Biophysical Chemistry 240 (2018) 34–41

Contents lists available at ScienceDirect

Biophysical Chemistry

journal homepage: www.elsevier.com/locate/biophyschem

Constrained dynamics of the sole tryptophan in the third intracellular loop of the serotonin$_{1A}$ receptor

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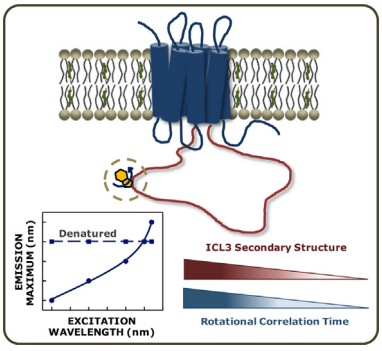
HIGHLIGHTS

- GPCR extramembranous regions are believed to be crucial for physiological functions.
- ICL3 of serotonin$_{1A}$ receptor is implicated in G-protein coupling and activation.
- Sole tryptophan of serotonin$_{1A}$ receptor ICL3 experiences constrained dynamics.
- This restricted dynamics is predominantly induced by peptide secondary structure.
- GPCR loop structure and dynamics may provide valuable insights into GPCR function.

GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
ICL3
Constrained tryptophan dynamics
REES
Serotonin$_{1A}$ receptor
GPCR

ABSTRACT

G protein-coupled receptors (GPCRs) are major signaling proteins in eukaryotic cells and are important drug targets. In spite of their role in GPCR function, the extramembranous regions of GPCRs are relatively less appreciated. The third intracellular loop (ICL3), which connects transmembrane helices V and VI, is important in this context since its crucial role in signaling has been documented for a number of GPCRs. Unfortunately, the structure of this loop is generally not visualized in x-ray crystallographic studies since this flexible loop is either stabilized using a monoclonal antibody or replaced with lysozyme. In this work, we expressed and purified the ICL3 region of the serotonin$_{1A}$ receptor and monitored its motional restriction and organization utilizing red edge excitation shift (REES) of its sole tryptophan and circular dichroism (CD) spectroscopy. Our results show that the tryptophan in ICL3 exhibits REES of 4 nm, implying that it is localized in a restricted microenvironment. These results are further supported by wavelength-selective changes in fluorescence anisotropy and lifetime. This constrained dynamics was relaxed upon denaturation of the peptide, thereby suggesting the involvement of the peptide secondary structure in the observed motional restriction, as evident from CD spectroscopy and apparent rotational correlation time. To the best of our knowledge, these results constitute one of the first measurements of motional constraint in the ICL3 region of GPCRs. Our results are relevant in the context of the

Abbreviations: CD, circular dichroism; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; GPCR, G protein-coupled receptor; ICL3, third intracellular loop; IPTG, isopropyl β-D-1-thio-galactopyranoside; PMSF, phenylmethylsulfonyl fluoride; REES, red edge excitation shift; TCSPC, time-correlated single photon counting

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https://doi.org/10.1016/j.bpc.2018.05.008
Received 1 May 2018; Received in revised form 24 May 2018; Accepted 27 May 2018
Available online 01 June 2018
0301-4622/

reported intrinsically disordered nature of ICL3 and its role in providing functional diversity to GPCRs due to conformational plasticity.

1. Introduction

G protein-coupled receptors (GPCRs) are a family of seven transmembrane domain proteins that allow the exterior of the cell to communicate with the cellular interior [1–3]. In molecular terms, signaling carried out by GPCRs involves their activation by ligands present in the extracellular environment, followed by the transduction of signals to the interior of the cell through concerted conformational changes in their transmembrane domain. This could imply that various domains in GPCRs act in a cooperative fashion for carrying out signal transduction. GPCRs act as highly versatile and dynamic membrane sensors and mediate cellular responses to a diverse variety of stimuli in several physiological processes. As a consequence of diverse signaling by GPCRs, they have emerged as major drug targets in all clinical areas [4, 5].

Serotonin receptors represent a major class of GPCRs involved in brain function [6, 7]. These receptors play a crucial role in the generation and modulation of cognitive and behavioral functions. Malfunctioning of serotonergic systems is implicated in disorders such as depression, anxiety, schizophrenia, obsessive compulsive disorder and migraine. According to the present classification, there are 16 subtypes of serotonin receptors [8]. The serotonin$_{1A}$ receptor is an important member of this family due to a number of reasons [9] and serves as an important target in drug discovery for neuropsychiatric disorders and cancer [8, 10]. Previous work from our laboratory has shown the crucial role of membrane cholesterol [11–13] and sphingolipids [13, 14] in the function of the serotonin$_{1A}$ receptor.

Typically, GPCRs consist of seven transmembrane domains (helices) interconnected by several extracellular and intracellular loops. Since GPCRs have odd number of membrane passes, the N- and C-termini are located on opposite sides of the membrane (see Fig. 1). Most of the focus on GPCRs till date has been centered on the transmembrane helices. This is in spite of the fact that the extramembranous regions of GPCRs have been reported to have important functions for cellular signaling and desensitization. For example, the third intracellular loop (ICL3), which connects transmembrane helices V and VI, has been reported to be crucial for function [16–20] and dynamics [21, 22] of a number of GPCRs. Yet, this flexible loop was either stabilized using a monoclonal antibody or replaced with lysozyme in a number of recent high resolution crystallographic analyses of GPCRs [23–26], since the inherent conformational flexibility of the loop poses a problem for x-ray crystallography. The lack of information from intracellular loops makes such crystallographic analyses somewhat limited. Another issue with many reported GPCR crystal structures is the fact that the receptor is crystallized in the lipidic cubic phase whose physiological significance is yet to be ascertained [27].

Although the number of reported crystal structures of GPCRs is continuously increasing [28], the challenge that remains is to generate a comprehensive understanding of the molecular mechanism underlying the conversion of ligand binding to GPCRs to receptor activation and signaling *via* a series of conformational changes. Keeping in mind this overall context, in this work, we have explored the organization and dynamics of the ICL3 of the serotonin$_{1A}$ receptor. Human serotonin$_1$ receptors are classified into five subtypes 1A, 1B, 1D, 1E and 1F [8]. The serotonin$_{1A}$ receptor is a prominent member of this subfamily. The receptor is estimated to have differentiated ~650 million years ago from the subfamily in the time period when vertebrates and invertebrates diverged, making this subtype an evolutionary marker [29, 30]. The intronless genomic clone for the human serotonin$_{1A}$ receptor (G-21) encodes a protein of 422 amino acids [31]. The serotonin$_{1A}$ receptor is predominantly localized in the hippocampal region in the brain [32] and has not been purified from native tissue due to relatively low amounts present in it. As such, no crystal or NMR structure is available for this receptor, in spite of its importance as a drug target [8, 10]. We previously built a homology model for the receptor using the β_2-adrenergic receptor template [33]. Due to the presence of three consensus sequences for N-linked glycosylation in the amino terminus, and ~48% homology of the receptor with β_2-adrenergic receptor in the transmembrane region, it is predicted that the membrane topology of the receptor is such that its amino terminus faces the extracellular region and the carboxy terminus faces the intracellular cytoplasmic region [33] (see Fig. 1).

A striking aspect of the serotonin$_{1A}$ receptor is the length (123 residues spanning from 221 to 343 residues, based on transmembrane helix prediction using TMHMM2 [15]) of its ICL3. This loop of the serotonin$_{1A}$ receptor is considerably longer than the corresponding loop of other members of the serotonin$_1$ receptor subfamily [33, 34]. ICL3 has been shown to bind calmodulin [34, 35] and is important for coupling to G-proteins [36], crucial for downstream signaling. Sequence alignment of the members of the serotonin$_1$ receptor subfamily exhibits conserved sequences in the transmembrane regions, but not in the ICL3 region [33, 34]. This suggests that the diversity of function exhibited by various subtypes of serotonin$_1$ receptors could be encoded

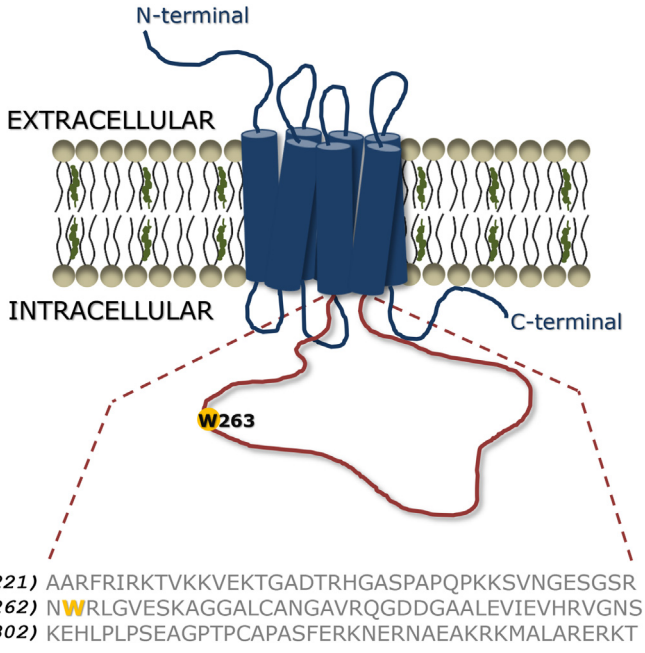

Fig. 1. A schematic representation of the topological features of the human serotonin$_{1A}$ receptor highlighting the third intracellular loop (ICL3). The seven transmembrane helices embedded in the membrane bilayer (composed of phospholipids and cholesterol, typical constituents of eukaryotic membranes), and the receptor termini and loop regions connecting the transmembrane domains are shown. The transmembrane regions of the receptor have been predicted as putative α-helices using TMHMM2 [15]. The ICL3 segment (in maroon) with its amino acid sequence and its sole tryptophan residue (highlighted in yellow) are shown. ICL3 consists of 123 residues (residues 221–343) connecting transmembrane helices V and VI and is believed to play an important role in the recruitment of effectors (such as G-proteins) critical for downstream signaling, and binds calmodulin. See text for more details. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)