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Review

Fluorescent approaches for understanding interactions of ligands with G protein coupled receptors☆

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ABSTRACT

G Protein Coupled Receptors (GPCRs) are responsible for a wide variety of signaling responses in diverse cell types. Despite major advances in the determination of structures of this class of receptors, the underlying mechanisms by which binding of different types of ligands specifically elicits particular signaling responses remain unclear. The use of fluorescence spectroscopy can provide important information about the process of ligand binding and ligand dependent conformational changes in receptors, especially kinetic aspects of these processes that can be difficult to extract from X-ray structures. We present an overview of the extensive array of fluorescent ligands that have been used in studies of GPCRs and describe spectroscopic approaches for assaying binding and probing the environment of receptor-bound ligands with particular attention to examples involving yeast pheromone receptors. In addition, we discuss the use of fluorescence spectroscopy for detecting and characterizing conformational changes in receptors induced by the binding of ligands. Such studies have provided strong evidence for diversity of receptor conformations elicited by different ligands, consistent with the idea that GPCRs are not simple on and off switches. This diversity of states constitutes an underlying mechanistic basis for biased agonism, the observation that different stimuli can produce different responses from a single receptor. It is likely that continued technical advances will allow fluorescence spectroscopy to play an important role in continued probing of structural transitions in GPCRs. This article is part of a Special Issue entitled: Structural and biophysical characterisation of membrane protein–ligand binding.

Contents

1. Introduction

Transmembrane receptors play critical roles in diverse cell signaling pathways that affect many aspects of cell behavior. Their functions in important physiological processes make them the targets of a large

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fraction of clinically useful drugs and candidate targets for the development of many new drugs [1]. Activation or modulation of downstream signaling pathways by receptors is generally initiated and controlled by interactions of the receptors with different classes of chemical ligands. These include agonists, which lead to activation of downstream pathways, antagonists, which do not activate the downstream pathways, but can inhibit activation by agonists, and inverse agonists, which act directly to inhibit receptor-mediated activation of pathways. Despite the importance of receptor–ligand interactions in controlling cell signaling pathways, the mechanisms by which such interactions elicit downstream responses remain poorly understood.

A major obstacle to understanding the molecular basis underlying receptor–ligand interactions has been the lack of structural information about receptors. Many receptors are transmembrane proteins for which structure determination by X-ray crystallography and NMR is extremely difficult. However, over the past few years, major advances have been made in determining structures of one particularly important class of receptors, the G Protein Coupled Receptors (GPCRs). These constitute a widely-distributed protein superfamily that is responsible for signaling responses to a wide variety of hormones, neurotransmitters, sensory stimuli, metabolites, and ions. GPCRs all consist of membrane proteins with seven transmembrane segments, an N-terminal extracellular portion that can vary considerably in size, and a C-terminal cytoplasmic tail, often involved in downregulation of signaling. Activation of GPCRs generally results in exchange of GTP for GDP bound to the α-subunit of a heterotrimeric G protein, followed by at least partial dissociation of the G protein α-subunit from the β- and γ-subunits. In some cases, activation of GPCRs may also result in signaling via mechanisms that do not involve G proteins, such as through interactions with arrestins [2,3].

To date, structures are available for rhodopsin [4,5] and the β2-adrenergic [6,7], A2a adenosine [8], dopamine D3 [9]; CXCR4 chemokine [10], histamine H1 [11], lyso-phospholipid S1P [12], M2 and M3 muscarinic acetylcholine [13,14], δ-, κ-, and μ-opioid [15–17], neurotensin [18], protease activated [19], serotonin [20], smoothened [21], glucagon [20], and corticotrophin releasing factor [22] receptors. With the exception of rhodopsin, all these structures have been obtained by fusing receptors to stable soluble proteins, or by introducing a variety of stabilizing mutations into the protein to render them stable enough to adopt a single state for crystallization. Such modifications can have significant effects on the functions of receptors [23,24]. However, availability of these structures provides critical information on GPCRs' overall topology, on the nature of their ligand binding sites, and, in some cases, on the nature of the conformational changes associated with receptor activation [25].

Despite the recent structural characterization of GPCRs, the specific ligand–receptor interactions that drive conformational changes of GPCRs that, in turn, result in activation or inhibition of receptor-mediated signaling pathways, are not yet defined. The diverse family of GPCRs apparently share common mechanisms for activating G proteins (for example, many different receptors can activate the same G proteins), but the molecular nature of the ligands that activate GPCRs is astonishingly diverse, ranging from large glycoproteins that interact with large extracellular domains of receptors to small molecules and ions, some of which appear to interact directly with transmembrane regions of the receptors. Classical models of receptor signaling postulated the existence of an active state of a receptor that is stabilized by binding of agonists and an inactive state stabilized by binding of inverse agonists. In this paradigm, ligands that act as antagonists bind with equal affinity to both active and inactive states, providing competition that inhibits activation by agonists, but resulting in no activation of receptors by antagonists added by themselves [26,27]. However, GPCRs appear to be more than simple two-state switches. A particularly intriguing aspect of GPCR signaling is the accumulating evidence for biased agonism, in which different ligands binding to similar sites on a particular receptor are capable of eliciting different downstream signaling responses [28].

Fluorescence-based techniques provide diverse ways of probing the chemical environments and intermolecular interactions that have been extensively applied to understanding receptor-mediated signaling. We focus in this review on applications in which these capabilities are used specifically to probe receptor–ligand interactions and associated conformational changes in GPCRs. Fluorescence has also been extensively used for other types of studies of GPCRs that will not be discussed here, including: 1) cell biological approaches in which fluorescence microscopy is used to characterize the subcellular locations of GPCRs under resting conditions and following stimulation; 2) examination of the dynamic nature of interactions between GPCRs and their cognate G proteins [29–32]; and 3) characterization of the oligomeric state of GPCRs, a complex and controversial topic that is beyond the scope of the present manuscript but has been reviewed in several contexts [29,33–39].

This review will also emphasize the usefulness of fluorescent ligands for studying GPCR signaling in the yeast pheromone response pathway. This signaling system has served as the basis for uncovering several aspects of GPCR signaling that have proved to be broadly relevant to such pathways in mammalian and other systems [40–42]. Haploid cells of the bakers' yeast *Saccharomyces cerevisiae* secrete the mating type-specific peptide pheromones a-factor and α-factor that bind to receptors on cells of the opposite mating type, reporting that a potential mating partner is nearby. Such signaling results in morphological changes, transcriptional reprogramming, and cell cycle arrest that prepare the haploid cell for mating to form a diploid zygote. The receptors for yeast mating pheromones are GPCRs that are, in some cases, functionally interchangeable with mammalian receptors, despite exhibiting very little sequence similarity to their mammalian counterparts [43–46]. In contrast, the sequences of trimeric G proteins in yeast are very similar to those of mammalian G proteins. The genetic approaches possible in yeast, along with the development of robust and diverse readouts for pheromone receptor activation, have resulted in a high level of characterization of this signaling system that has been complemented by the application of quantitative systems-based approaches for detailed analyses of pheromone signaling responses [47–49].

2. Fluorescent ligands

The usefulness of fluorescently labeled ligands for the study of GPCRs has been recognized for several decades [50–54]. Fluorescent GPCR ligands have been used for studies ranging from localization of receptors in tissues and cells (including an early demonstration of the internalization of ligand-bound receptors in cells [53]), to simple binding assays (in many cases, as replacements for radioligands), to sophisticated probing of the geometry and mechanisms of ligand–receptor interactions and receptor–receptor interactions. Several previous reviews have provided compendia of fluorescent ligands for GPCRs that have been reported in the literature [29,55–61]. Table 1 presents an updated list of published fluorescent ligands for GPCRs, including information from these previous reviews.

Since most GPCR ligands are not inherently fluorescent, the use of fluorescent ligands to study GPCRs requires modification of normal ligands to render them fluorescent. A significant problem in the field is the fact that such modifications can alter the ligands' properties, including, importantly, the nature of their interactions with receptors. Alteration of ligand properties is obviously a major issue in creating fluorescent derivatives of small molecule ligands, such as biogenic amines (see [59]), where the native ligands are smaller than any fluorescent moiety to which they can be conjugated. However, the introduction of a fluorophore can also lead to major alterations of the properties of larger ligands, such as peptides [57]. For example, upon testing of fifteen different analogs the yeast peptide mating pheromone, α-factor in which the small NBD (7-nitrobenz-2-oxa-1,3-diazol-4-yl) fluorophore was attached at 7 out of 13 possible amino acids in the peptide, each of the analogs exhibited at least moderately reduced binding affinity for receptor, and several of the analogs had binding affinities that

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