involved in the coupling to the G proteins has been an area of active research (Mukhopadhyay and Howlett 2001; Nie and Lewis

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Abbreviations: AL-IC3, peptide sequence of IC3 with AL swap, DIRALKTLV; CB1, cannabinoid receptor one; CHAPS, 3-[(3-Cholamidopropyl)dimethylammonio]-1-propane-sulfonate; DG, distance geometry; DTT, dithiothreitol; EM, energy minimization; GPCR, G-protein coupled receptor; IC3, intracellular loop 3; IPTG, isopropyl- β -D-thio-galactopyranoside; IRMA, iterative relaxation matrix approach; MD, molecular dynamics; NOE, nuclear overhauser effect; PMSF, phenylmethanesulfonyl fluoride; RMSD, root mean squared deviation; ROE, rotating frame Overhauser effect; TM, transmembrane segment; TSP, 3-(trimethylsilyl) tetraduetero sodium propionate; WT-IC3, wild-type sequence of IC3, DIRLAKTLV.

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2001b). The C terminus of CB1 has been shown to play an important role in G protein coupling (Nie and Lewis 2001a), as well as in the desensitization and internalization of CB1 (Hsieh et al. 1999). Phosphorylation (Garcia et al. 1998) and peptide studies (Howlett et al. 1998) of the CB1 receptor, along with a number of studies in analogous regions of other GPCRs (Cotecchia et al. 1990; Ren et al. 1993; Samama et al. 1993), have shown that the C-terminal region of the third intracellular loop plays a significant role in G-protein activation. Previously, we have shown that the third cytoplasmic loop of CB1 is involved in coupling and activation of the G protein, and that exchange of the position of Leu³⁴¹ and Ala³⁴² resulted in altered specificity to constitutive G_s coupling (Abadji et al. 1999). Here, we examine the structure and function of this region of CB1. The structure of the wild-type sequence (WT-IC3: D³³⁸IRLAKTLV³⁴⁶) and mutant containing an inversion of residues 341 and 342 (AL-IC3: D³³⁸IRALKTLV³⁴⁶) while associated with the G protein is solved. This is the first example of a GPCR domain structure associated with G protein. Correlating these findings with the functional assays (stimulation of GTPase) illustrates important features for efficient receptor coupling and activation of the G protein.

Results

To reveal structural features of CB1 important for coupling with G protein, we exploited the differences in G_α subunit specificity exhibited *in vivo* by the wild type and the mutant Ala³⁴¹-Leu³⁴² receptor (Abadji et al. 1999). Peptides corresponding to the IC3 region were examined for GTPase stimulatory activity *in vitro* using G_{αi1}. The G_{αi1} subunit, which coimmunoprecipitates with CB1 (Mukhopadhyay et al. 2000), exhibits endogenous GTPase activity (Fig. 1A) (Kleuss et al. 1994). Previous studies have demonstrated that the toxin mastoparan (Higashijima et al. 1990) and peptides from the α₂-adrenergic receptor (Wade et al. 1996) stimulate this activity by promoting GDP release in a manner that reflects the response to agonist-induced activation of an intact GPCR (Dalman and Neubig 1991; Taylor et al. 1994). We observed a small enhancement in the GTPase activity of G_{αi1} with mastoparan consistent with a previous report (Higashijima et al. 1990) and a comparable effect with the flAL-IC3 peptide (Fig. 1A). In contrast, the stimulation with the flWT-IC3 peptide is marked, a more than fourfold enhancement compared to the latter peptides. The GTPase stimulation is dependent on peptide concentration (Fig. 1B), GTP concentration (Fig. 1C), and is saturable. Remarkably, the flAL-IC3 peptide differs from the flWT-IC3 peptide in sequence only by the conversion of Leu³⁴¹-Ala³⁴² to Ala³⁴¹-Leu³⁴² yet the impact on G_{αi1} stimulation is dramatic, underscoring the critical nature of this region of the receptor.

To establish a structural basis for the functional differ-

Fig. 1. GTPase activity of G_{αi1}. The enzyme assays were conducted as described in Materials and Methods. Each data point represents an average of triplicate assays $\pm$ SE. (A) G_{αi1} GTPase activity in the absence and presence of 1 mM peptides and 200 μ M GTP. (B) The dependence of G_{αi1} GTPase activity on the concentration of the wild-type IC3 peptide (filled circles) and the Ala³⁴¹-Leu³⁴² variant (open circles). (C) The dependence of the peptide-stimulated G_{αi1} GTPase activity on GTP concentration. The concentration of the wild-type IC3 peptide (filled circles) and the Ala³⁴¹-Leu³⁴² variant (open circles) was 1 mM.

ences, transferred nuclear Overhauser effect (NOE) experiments were carried out with the G_{αi1} subunit and peptides containing the C terminus of IC3, centered on Leu³⁴¹-Ala³⁴², WT-IC3 and AL-IC3. Both WT-IC3 and AL-IC3 displayed no ordered structure in aqueous solution. All of the nuclear magnetic resonance (NMR) parameters, including chemical shift, coupling constants, and NOEs (or ROEs) indicated the lack of secondary structural elements.

The peptide-to-protein ratio for the trNOE experiments was determined by titrating $G_{\alpha i1}$ into solutions containing the peptides and monitoring line widths in one-dimensional proton spectra. The peptides proved to be very soluble at high concentrations (4 mM peptide), even upon addition of G protein. A series of NOESY spectra (mixing times of 50, 80, 100, and 120 ms) was collected at the optimal conditions of a G-protein:peptide ratio of 1:20 (200 μ M $G_{\alpha i1}$).

The initial ensemble of 20 peptide structures generated by metric matrix distance geometry (DG) calculations using NOE-derived distance restraints are shown superimposed in Fig. 2. For **WT-IC3**, a helical structure with some fraying at the terminal Asp¹ and Val⁹ is observed. A number of the 93 trNOEs observed are consistent with a helix (Table 1) including $\alpha N(i)(i+3)$ $i = 1,5,6$, $\beta N(i)(i+3)$ $i = 1,2,3,4,6$ and $NN(i,i+1)$ $i = 2,3,4$. The average pairwise RMSD for the backbone atoms of residues 2–8 is 0.62 Å. The conformation of the side chains is well defined, with an average RMSD value of 1.4 Å for the heavy atoms of residues 2–8. The side chains of Leu⁴ and Ala⁵ are particularly well resolved with RMSD values of 0.79 and 0.71 Å, respectively (Table 1).

In contrast to the helical structure observed for the wild-type IC3, the center of **AL-IC3** adopts a well-defined turn involving Arg³-Lys⁶ (Fig. 2). Out of 93 total NOEs, eight are determinant in defining the structure of the turn, including Arg³ HN-Ala⁴ HN, Arg³ HN-Leu⁵ HN, Arg³ HN-Ala⁴ H β Arg³ H β -Ala⁴ HN, Ala⁴ H β -Leu⁵ HN, and Ile² H α -Leu⁵ HN. The average pairwise RMSD for the ensemble of structures is 0.81 Å for the backbone atoms of residues 2–8, and 1.7 Å for the side-chain atoms of residues 2–8.

Fig. 2. Superposition of the ensemble of 20 structures from the DG calculations: (a) WT-IC3 and (b) AL-IC3. The heavy backbone atoms were used in the superposition.

Table 1. Distribution of trNOEs and structural statistics of WT-IC3 and AL-IC3

Distribution of NOEs	WT-IC3	AL-IC3
NOEs	93	93
intraresidue	66	64
i, i + 1	16	19
i, i + 2	1	2
i, i + 3	10	8

Atomic RMSD values (heavy atoms) of the ensemble of 20 DG structures		
	WT-IC3	AL-IC3
Backbone, side chain (Å)		
Asp ¹	1.05, 2.07	1.48, 2.62
Ile ²	0.64, 1.39	0.86, 1.58
Arg ³	0.54, 2.37	0.88, 2.37
Leu/Ala ⁴	0.36, 0.79	0.92, 1.28
Ala/Leu ⁵	0.43, 0.71	0.71, 1.71
Lys ⁶	0.67, 1.53	0.72, 2.19
Thr ⁷	0.81, 1.64	0.81, 1.29
Leu ⁸	0.68, 1.41	0.82, 1.76
Val ⁹	1.00, 1.85	1.18, 2.31

NMR R-factor from IRMA Calculations (Gonzales et al., 1991)		
Iteration	WT-IC3	AL-IC3
1	0.84	0.86
2	0.75	0.88
3	0.74	0.81
4	0.74	0.79
5	0.74	0.79

The ensemble of DG structures was utilized as a starting point for the ensemble-IRMA calculations (Boelens et al. 1989; Bonvin et al. 1993). Extensive MD simulations with the ensemble-IRMA generated distance restraints based on the integrated NOE cross-peak volumes and the starting ensemble produced the next generation ensemble of structures. This procedure was iterated until the R-factor (Gonzales et al. 1991) did not further decrease. The resulting structures are illustrated in Figure 3.

Discussion

We have examined the structure and function of the interaction of the third intracellular loop of the CB1 receptor with $G_{\alpha i1}$. In the peptides corresponding to the C-terminus of IC3, the conservative mutation, L341A/A342L, reduces the stimulation of $G_{\alpha i1}$ GTPase compared to wild type. This correlates with the results from the corresponding mutation in vivo in which we observe a shift in preference toward G_s when both G_i and G_s α subunits are available in the cell (Abadji et al. 1999). It should be noted that when cells

Fig. 3. Structure of (a) WT-IC3 and (b) AL-IC3 after IRMA refinement. The Connolly surface representation of the molecules are shown color coded by hydrophobicity (red represents hydrophobic, blue represents hydrophilic).

harboring the mutant receptor are treated with cholera toxin so that coupling via G_s is not possible, inhibition of cAMP accumulation via G_i coupling is observed. This is consistent with the NMR analysis, which demonstrates that the peptide with the mutant sequence does bind G_i, and the GTPase results that show stimulation to a level comparable to mastoparan. However, the strong parallelism observed between the diminished preference for G_i in vivo, the reduced extent of GTPase stimulation relative to the wild type, and the nonhelical structure of the G_i-associated form argue that the mutant sequence diminishes the efficiency of the interaction with G_i though it does not abrogate it altogether. The L341A/A342L mutation disrupts the LAKT motif important for receptor-G protein interaction. This motif is 100% conserved in the 11 cannabinoid receptors that have been characterized to date. Examining the database of 1026 GPCRs, a sequence of AAKM is observed ~50% of the time, underscoring the critical nature of this segment of the receptor (Horn et al. 1998). Transferred NOE experiments have allowed us to directly observe the structure of this motif and the AL mutation when bound to the G_{αi1} protein.

When bound, WT-IC3 adopts a distorted helical conformation, which orients the side chains of Leu⁴ and Ala⁵ in the LAKT motif to form a hydrophobic ridge along one face of the helix (Fig. 3). In contrast, AL-IC3 is not helical but instead forms a turn at Ala⁴-Leu⁵. This may result from distortion of the side chains of leucine and alanine to fit into a binding pocket that is sterically optimized for the wild-type sequence. The structural difference has a major consequence of the relative topological display of the two positively charged residues, Arg³ and Lys⁶. As illustrated in Figure 3, the helical structure of WT-IC3 produces an amphipathic arrangement, with the Asp¹, Arg³, and Lys⁶ all on one face. The turn structure observed for AL-IC3 disrupts this arrangement (Fig. 3b). The dramatic alteration of the

bound peptide structure consequent to a conservative mutation (two hydrophobic residues with only a slight difference in size) indicates that this part of the receptor makes very close contacts with the G protein. It is unlikely that such a small change would be able to induce a major shift in a distant binding site.

Based on homology with rhodopsin, the peptide sequence examined here is at the interface of IC3 and transmembrane helix (TM) 6 of the CB1 receptor (WT-IC3 corresponds to ²⁴⁷EKEVTRMVI²⁵⁵ of bovine rhodopsin); this region is α-helical in the X-ray structure of rhodopsin (Palczewski et al. 2000). Based on studies incorporating spin and fluorescent labels into this region of rhodopsin and the β₂-adrenergic receptor (Farrens et al. 1996; Ghanouni et al. 2001), activation leads to a rearrangement of the TM helices, particularly TM6, possibly leading to accessibility of the LAKT domain. Our NMR data substantiate that, indeed, this critical region changes conformation upon interaction with G protein. The characterization of these structural changes with different G proteins will provide greater insight into the mechanism of specific G protein activation.

Materials and methods

Sample preparation

Using a pGEX fusion system (Amersham Biosciences), G_{αi1} and IC3 peptides were expressed in BL21(DE3) (Invitrogen) from plasmids pGEX-6P1.G_{αi1} (a gift from K. Wakanatsu and T. Kohno of Mitsubishi Kasei Institute of Life Science, Tokyo), pGEX-2T.CB1IC3 and pGEX-2T.CB1IC3AL (encoding full-length CB1 IC3, designated fWT-IC3, and that with the L341A/A342L mutation, designated fAL-IC3, respectively). The GST fusions were induced with IPTG (30°C for G_{αi1} and 37°C for IC3 peptides) and purified using glutathione Sepharose 4B resin according to the manufacturer's protocol with minor modification. After PreScission protease cleavage, G_{αi1} was isolated by Q-Sepharose chromatography followed by a second round of glutathione Sepharose 4B chromatography to >98% purity confirmed by SDS-PAGE. The IC3 peptides were separated from GST after thrombin cleavage, which results in a Gly and Ser amino terminal to the CB1 sequences, using a SP-Sepharose fast flow column and a linear gradient of ammonium acetate (0 mM to 500 mM). Pure peptide-containing fractions, monitored via SDS-PAGE, were pooled, lyophilized, dissolved in 5% acetic acid, and lyophilized again three times.

Peptides containing the C terminus of the IC3 of CB1, WT-IC3: DIRLAKTLV-NH₂ and AL-IC3: DIRALKTLV-NH₂, were synthesized and purified by reverse-phase HPLC (Tufts Analytical Core Facility). Purity was verified by mass spectrometry and NMR.

GTPase assays

GTPase activity was determined essentially as described previously (Higashijima et al. 1987, 1990; Wade et al. 1996). Reactions contained 50 mM Na HEPES (pH 8.0), 1 mM EDTA, 1.1 mM MgCl₂, 1 mM DTT, 120 nM G_{αi1}, peptides, and GTP (Sigma)

concentrations as indicated in the figure legends, and 20% glycerol. Nonenzymatic hydrolysis of [γ - 32 P]GTP, which was generally <5% of the total added, was subtracted from all responses.

Nuclear magnetic resonance spectroscopy

NMR samples for the transferred NOE experiments were prepared by exchanging $G_{\alpha 1}$ into 10 mM acetate buffer, pH 6.0, containing 1 mM deuterated DTT and TSP as an internal chemical shift reference and adding the protein in solution to the lyophilized peptide. All spectra were acquired on a 600 MHz Bruker AVANCE spectrometer at 25°C. The transferred NOE experiments of 4 mM WT-IC3 or AL-IC3 and 200 μ M $G_{\alpha 1}$ were carried out using mixing times of 50, 80, 120, 150, and 200 ms. Sequential assignments of the NOESY spectra were made using TOCSY spectra (mixing times 30 and 60 ms; 10 kHz spin locking field obtained with MLEV17). Both NOESY and TOCSY experiments used the WATERGATE 3–9–19 pulse sequence for water suppression (Piotto et al. 1992; Sklenar et al. 1993). Spectra were processed using Bruker XWIN-NMR software or NMRPipe (Delaglio et al. 1995), and Sparky (Goddard and Kneller 2001) was used to view and assign spectra, and to measure peak volumes and intensities.

Distance geometry calculations

Peak volumes from the assigned NOESY spectra were divided into strong, medium, and weak peaks to generate distance restraints (2.0–3.0 Å, 2.0–4.0 Å, and 2.0–5.0 Å, respectively). Pseudoatoms were used for methylene protons that could not be stereospecifically assigned. Distance geometry calculations were carried out employing the random metrization method of Havel (1991) to generate an ensemble of structures to be refined using IRMA (Boelens et al. 1989; Bonvin et al. 1993) following published procedures (Mierke et al. 1994; Pellegrini et al. 1996).

IRMA calculations

The ensemble IRMA (Bonvin et al. 1993) calculations were run from the InsightII software suite (Molecular Simulations, Inc.); the MD simulations were run using Discover (Molecular Simulations, Inc.). In the first iteration of IRMA, the 20 DG structures with the lowest penalty function were used as a starting ensemble. The result of the IRMA calculations is a set of distance restraints based on the integrated volumes of the NOEs and the starting ensemble. The distance restraints were then applied during NOE-restrained MD simulations and energy minimization to a randomly chosen set of ten structures from the original ensemble. These resulting structures then formed the starting ensemble for a second IRMA iteration. This procedure of IRMA followed by MD and EM was repeated for a total of five iterations, until the NOE R-factor (Gonzales et al. 1991) did not decrease further. The resulting structures and the distance restraints have been submitted to the PDB databank (access code 1LVQ and 1LVR).

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