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Structural biology of human cannabinoid receptor-2 helix 6 in membrane-mimetic environments ☆

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ABSTRACT

We detail the structure and dynamics of a synthetic peptide corresponding to transmembrane helix 6 (TMH6) of human cannabinoid receptor-2 (hCB2) in biomembrane-mimetic environments. The peptide's NMR structural biology is characterized by two α-helical domains bridged by a flexible, nonhelical hinge region containing a highly-conserved CWFP motif with an environmentally sensitive, *Pro*-based conformational switch. Buried within the peptide's flexible region, W^{258} may hydrogen-bond with L^{255} to help stabilize the *Pro*-kinked hCB2 TMH6 structure and position C^{257} advantageously for interaction with agonist ligands. These characteristics of hCB2 TMH6 are potential structural features of ligand-induced hCB2 activation *in vivo*.

Introduction

G protein-coupled receptors (GPCRs) comprise a superfamily of cell-surface proteins whose iconic structural signature includes seven hydrophobic transmembrane helices (TMHs) [1]. As components of the information-transducing machinery of eukaryotic cells, GPCRs are activated by exogenous agonists whose binding elicits GPCR structural changes that transition the receptor from inactive to active state(s). A "rotamer toggle switch" involving the bend angle or "kink" around a conserved proline in TMH6 and an "ionic lock" involving salt-bridge formation between charged amino acids in TMH3 and TMH6 have been implicated in the structural changes upon ligand-induced GPCR activation, largely from data on those few GPCRs whose high-resolution crystal structures have been solved [2–4]. Isolated successes with GPCR crystallization notwithstanding, direct experimental information on GPCR structural biology remains very limited.

Cannabinoid (CB) receptors are class-A, rhodopsin-like GPCRs that are activated by intrinsic lipid mediators (endocannabinoids) and exogenous cannabimimetic agents [5]. The (patho)physiological importance of cannabinergic signaling has prompted the pharmacotherapeutic evaluation of designer ligands (agonists/antagonists) selective for one of the two CB receptor subtypes, CB1 and CB2, which have been cloned, expressed, and sequenced [6]. Because of CB2's largely peripheral localization, CB2-targeted ligands as potential drugs for pain and inflammatory and neurodegenerative disorders have an inherently low risk of inducing centrally-mediated side effects, which mainly reflect CB1 activation [7]. Although CB1 and CB2 have been the focus of mutation and homology-modeling studies, their crystal structures are lacking [8,9]. Consequently, CB-receptor structural properties critical to ligand docking and pharmacological activation remain experimentally ill-defined. Our previous work has implicated a flexible TMH6 CWxP motif in the structural changes critical to agonist-induced CB1 activation [10]. Molecular dynamics simulations suggest the existence of at least two CB1 TMH6 conformations differing in the degree of *Pro*-kink in that flexible-hinge region [11]. Such findings cannot be extrapolated readily to CB2, however, for human CB1 and CB2 (hCB2) differ considerably in their primary sequence, modeled tertiary conformations, and agonist structure–activity profiles [12,13].

☆ Atomic coordinates have been deposited in the Research Collaboratory for Structural Bioinformatics Protein Data Bank (pdb id:2ki9), and NMR data have been deposited in BioMagResBank (accession number 16268).

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The present work is focused on defining experimentally the structural features of CB2 TMH6 and identifying motifs potentially involved in the conformational switching of CB2 TMH6 upon ligand-induced activation. We have utilized a synthetic peptide representing hCB2 TMH6 [9], high-resolution solution nuclear magnetic resonance (NMR) spectroscopy (including 2D-^{1}H TOCSY and NOESY experiments), and spectrofluorometry. This approach is well validated for complete ^{1}H assignment with small nonlabeled peptides [14,15]. For our NMR experiments, the hCB2 TMH6 peptide was studied in a compatible solvent system that simulates a biomembrane environment [16]. Tryptophan is considered to play a special role in helping anchor and stabilize protein α-helical regions [17]. For our spectrofluorometry studies, we have exploited the single hCB2 TMH6 tryptophan residue (W^{258}) modeled as being centrally disposed within the plasma membrane *in situ* [9] to monitor local structural responses of hCB2 TMH6 when the peptide is reconstituted in micelle membrane-mimetics [18]. To our knowledge, this study provides the first direct experimental characterization of hCB2 TMH6 structure and implicates a motif around P^{260} in agonist-induced TMH6 conformational switching.

Materials and methods

Peptide synthesis and purification. The 33-mer [^{240}DVRLAKTLGLV LAVLLICWFPVLALMAHSLATT^{272}] corresponding to hCB2 TMH6 [9] was synthesized by a standard 9*H*-fluoren-9-ylmethoxycarbonyl (Fmoc)–polyamide method at the Molecular Biology Core Facility, Dana-Farber Cancer Institute (Boston, MA, USA). The peptide was isolated by reverse-phase LC to >95% purity according to LC and MALDI-TOF mass spectrometry (MS) analyses.

Sample preparation and NMR experiments. For 1-D and 2-D NMR, the hCB2 TMH6 peptide was dissolved (1.0 mM final conc.) in 30% (v/v) aqueous trifluoroethanol-d_2 (TFE). 2-Dimethyl-2-silapentane-5-sulfonic acid was added as reference standard. All experiments were conducted at 27 or 37 °C on a 700-MHz NMR spectrometer (Bruker BioSpin, Billerica, MA, USA). TOCSY (with 70, 80 and 90 ms mixing times) and NOESY (with 200 and 250 ms mixing times) spectra were acquired in phase-sensitive mode. Virtually complete proton chemical shift assignments have been obtained for this TFE/H_2O mixture [16,19].

Circular dichroism (CD) spectropolarimetry. Small micelles composed of either 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine (D-6-PC) or dodecylphosphocholine (DPC) were prepared in 10 mM Tris buffer, pH ~ 7.5, containing 0.5 mM EDTA and 10 mM NaCl. The hCB2 TMH6 peptide was dissolved in 30% TFE/H_2O or incorporated into micelles at a 100:1 lipid-to-peptide molar ratio. Analysis of peptide secondary structure was then performed with a nitrogen-flushed J-810 spectropolarimeter controlled by Spectra Manager (version 1.15.00) (Jasco Instruments, Easton, MD, USA) [20]. Spectra were recorded from 280 to 185 nm at 23 °C using a 1-cm cuvette, a scan rate of 1 nm/min, and an average of three scans per sample. Percent α-helical content was calculated as described [21].

Fluorescence spectroscopy. Spectrofluorometry experiments were performed at 30 °C using a QuantaMaster QM-1/2005 spectrofluorometer (Photon Technology International, Birmingham, NJ, USA) in a quartz cuvette. The samples were excited at 295 nm, and emission spectra were collected between 300 and 400 nm. The bandwidth for both excitation and emission monochromators was 5 nm. Samples were prepared by mixing D-6-PC or DPC with hCB2 TMH6 peptide in TFE/chloroform at a molar lipid-to-peptide ratio of 100:1. The mixture was dried to a film that was rehydrated with 25 mM Tris buffer, pH 7.4, containing 100 mM NaCl to give a stock solution of 1 mM peptide, which was then suitably diluted with the Tris–HCl solution to a final peptide concentration of 2.5 μM in the analyzed sample.

Collisional quenching experiments with acrylamide were performed by adding increasing amounts of a 10 M acrylamide stock solution in 25 mM Tris buffer (pH 7.4) to hCB2 TMH6 peptide samples reconstituted either in 30% TFE/H_2O or in D-6-PC or DPC micelles up to a maximal acrylamide concentration of 5 mM. The difference between the peptide's (i.e., the peptide's sole tryptophan residue, W^{258}) net fluorescence in the presence and absence of a given acrylamide concentration in each respective membrane-mimetic environment was determined. Quenching of the fluorescence intensities at maximum emission was calculated with the Stern–Volmer equation,

$$F_0/F = 1 + K_{sv}[\text{acrylamide}]$$

where F_0 is the unquenched fluorescence intensity; F is the fluorescence intensity at [acrylamide]; K_{sv} is the Stern–Volmer quenching constant, which was determined as a function of [acrylamide].

Structure determination. Observed nuclear Overhauser effects (NOEs) were classified according to three categories: short, medium, and long range. A total of 830 inter-residue distance restraints were used in the structure calculations. All the spectra were processed with Topspin (Bruker BioSpin) and visualized using CARA software (http://www.nmr.ch/). NOE assignments were improved by a KNOWNOE protocol [22]. Structure calculations were performed by Xplor-NIH [23]. Fifteen lowest-energy conformations were subjected to molecular dynamics simulations in explicit water using the Crystallography and NMR System software suite [24–26]. Structures were validated by PROCHECK-NMR and visualized with MOLMOL [27,28].

Results

hCB2 TMH6 solution structure

Superimposition of the fifteen lowest-energy hCB2 TMH6 NMR conformers in membrane-mimetic solvent (30% TFE/H_2O) is depicted in Fig. 1A. The α-helical secondary elements of the averaged hCB2 TMH6 structure are depicted as the solid ribbon in Fig. 1B. The ensemble statistics are presented in Table 1. The solution structure of hCB2 TMH6 is characterized by two helical segments of different lengths separated by a discrete unstructured region with a pronounced *Pro*-based kink. The kink, well defined by numerous NOEs, seems quite rigid in nature and orients the two helical segments at almost 45° with respect to each another. The two hCB2 TMH6 terminal regions, i.e., from the N-terminus to K^{245} and from A^{264} to the C-terminus, are dynamically unstructured.

CD structural analysis of hCB2 TMH6

In order to assess the structural responsiveness of hCB2 TMH6 to its immediate environment, we conducted CD studies of the peptide as reconstituted in three biomembrane-mimetic environments: the 30% TFE/H_2O mixture used in the NMR analysis (above) and detergent micelles composed of either D-6-PC or DPC [18]. The shapes and intensities of representative CD spectra (Fig. 2) are indicative of the peptide's having significant α-helical content in all three membrane-mimetics. The average α-helical content of hCB2 TMH6 was 44, 48, and 36% in D-6-PC micelles, DPC micelles, and 30% TFE/H_2O, respectively. The convergence (isodichroic) point among the three traces reflects a two-state system associated with environmentally-sensitive changes in hCB2 TMH6 conformation.

Involvement of W^{258} in hCB2 TMH6 structural dynamics

To determine whether W^{258} is involved in any local conformational changes of hCB2 TMH6 when the peptide interacts with li-