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Hot spots for GPCR signaling: lessons from single-molecule microscopy

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Abstract

G protein-coupled receptors (GPCRs) are among the best-studied membrane receptors, mainly due to their central role in human physiology, involvement in disease and relevance as drug targets. Although biochemical and pharmacological studies have characterized the main steps in GPCR signaling, how GPCRs produce highly specific responses in our cells remains insufficiently understood. New developments in single-molecule microscopy have made it possible to study the protein–protein interactions at the basis of GPCR signaling in previously inconceivable detail. Using this approach, it was recently possible to follow individual receptors and G proteins as they diffuse, interact and signal on the surface of living cells. This has revealed hot spots on the plasma membrane, where receptors and G proteins undergo transient interactions to produce rapid and local signals. Overall, these recent findings reveal a high degree of dynamicity and complexity in signaling by GPCRs, which provides a new basis to understand how these important receptors produce specific effects and might pave the way to innovative pharmacological approaches.

Highlights

- Single-molecule microscopy provides unique opportunities to study GPCR signaling
- New single-molecule data show that receptor–G protein interactions last 1-2 s
- Agonists increase the association rate between receptors and G proteins
- The receptor–G protein association rate correlates with agonist efficacy
- Receptors interact with G proteins at hot spots on the plasma membrane

Introduction

G protein-coupled receptors (GPCRs) constitute the largest superfamily of membrane receptors, encoded by about 800 separate genes in the human genome [1]. They mediate the effects of a large variety of extracellular cues, including several hormones and neurotransmitters, odorants and light. Moreover, they are the targets of at least 30% of all drugs currently on the market [2]. GPCRs have been traditionally investigated in membrane or crude cell preparations using classical pharmacological and biochemical assays. This approach has been very successful in characterizing the key biochemical steps of GPCR signaling, which are now known to involve a series of well-defined protein–protein interactions and enzymatic reactions. Our understanding of these basic mechanisms has received tremendous impetus from recent structural biology and biophysical studies on purified proteins. In spite of these major achievements, how GPCRs work in the complexity of a living cell or organism to produce specific biological effects remains insufficiently understood.

Classical models of GPCR signaling

Since the discovery that GPCRs mediate their effects via interacting with other proteins on the plasma membrane, several hypotheses have been made on the nature of these interactions. A first model, generally known as ‘collision coupling’, postulated that receptors and G proteins are freely diffusing on the plasma membrane and, once the receptors are activated by agonists, interact via pure random collisions [3,4]. Subsequently, discrepancies between predictions and experimental data led to the formulation of the ternary complex model [5-7], which hypothesizes that receptors are in equilibrium between an active conformation stabilized by agonists and an inactive one, stabilized by inverse agonists. According to this model, receptors in both conformations would be able to interact with G proteins, although with different probabilities. Importantly, the ternary complex model implies that a fraction of receptors and G proteins might be ‘pre-coupled’ (i.e. pre-bound) in the absence of agonist. Whereas the development of the ternary complex model marked a milestone in the GPCR field, it did not fully solve the question about the nature of receptor–G protein interactions, as it allows very different scenarios depending on their stability. At one extreme of the spectrum, receptors and G proteins would undergo very transient interactions, maximizing signal amplification. At the other extreme, receptors and G proteins would form virtually stable complexes in the absence of agonist, maximizing signaling speed and allowing for signals to stay local, but at the expense of signal amplification. Moreover, all these models did not take into account the complexity of the plasma membrane, where interactions involving integral membrane proteins, membrane lipids and the cortical

cytoskeleton have been shown to strongly influence the localization and diffusion of membrane proteins (see [8] for a recent review).

An unexpected structural dynamicity in GPCR signaling

The last ten years have seen an enormous progress in our understanding of the structural basis of GPCR signaling, thanks to the determination of high-resolution three-dimensional structures of GPCRs, G proteins and β-arrestins in different conformations [9] as well as of receptors in complex with either G proteins [10-19] or arrestins [20-22]. Together with results obtained by electron microscopy and biophysical methods on purified proteins, these studies have revealed that GPCRs possess a high structural plasticity and are in dynamic equilibrium among multiple conformations (for a comprehensive review see [23]). This high plasticity might be crucial for GPCRs to be able to interact with more than one signaling partner, and, thus, expand their signaling repertoire. Moreover, these studies have revealed that the formation of receptor–G protein and receptor–arrestin complexes involve large conformational rearrangements that likely proceed through a series of intermediate steps. Since different agonists appear to stabilize receptors in different conformations, this could pave the way to a new generation of 'biased' drugs [24].

The first conformational rearrangements for nonvisual GPCRs likely occur within a few microseconds after binding of an agonist, however these changes are subtle and GPCRs seem to be able to rapidly switch among multiple pre-active conformations [25,26]. Remarkably, GPCRs seem to adopt a fully active conformation only upon binding of a G protein [10,27,28]. The changes leading to a fully active receptor conformation include an outward movement of the 6^{th} transmembrane helix (TM6) of up to 14 Å, which opens up a cavity on the intracellular side of the seven transmembrane domain where the G protein can interact [10] (Figure 1). This is accompanied by a large conformational change in the G protein, with a rotation of approximately 130° of the α-helical domain (GαAH) relative to the Ras-like domain (GαRas), which appears required for GDP release [10,27-29]. This idea is further supported by a recent study with purified receptors and G proteins, where the conformational changes in individual β_2-adrenergic receptors were followed by single-molecule fluorescence resonance energy transfer (FRET) [30].

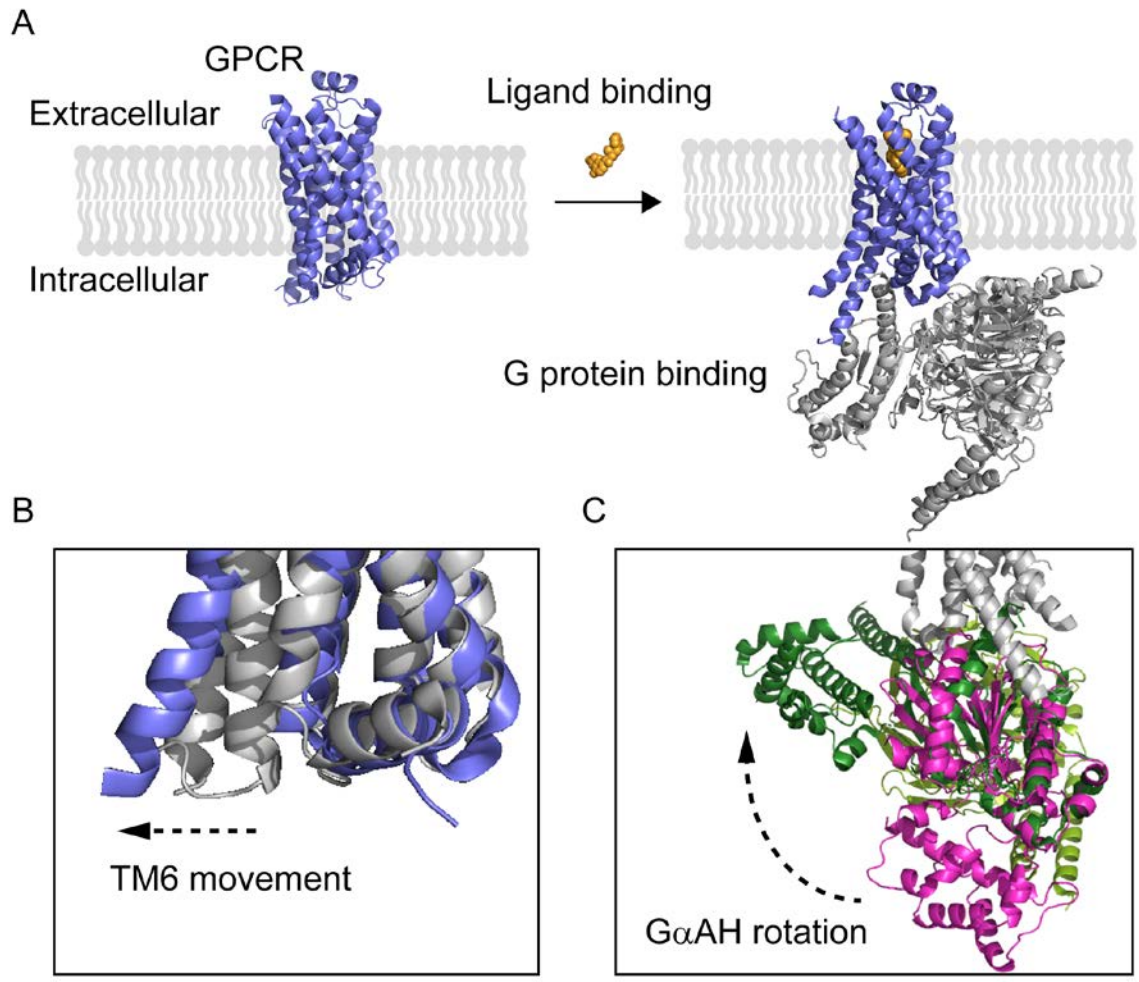

Figure 1. Overview of the conformational changes occurring during the formation of an active receptor–G protein complex. A, transition from inactive to active receptor. Binding of both agonist and G protein is required for the receptor to reach a fully active conformation. B, Zoom-in view showing the outward movement of TM6 as the receptor switches from inactive (grey) to active (blue) state. C, Zoom-in view showing the conformational change in the G protein, including the large rotation of the α-helical domain (GαAH). Magenta, inactive G protein; green, active G protein. Data are based on the high-resolution crystal structures of the inactive β_2-adrenergic receptor bound to an inverse agonist (PDB 2RH1), $G\alpha_s$ in complex with GTPγS (PDB 1AZT) and active β_2-adrenergic receptor–G_s protein complex (PDB 3SN6).

Single-molecule methods to investigate receptor dynamics at the plasma membrane

Single-molecule microscopy methods have been instrumental to investigate the complex organization of the plasma membrane and the dynamics of membrane proteins and lipids [8]. In contrast to classical biochemical and imaging methods, which give only average information about the molecules under investigation, single-molecule methods allow directly

monitoring the behavior of individual molecules with very high spatiotemporal resolution – typically about 10 ms and 10 μm, which is approximately 20-times better than with standard fluorescence microscopy. This has major advantages over classical methods, as it allows directly investigating the kinetics of biological events, which usually occur in mixed and non-synchronized populations of molecules, as well as identifying rare or short-lived states that are hidden in ensemble measurements.

Single particle tracking studies pioneered by Akihiro Kusumi's lab and aimed at investigating the diffusion of fluorescently labelled membrane proteins and lipids in living cells have given important insights into the complex organization of the plasma membrane (see [31,32] for a comprehensive review). The results of these studies led to the conclusion that both proteins and lipids are not freely diffusing on the plasma membrane but rather 'jump' across small nanodomains of 40–300 nm, where they are loosely trapped by barriers mainly provided by the cytoskeleton and the membrane proteins associated with it. This led to the formulation of the so-called 'fence-and-picket' model of the plasma membrane, according to which the cytoskeleton underneath the plasma membrane ('fences') and the associated integral membrane proteins ('pickets') provide physical barriers to the diffusion of membrane molecules, leading to their compartmentalization in small nanodomains [31,32]. Moreover, there is evidence that the cytoskeleton does not only provide barriers to diffusion but also anchors for receptors and other membrane proteins. Although this has been better investigated for ionotropic receptors [33], there is growing evidence that this might also be the case for GPCRs. For instance, single-molecule work by our group has shown that the $GABA_B$ receptor [34] and, to a lesser degree, the somatostatin receptor type 2 (SSTR2) [35] interact with the actin cytoskeleton, leading to their preferential arrangement along actin fibers. In the case of SSTR2, this involves its interaction with the acting-binding scaffold protein filamin A [35].

Moreover, single-molecule microscopy has given an important contribution to clarifying the highly debated issue of GPCR dimerization. Two initial studies investigated M_1 muscarinic [36] and *N*-formyl peptide [37] receptors using a fluorescently labeled antagonist or agonist, respectively. Subsequently, a study by our group compared the supramolecular organization of three prototypical GPCRs, i.e. β_1-adrenergic, β_2-adrenergic and $GABA_B$ receptors, which were labeled with small organic fluorophores via introduction of SNAP tags at their N-termini [34]. More recently, Tabor *et al.* has used single-molecule imaging to study the dimerization of the dopamine D_2 receptor [38]. Overall, these studies suggest that GPCRs exist on the plasma membrane in dynamic equilibrium among monomers, dimers and, at least in the case of $GABA_B$ receptors, higher order oligomers. Whereas these studies were able to

capture the formation of transient receptor dimers and higher order oligomers, they do not support the existence of stable complexes as suggested by some previous investigations.

Hot spots for GPCR signaling

Single-molecule microscopy has not only helped to clarify the organization of the plasma membrane and the nature of receptor dimerization but is also emerging as a general, powerful method to precisely investigate protein–protein interactions, such as those involved in receptor–G protein interactions. By taking advantage of direct protein labelling via SNAP [39] and CLIP [40] tags and fast multicolor total internal reflection fluorescence (TIRF) microscopy, our group recently succeeded in visualizing individual GPCRs and G proteins as they diffuse, interact and signal on the surface of living cells [41]. Two prototypical GPCRs, α_{2A}- and β_2-adrenergic receptors, and the main G proteins coupled to them (G_i and G_s, respectively) were investigated. Experiments were conducted both in a simple cell system and in human primary endothelial cells, were α_{2A}- and β_2-adrenergic receptors are involved in the control vascular tone. This study revealed a high heterogeneity and complexity in the diffusion of receptors and G proteins, with both receptors and G proteins rapidly switching among phases of rapid and slow diffusion. Remarkably, both receptors and G proteins were found to undergo transient trapping at shared sites on the plasma membrane and largely avoid other areas, leading to their preferential accumulation in small nanodomains. By simultaneously labelling actin filaments and microtubules underneath the plasma membrane, it was possible to demonstrate that such trapping is at least partially caused by barriers provided by the cortical cytoskeleton, in agreement with the 'fence-and-picket' model. Importantly, this leads to the formation of signaling 'hot spots' where receptors and G proteins accumulate and preferentially interact with each other (Figure 2). Based on measurements of local G protein activation with a nanobody-based biosensor [42] and the results of computer simulations, we hypothesized that these hot spots might have a relevant impact on the efficiency and specificity of GPCR signaling. On the one hand, by increasing the local concentration of receptors and G proteins and keeping them near to each other after a previous interaction, they likely increase both the efficiency and speed of GPCR signaling. On the other hand, they allow GPCRs to produce local signals. As the location of these signals might differ depending on the receptors and G proteins involved, this might help to explain how GPCR can produce a variety of specific and distinct biological responses despite converging on just few downstream signaling pathways.

New single-molecule approaches are likely going to play a crucial role in further elucidating the organization of GPCR signaling nanodomains on the plasma membrane. For instance, Mo *et al.* recently developed a new type of biosensor that enables fast and reversible

superresolution imaging of dynamic enzymatic activities [43]. Using this approach, they were able to directly visualize protein kinase A (PKA) signaling microdomains on the plasma membrane.

Another aspect that will require further investigation is the impact of the local membrane composition and geometry on GPCR signaling. Whereas the existence of stable lipid domains remains a matter of debate, interactions between receptors as well as other signaling proteins and membrane lipids likely affects their activity, as has been recently shown for β_2-adrenergic receptors *in vitro* [44]. Moreover, the local curvature of the plasma membrane might also affect the localization of GPCRs as well as their trafficking and/or signaling [45].

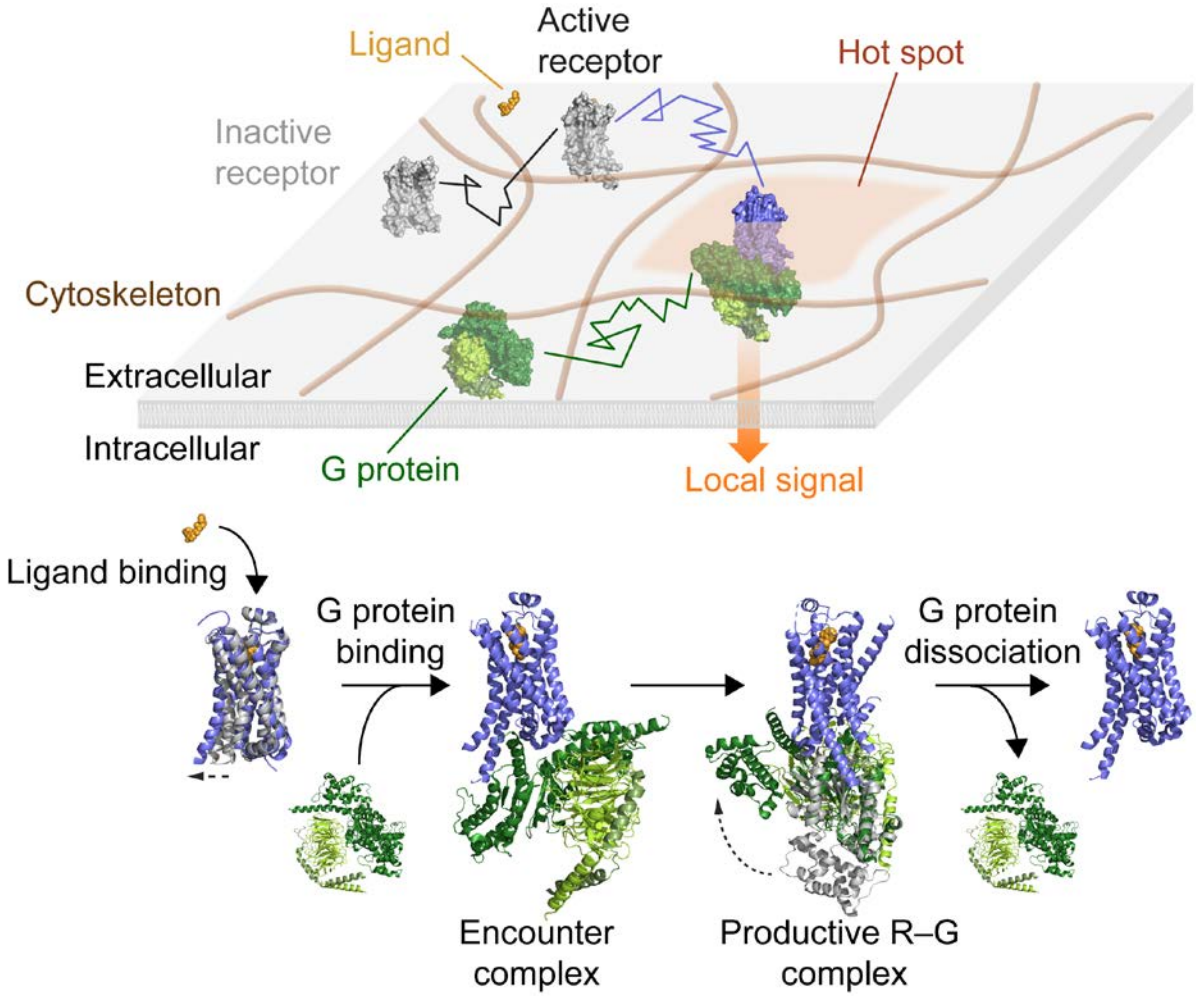

Figure 2. Dynamics of GPCR signaling at the plasma membrane as revealed by single-molecule microscopy. The plasma membrane is compartmentalized in small nanodomains with important contribution of the cytoskeleton, which provides barriers to the diffusion of receptors and G proteins. This leads to the formation of 'hot spots', where receptors and G proteins preferentially interact and produce local signals. Within such nanodomains,

receptors and G proteins undergo transient interactions lasting approximately 1–2 seconds. Agonists mainly act by increasing the probability that an encounter complex between a receptor and a G protein leads to a productive interaction, which is consistent with the large conformational changes that occur during the formation of receptor–G protein complexes.

K**inetics of receptor–G protein interactions in living cells**

Given the fundamental role of protein–protein interactions in virtually all biological processes, much attention has been given to the study of their mechanisms and kinetics – see [46] for a comprehensive review. Although this might represent a simplification, it is often convenient to imagine protein–protein interactions to occur via formation of a very short-lived intermediate state (typically lasting for a few nanoseconds), known as encounter complex. In the encounter complex, the interacting proteins have a near-correct orientation and distance, but have yet to undergo the conformational changes required for the formation of a fully assembled complex, often termed productive complex [46]. In the case of proteins that undergo only minor conformational changes during their interaction, the conformational changes are faster than the dissociation of the encounter complex. Thus, there is a high probability that the encounter complex is converted into a productive one. Under these conditions, the overall association rate (k_{on}) is high and is mainly controlled by diffusion of the interacting proteins [46]. In contrast, if the formation of the productive complex requires conformational changes that are relatively large and slower than the dissociation of the encounter complex, the conformational rearrangement becomes rate-limiting and the overall association rate is low [46].

The recent single-molecule data obtained investigating receptor–G protein interaction on the surface of living cells do not only provide important information about these critical interactions in GPCR signaling but also gives us a glimpse into how a typical protein–protein interaction involved in cell signaling takes place and is controlled over space and time. By applying a novel analysis based on deconvolution of the apparent colocalization times, it was possible to precisely estimate both the association (k_{on}) and dissociation (k_{off}) rates of receptor–G protein interactions, which were previously unknown. A first interesting finding is that receptors and G proteins undergo transient interactions that last approximately 1–2 seconds and occur, albeit at a low frequency, also in the absence of agonist. This helps clarifying a fundamental and long-debated aspect of GPCR signaling, i.e. whether receptors and G proteins form stable complexes in the absence of agonist (i.e. are pre-coupled) or rather interact via random collisions leading to the formation of short-lived complexes. The recent results obtained by single-molecule microscopy confirm the occurrence of receptor–G protein interactions in the absence of agonist, but at the same time do not support the

formation of stable complexes. The occurrence of transient interactions in the absence of agonist and the trapping effect provided by the cytoskeleton likely cooperate to keep the receptors and G proteins near to each other so that they can rapidly and efficiently respond to a stimulus. At the same time, this allows the signal to be amplified, enabling one receptor to activate multiple G proteins, which would not be possible in the case of stable receptor–G protein complexes. Moreover, since the estimated average duration of receptor–G protein interactions is longer than the time required to activate effectors such as GIRK channels – which can happen as fast as approximately 40 ms [47] – it is tempting to speculate that G proteins might be able to stimulate effectors while still bound, perhaps in a lose form, to a receptor.

Another interesting observation regards to effect of agonists on the formation and stability of receptor–G protein complexes. Remarkably, stimulation with agonists was found to have only modest effects on the dissociation rate of receptor–G protein complexes, while increasing up to 10 times their association rate [41]. Moreover, the measured rates of G protein binding to receptors were lower than would be expected for diffusion-governed protein–protein interactions. These findings are in good agreement with independent measurements obtained with purified receptors and G proteins [30]. Overall, these data indirectly suggest that receptor–G protein interactions are limited by conformational changes rather than diffusion, which is in very good agreement with the growing body of evidence from structural and biophysical studies with purified proteins indicating that the formation of receptor–G protein complexes proceed through a series of complex conformational rearrangements [23].

Finally, these recent single-molecule studies have revealed that the rate of receptor–G protein association correlates positively with agonist efficacy [30, 41]. This indicates that efficacy operates at least partially at the level of the probability that collisions between receptors and G proteins lead to a productive complex. Moreover, since the association and dissociation rates seem to be independent from each other, this suggest the possibility of designing kinetically ‘biased’ agonists endowed with unique combinations of k_{on} and k_{off} for receptor–G protein interactions and, thus, potentially, distinct pharmacological profiles.

Concluding remarks

The most recent studies investigating the spatiotemporal dynamics GPCRs *in vitro* and in living cells have revealed an exceptionally high degree of dynamicity and complexity across a wide range of spatial and temporal scales. Thoroughly investigating this complexity appears crucial to fully understand how GPCRs produce specific effects and, hopefully, learn to modulate their function for therapeutic purposes in ways that are currently not possible

with the available drugs. Whereas we are beginning to understand how the complex interactions occurring within the plasma membrane critically contribute to determining the location, duration and quality of GPCR signaling, several fundamental questions remain to be answered. New developments in the rapidly expanding field of single-molecule microscopy are likely to play a relevant part in the current and future endeavors aimed at answering these questions and, ultimately, providing a full dynamic description of how GPCRs function and signal in the complexity of a living cell and organism.

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Conflict of Interest

The authors declare no conflict of interest.

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