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Current advances in the development of G-protein-coupled receptor structure and their future application in drug design

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G-protein-coupled receptors (GPCRs) are seven-transmembrane proteins that play important roles in signal transduction from the extracellular compartment to the cytoplasm. GPCR malfunction has been implicated in various diseases such as cancer, allergy, neuronal disease, rheumatism, and obesity. Because of the importance of GPCRs, GPCR-targeting drugs occupy approximately 40% of the total drug market. Recently, three-dimensional structures of many GPCRs have been successfully ascertained on the basis of various developed methods that improve protein stabilization, crystallization, and diffraction. Some GPCR structures have elucidated the underlying activation mechanism by allowing comparison between the receptor's inactive and active states. β-adrenergic receptor bound with G-protein trimeric complex ($G_{\alpha\beta\gamma}$) structure has also been determined. To date, XFEL technology is the latest technology used to determine GPCR structures. In this review, we describe GPCR structures, structure-based activation mechanisms and principles, and the application of XFEL in determining GPCR structures and development of GPCR-based drugs.

INTRODUCTION

G-protein-coupled receptors (GPCRs) are membrane-protein receptors that consist of seven-transmembrane helices and both N-terminal and C-terminal loops. Each transmembrane helix connects the loops, known as extracellular loop (ECL) 1, 2, and 3 and intracellular loop (ICL) 1, 2, and 3. GPCRs localize at the cellular membrane around the lipid bilayer. They are found only in eukaryotes, including yeast, insects, and animals (King, Hittinger et al. 2003). GPCRs can quickly respond to the environmental factors, such as flavor, odor, light, and shock (Hermans 2003). At the cellular level, GPCRs play important roles in signal transduction by transferring signals from various extracellular factors, such as cytokines, hormones, neurotransmitters, and chemicals, to intracellular components of the signaling pathway (Wong 2003). GPCR-targeting agents account for approximately 40% of current drugs, and GPCRs are considered good targets for the development of new drugs (Overington, Al-Lazikani et al. 2006). During the last 5 years, in order to develop GPCR-targeting drugs, X ray crystallographers and NMR researchers have determined many GPCR structures, including the beta-1 and beta-2 adrenergic, Adenosine A2A, dopamine 2, CXCR4, histamine, three opioid, muscarinic, and S1P1 receptors (Venkatakrishnan, Deupi et al. 2013). In 2012, Brian Kobilka's group succeeded in crystallizing and determining the structure of the active GPCR and complex, composed of the trimeric G-protein complex and nanobody, from a camel. These results provided information about the mechanism of GPCR activation (Rasmussen, DeVree et al. 2011). Physicists have developed new technology, known as XFEL (X-ray Free Electron Laser) (Chapman, Fromme et al. 2011). The first GPCR structure determined using XFEL was the serotonin receptor. Two major advantage of XFEL are that crystals are not limited by size and do not require freezing (Liu, Wacker et al. 2013).

STRUCTURES OF GPCRS

GPCR structures comprise three distinct parts. The first part is the extracellular domain, which is the N-terminal and includes ECLs 1-3, and which recognizes and interacts with ligands as extracellular signals. The second part is the seven-transmembrane domain (7-TM), which consists of hydrophobic 7 α-helices stabilized by the lipid bilayer. 7-TM has a canonical structural scaffold that is evolutionally conserved, especially in the class A GPCR family, and plays role in signal transduction via slight conformational changes, such as tilting of the alpha helices. The final part of a GPCR is the intracellular domain, which contains the C-terminal loops and ICLs 1-3. The C-terminal domain is essential for the determination of GPCR-fate, which is mediated via phosphorylation by GPCR kinase (GRK) and consequently accesses the β-arrestin and is

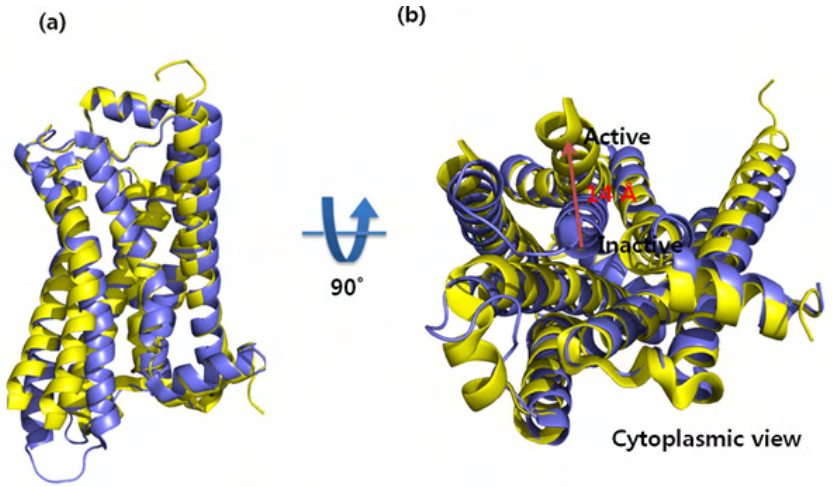

FIGURE 1 | Superposition of the active (yellow) and inactive (cyan) β2-AR structure. (a) The superimposed β2-AR structures show a lot of similarity between the active (3POG) and inactive (4GBR) structures. (b) The major difference between the two structures is the location of TM6, with approximately 14Å difference between the locations. In the cytoplasmic domain of GPCRs, TM6 undergoes a conformational change to be more forward and outward in the active state.

internalized to cytoplasm. (Yang and Xia 2006). ICL-3 has been known to be a binding site for the G-protein trimeric complex, but the molecular details of this interaction are unclear.

Most GPCR structures determined to date have been observed to be in the inactive state. There are some reasons for this preference of inactive structure over the active structure. When bound to antagonists, GPCRs show increased rigidity and stability, making them more successfully crystallized than their active structures (Venkatakrishnan, Deupi et al. 2013).

Interestingly, the structure of beta-2 adrenergic receptor (β2-AR) was shown to exist in both the inactive and active states in complex with two anti-polar ligands, an agonist (Rasmussen, Choi et al. 2011) and an antagonist (Cherezov, Rosenbaum et al. 2007). The major difference between inactive and active β2-AR is a conformational change of TM6. In the active state, TM6 expands, such that its location is about 14Å removed from its position in the inactive state. This is caused by steric hindrance as a result of the antagonist binding and breaking the ionic lock (Rosenbaum, Rasmussen et al. 2009) (Figure 1).

The active state of β2-AR with the trimeric G protein complex was also ascertained (Rasmussen, DeVree et al. 2011). This structure displays how activated GPCRs bind to the trimeric G protein complex at the molecular level. In the presence of agonist, β2-AR undergoes the conformational change that moves the TM6 helix outward and thus opens up enough space for G-protein binding. Following the conformational change of TM6, β2-AR favors the binding of the G-protein trimeric complex (Figure 2). The G_{α}-subunit consists of two domains, the Ras and G alpha helical (Gah) domains, and GDP binds between these two domains. When β2-AR and the G-protein trimeric complex are bound, the Ras and Gah domains loosen binding between the two domains. Then, GDP is released from the G_{α}-subunit, and GTP is substituted in the same position. Consequently, GTP binds to the G_{α}-subunit, and the G_{α}-subunit is released from β2-AR and the $G_{\beta\gamma}$-subunit. Following activation of the G_{α}-subunit, it activates adenylate cyclase to increase the cAMP level (Rasmussen, DeVree et al. 2011).

ADVANCES IN GPCR STRUCTURE DETERMINATION

In the last 5 years, 20 GPCR structures have been determined. Several advances have been made that are significant for

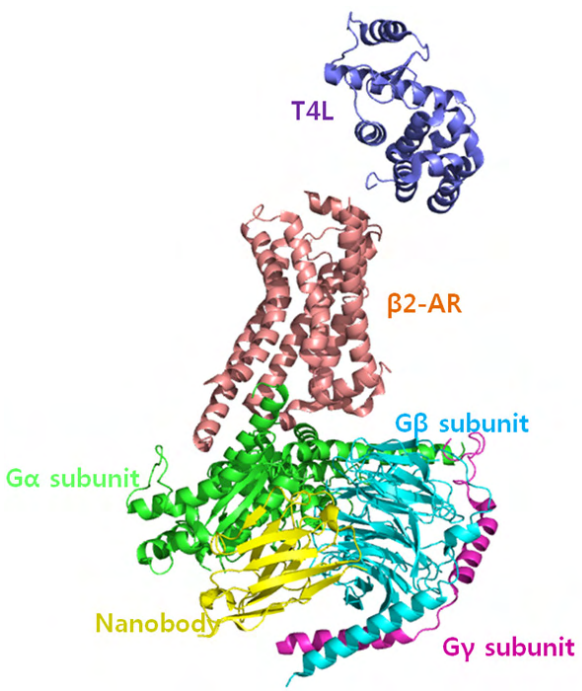

FIGURE 2 | The overall structure of the complex with β2-Adrenergic receptor (pink) and the G_{α} (green), Gβ (blue), and Gγ (magenta) trimeric complex, with stabilizing proteins, such as T4-lysozyme (violet) and nanobody (yellow) (PDB:3SN6). The complex structure shows that agonist-activated β2-AR triggers the binding of the G-protein trimeric complex.

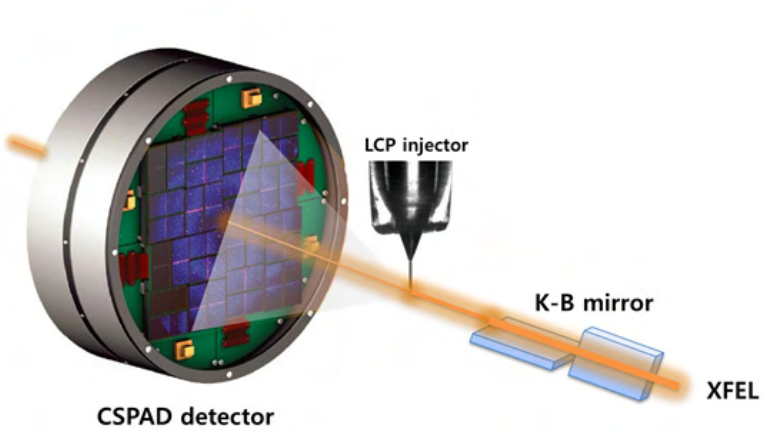

FIGURE 3 | A schematic view of an XFEL experiment for data collection using an LCP injector. The XFEL beam is focused with Kirkpatrck-Baez (K-B) mirrors. LCP crystals are dispersed by the LCP injector as a continuous column, 20 to 50 μm in diameter. Single-pulse diffraction patterns are collected at 120 Hz by using a CSPAD detector.

successful determination of GPCR structures. One of them involves the use of successful fusion partners, such as T4-lysozyme and BRIL, inserted in ICL-3, which is between TM5 (transmembrane) and TM6 (Chun, Thompson et al. 2012). This region is regarded as a very flexible domain. Secondly, the method of thermostabilizing mutagenesis (StaR) has been useful (Warne, Serrano-Vega et al. 2008, Shibata, White et al. 2009, Robertson, Jazayeri et al. 2011). The third advancement is the use of an anti-GPCR monoclonal antibody or nanobody, which results in better crystal packing (Rasmussen, Choi et al. 2007, Rasmussen, Choi et al. 2011, Rasmussen, DeVree et al. 2011). In addition, other factors—such as using nanomolar affinity ligands and proper detergents to improve the stabilization of GPCRs (Chae, Rasmussen et al. 2010, Hong, Baker et al. 2010), the LCP crystallization method (Landau and Rosenbusch 1996), and strong nano-sized X-ray beamlines—have allowed more structures to be determined.

X-ray free electron laser (XFEL) is an X-ray that combines the features of lasers at the free electron state. It is one of the most promising light sources for the next generation of scientific exploration and discovery in fourth generation synchrotron (Barty, Kupper et al. 2013). XFEL was optimized for LCP GPCR crystals, which are very small (Figure 3). In XFEL diffraction, nanocrystals are destructed by the strong XFEL beam, but at the same time, the diffraction pattern is recorded in the CSPAD detector (Figure 3). Serial femtosecond crystallography that takes advantage of x-ray free electron lasers (XFELs), has recently demonstrated promise for obtaining data at room temperature with high-resolution. XFEL technology also has another advantage over protein crystallographers as XFEL overcomes the limitation of crystal size due to its strong laser beam, and it also does not require mounted tools because the injector disperses even in viscous LCP (Liu, Wacker et al. 2013).

Recently, the serotonin receptor structure was ascertained at room temperature using XFEL technology (Figure 4). This structure shows no structural difference from the structure of serotonin receptor determined by synchrotron X-ray using a cryosystem, except for minor differences in the B-factor (Liu, Wacker et al. 2013, Wang, Jiang et al. 2013). Additionally, experimental comparisons between synchrotron and XFEL are interesting. Temperature, beam size, average crystal size, number of crystals, number of collected images, and completeness all vary (Table 1). These differences between synchrotron and XFEL imply that XFEL data quality is better than that of synchrotron data because the diffracted image data is about a terabyte, which increases the redundancy and can enhance the

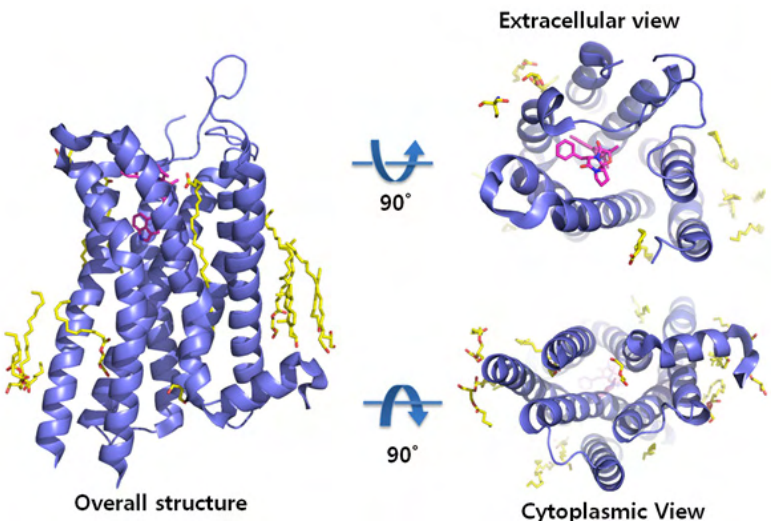

FIGURE 4 | The structure of the serotonin receptor that was solved by X-ray Free Electron Laser (XFEL). The serotonin receptor is depicted in cyan, lipid and detergent are in yellow, and the antagonist, ergotamine, is pink. This structure is first structure of a GPCR using XFEL, and no significant structural difference was seen when compared with other GPCR structures. The depicted lipid and detergent (yellow) are much more shown compared to previous structure of serotonin receptor from synchrotron X-ray.

TABLE 1 | The major differences in XFEL and synchrotron (SYN) data collection statistics. Although the sizes of the crystals used in XFEL are smaller, the data quality is better than for the synchrotron.

Data collection	Serotonin receptor XFEL	Serotonin receptor Synchrotron
Temperature, K	294	100
Beam size, μm	1.5	10
Crystal size, μm	5 x 5 x 5	80 x 20 x 10
No. collected image	4,217,508	91
Completeness, %	100	90.5
Multiplicity	1,150	3.2
$R_{split(XFEL)}$ or $R_{merge(SYN)}$	9.5	15.0

data quality. Although XFEL diffracts the nanocrystals during a very short time (femtosecond), the serotonin receptor structure from XFEL shows an electron density map that includes additional cholesterol, one palmitoyl chain, and 9 lipid molecules.

DRUG DISCOVERY TARGETING GPCRS

GPCRs are attractive targets for pharmacological modulation due to their essential roles in many biological processes. As mentioned earlier, about 40% of the currently marketed drugs target GPCRs (Congreve, Dias et al. 2014). The kind of selling drug is Zyprexa for schizophrenia (D2/D1/5-HT2 receptors), Risperdal for psychosis (D2/5-HT2A receptors), Imigran/Imitex for migraine (5-HT1 receptor) and Claritin for allergies (H1 receoptor), Serevent for asthma (β2 receptor) and Zantac/Pepcidine for ulcers (H2 Receptor) etc. (Evers, Hessler et al. 2005). Of these, Abilify is a best selling drug for depressive disorder (dopamine receptor) and sold about $6,293,801 per year (2013) in the world (www.drugs.com/stats/top100/2013/sales). For this reason, pharmaceutical companies aggressively invest in the research and development of GPCR-targeting drugs (Lappano and Maggiolini 2011). However, drug discovery targeting GPCRs remains challenging. In contrast to classes of soluble protein drug targets, such as kinases and proteases, the understanding of GPCRs has been severely hampered by the lack of understanding of GPCR structure and mechanism in addition to how agonists and antagonists interact with GPCRs. The new structural information strongly suggests that GPCRs have a pocket that is intrinsically a drug-binding site for small molecules or biologics (Heptares Therapeutics).

There are two major methods of GPCR-based drug development. Fragment-based drug discovery (FBDD) is a prevailing method used for finding lead compounds as a part of the drug discovery process. FBDD starts by identifying small chemical fragments, which may bind to the biological target weakly, and those fragments are modified or combined to produce a lead with a higher affinity (Scott, Coyne et al. 2012). FBDD identifies low-molecular-weight ligands that bind to biologically important macromolecules (Murray and Rees 2009).

Structure-based drug discovery (SBDD) is a method based on the resolved GPCR structure combined with protein-ligand docking, cell biology assays, and kinetics. SBDD uses therapeutic antibodies shown to be very precise monoclonal antibodies for receptors and ligands, such as receptor tyrosine kinases and growth factors. Anti-GPCR antibodies are believed to be difficult to obtain. This may be due to several factors, including immune tolerance for GPCR proteins, a variety of flexible states, and the relatively small amount of exposed hydrophilic surface of many GPCRs (Conformetrx). However, due to the development of new antibody production techniques and available GPCR proteins, the development of therapeutic monoclonal antibodies for GPCRs is rapidly growing, and now these antibodies are a significant part of GPCR-based drug discovery. The structures of the β2-AR, histamine, adenosine, CXCR4, and CRF1 receptors conjugated with ligands have facilitated virtual screening and hit generation for these targets (Congreve, Dias et al. 2014). Pharmacophores, defined by the complex structure with ligands, can provide a foundation to screen and dock the various chemical structures *in silico*, and screening hits cam be identified for binding affinity by using a competitive binding assay. Despite much research, GPCR-based drug discovery has not yet been utilized successfully in clinical trials. Still, efforts to create GPCR-based drugs are just beginning, and more studies and time are needed.

CONCLUSIONS

GPCRs have been recognized as important therapeutic targets. During the last 5 years, GPCR studies have actively progressed, and many advanced methods have been developed to determine structure, discover of therapeutic drugs, and to develop new techniques such as XFEL, LCP, and stabilizing mutagenesis. Among them, XFEL technology might be one of the most advanced and promising techniques. Because XFEL technology has room for more development, including in the areas of as the processing algorithm and program, injection tools, and database for mass storage, XFEL will dramatically increase the opportunity to determine various GPCR structures.

We know that GPCRs are key players in the regulation of various physiological responses, and that GPCRs are implicated in many diseases such as cancer. Despite their importance, crystallographers have been reluctant to study the GPCR structure because the process is very expensive. In addition, membrane proteins are notoriously difficult to study. However, following the development of XFEL, the threshold of effort

required to determine a GPCR structure is lower. We expect that more GPCR structures will be determined using XFEL technology, and that more studies will be undertaken to develop GPCR-based drugs.

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