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

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20 **Abstract**

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

34 **Highlights**

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

40 **Introduction**

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

54

55 **Classical models of GPCR signaling**

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

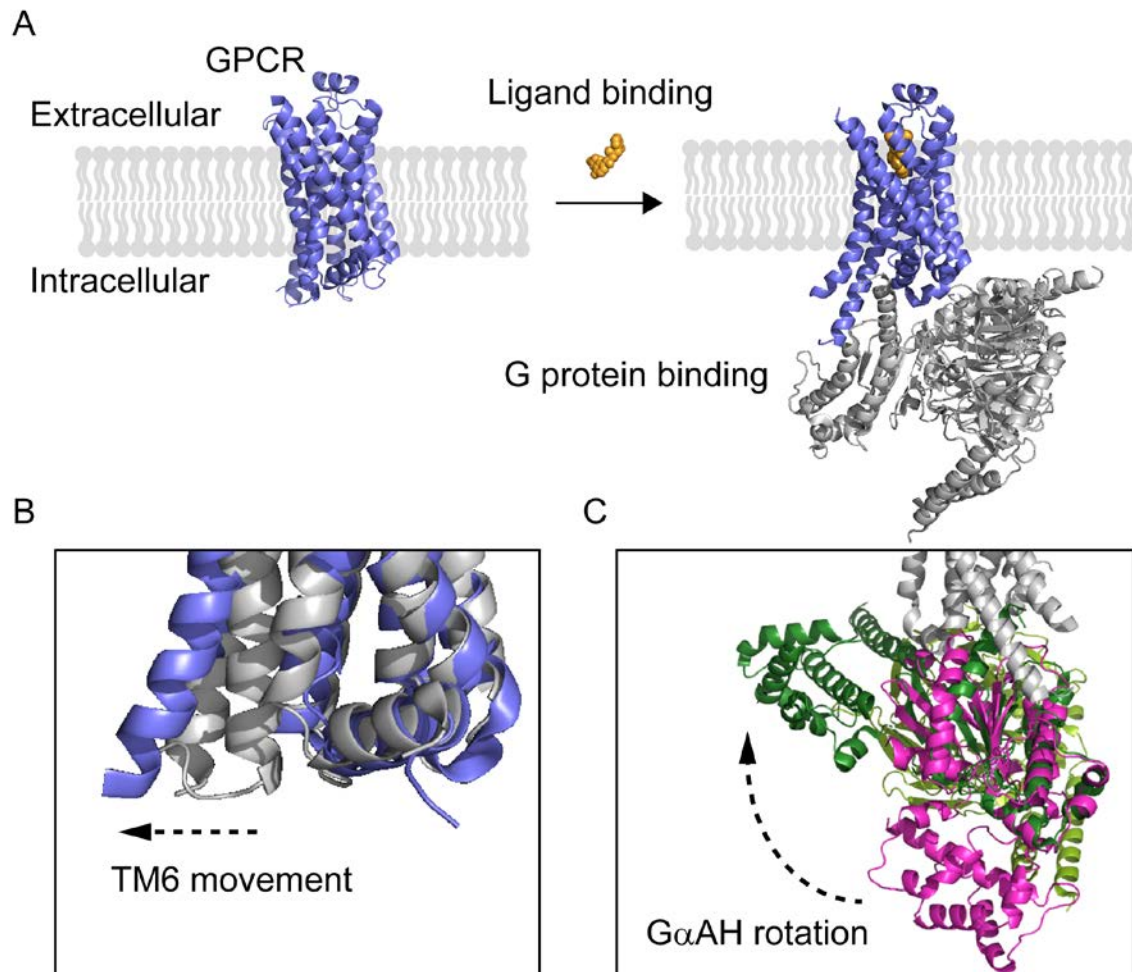
76 cytoskeleton have been shown to strongly influence the localization and diffusion of
77 membrane proteins (see [8] for a recent review).

78

79 **An unexpected structural dynamicity in GPCR signaling**

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

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



106

107 **Figure 1. Overview of the conformational changes occurring during the formation of**

108 **an active receptor–G protein complex.** A, transition from inactive to active receptor.

109 Binding of both agonist and G protein is required for the receptor to reach a fully active

110 conformation. B, Zoom-in view showing the outward movement of TM6 as the receptor

111 switches from inactive (grey) to active (blue) state. C, Zoom-in view showing the

112 conformational change in the G protein, including the large rotation of the α -helical domain

113 ($G\alpha$ AH). Magenta, inactive G protein; green, active G protein. Data are based on the high-

114 resolution crystal structures of the inactive β_2 -adrenergic receptor bound to an inverse

115 agonist (PDB 2RH1), $G\alpha_s$ in complex with $GTP\gamma S$ (PDB 1AZT) and active β_2 -adrenergic

116 receptor– G_s protein complex (PDB 3SN6).

117

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

119 Single-molecule microscopy methods have been instrumental to investigate the complex

120 organization of the plasma membrane and the dynamics of membrane proteins and lipids

121 [8]. In contrast to classical biochemical and imaging methods, which give only average

122 information about the molecules under investigation, single-molecule methods allow directly

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

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

148 Moreover, single-molecule microscopy has given an important contribution to clarifying the
149 highly debated issue of GPCR dimerization. Two initial studies investigated M₁ muscarinic
150 [36] and *N*-formyl peptide [37] receptors using a fluorescently labeled antagonist or agonist,
151 respectively. Subsequently, a study by our group compared the supramolecular organization
152 of three prototypical GPCRs, i.e. β_1 -adrenergic, β_2 -adrenergic and GABA_B receptors, which
153 were labeled with small organic fluorophores via introduction of SNAP tags at their N-termini
154 [34]. More recently, Tabor *et al.* has used single-molecule imaging to study the dimerization
155 of the dopamine D₂ receptor [38]. Overall, these studies suggest that GPCRs exist on the
156 plasma membrane in dynamic equilibrium among monomers, dimers and, at least in the
157 case of GABA_B receptors, higher order oligomers. Whereas these studies were able to

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

160

161 **Hot spots for GPCR signaling**

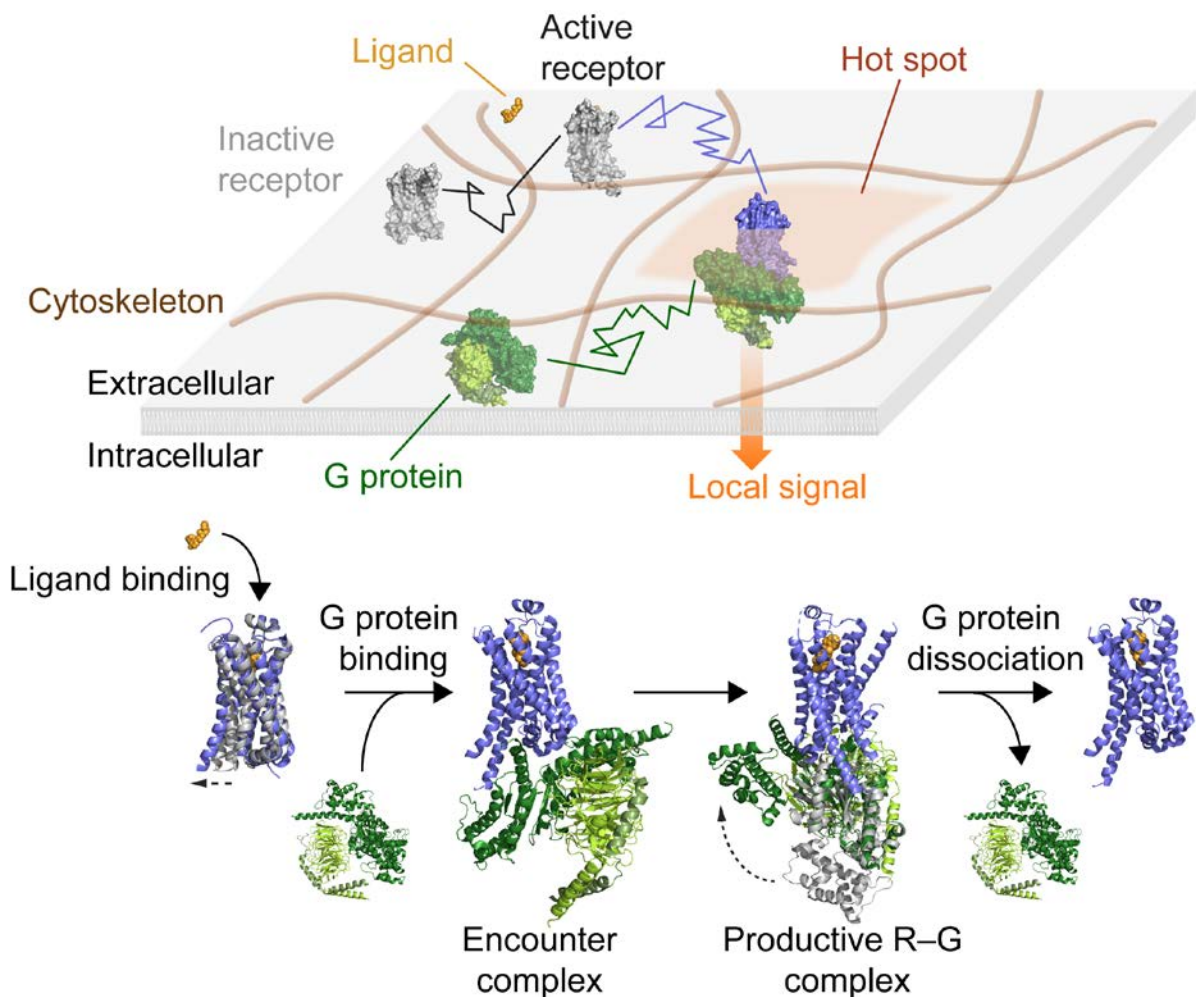
162 Single-molecule microscopy has not only helped to clarify the organization of the plasma
163 membrane and the nature of receptor dimerization but is also emerging as a general,
164 powerful method to precisely investigate protein–protein interactions, such as those involved
165 in receptor–G protein interactions. By taking advantage of direct protein labelling via SNAP
166 [39] and CLIP [40] tags and fast multicolor total internal reflection fluorescence (TIRF)
167 microscopy, our group recently succeeded in visualizing individual GPCRs and G proteins as
168 they diffuse, interact and signal on the surface of living cells [41]. Two prototypical GPCRs,
169 α_{2A} - and β_2 -adrenergic receptors, and the main G proteins coupled to them (G_i and G_s ,
170 respectively) were investigated. Experiments were conducted both in a simple cell system
171 and in human primary endothelial cells, where α_{2A} - and β_2 -adrenergic receptors are involved
172 in the control vascular tone. This study revealed a high heterogeneity and complexity in the
173 diffusion of receptors and G proteins, with both receptors and G proteins rapidly switching
174 among phases of rapid and slow diffusion. Remarkably, both receptors and G proteins were
175 found to undergo transient trapping at shared sites on the plasma membrane and largely
176 avoid other areas, leading to their preferential accumulation in small nanodomains. By
177 simultaneously labelling actin filaments and microtubules underneath the plasma membrane,
178 it was possible to demonstrate that such trapping is at least partially caused by barriers
179 provided by the cortical cytoskeleton, in agreement with the ‘fence-and-picket’ model.
180 Importantly, this leads to the formation of signaling ‘hot spots’ where receptors and G
181 proteins accumulate and preferentially interact with each other (Figure 2). Based on
182 measurements of local G protein activation with a nanobody-based biosensor [42] and the
183 results of computer simulations, we hypothesized that these hot spots might have a relevant
184 impact on the efficiency and specificity of GPCR signaling. On the one hand, by increasing
185 the local concentration of receptors and G proteins and keeping them near to each other
186 after a previous interaction, they likely increase both the efficiency and speed of GPCR
187 signaling. On the other hand, they allow GPCRs to produce local signals. As the location of
188 these signals might differ depending on the receptors and G proteins involved, this might
189 help to explain how GPCR can produce a variety of specific and distinct biological responses
190 despite converging on just few downstream signaling pathways.

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

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

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

204



205

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

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

215

216 **Kinetics of receptor–G protein interactions in living cells**

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

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

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

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

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

276

277 **Concluding remarks**

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

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

290

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294

295 **Conflict of Interest**

296 The authors declare no conflict of interest.

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