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Signalling by G protein-coupled receptors: why space and time matter

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Abstract

G protein-coupled receptors (GPCRs) are the largest family of membrane receptors and major drug targets. They play a fundamental role in the endocrine system, where they mediate the effects of several hormones and neurotransmitters. As a result, alterations of GPCR signalling are a major cause of endocrine disorders such as congenital hypothyroidism or Cushing's syndrome. My group develops innovative optical methods such as fluorescence resonance energy transfer (FRET) and single-molecule microscopy, which allow us to investigate GPCR signalling in living cells with unprecedented spatiotemporal resolution. Using this innovative approach, we have contributed to elucidate some long-debated questions about the mechanisms of GPCR signalling and their involvement in human disease. Among other findings, these studies have led to the unexpected discovery that GPCRs are not only signalling at the cell surface, as previously assumed, but also at various intracellular sites. This has important implications to understand how hormones and neurotransmitters produce specific responses in our cells and might pave the way to innovative treatments for common diseases like diabetes or heart failure.

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Introduction

G protein-coupled receptors (GPCRs) are largest and most assorted family of cell receptors in eukaryotes. Of the approximately 800 GPCRs in the human genome, around

460 sense odorants and are engaged in olfaction. The remainder mediates the effects of a variety of internal and external cues, including light, ions, small metabolites,

Invited Author's profile

Davide Calebiro is Professor of Molecular Endocrinology, Wellcome Trust Senior Research Fellow at the Institute of Metabolism and Systems Research (IMSR) and Co-Director of the Centre of Membrane Proteins and Receptors (COMPARE) of the Universities of Birmingham and Nottingham, UK. His group investigates the basic mechanisms of G protein-coupled receptor (GPCR) signalling in physiology and disease, which they elucidate using innovative microscopy methods such as FRET and single-molecule microscopy. His major contributions include the discoveries that GPCRs signal in the endosomal compartment and interact among themselves and with other membrane proteins to form dynamic signalling hot spots at the plasma membrane.

hormones and neurotransmitter (1, 2). GPCRs play a particularly important role in the endocrine system, where they mediate the effects of several hormones and hypothalamic releasing factors. These include all major hypothalamic releasing factors – thyrotropin-releasing hormone (TRH), gonadotropin-releasing hormone (GnRH), corticotropin-releasing hormone (CRH), growth hormone-releasing hormone (GHRH), somatostatin and dopamine – as well as most anterior – thyroid-stimulating hormone (TSH), luteinising hormone (LH), follicle-stimulating hormone (FSH), adrenocorticotrophic hormone (ACTH), melanocyte-stimulating hormone (MSH) – and posterior, that is, vasopressin and oxytocin, pituitary hormones (3). Because of this, alterations in GPCR signalling, for example, due to germ-line or somatic gene mutations, are responsible for a wide range of endocrine disorders, including, among others, diseases of the pituitary, thyroid, ovary, parathyroid and adrenal glands as well as central obesity (4). Given their accessibility and involvement in a wide range of pathophysiological processes, GPCRs play a major role in drug discovery, being the target of at least one-third of all drugs currently on the market (5).

Basic mechanisms of GPCR signalling

All GPCRs share a common structural signature, characterized by the presence of a seven-transmembrane domain (TMD), and signalling themes. The core mechanisms of GPCR signalling have been extensively investigated using classical biochemical and pharmacological approaches (1). Upon binding of an agonist, GPCRs undergo a series of conformational changes that lead to the exposure of a binding cavity on their cytoplasmic side, allowing them to interact with and activate heterotrimeric G proteins, composed of an α , β and γ subunit. The activated heterotrimeric G proteins then relay the signal to effectors located on the plasma membrane, which include both enzymes like adenylyl cyclase, responsible for the production of the second messenger cyclic AMP (cAMP), and ion channels. These events ultimately lead to a biological response in the stimulated cell. Like for other receptor types, prolonged agonist stimulation leads to rapid signal desensitisation and receptor internalisation, primarily via clathrin-mediated endocytosis (1, 6, 7, 8, 9). Arrestins, which decouple the receptors from their interacting G proteins and promote the recruitment of GPCRs in clathrin-coated pits (10, 11), play a key role in these processes.

Old and new paradigms in GPCR signalling

When I started working in the GPCR field as a young endocrinologist, two tenets of GPCR signalling were commonly found in textbooks. First, GPCRs were generally depicted as rather simple on-off switches that, once activated, produce a generalized response in the stimulated cell. Second, they were strongly believed to signal exclusively from the cell surface and stop doing so after agonist-induced internalisation.

At a deeper look, though, the picture was more complex. A long-debated question in the field was how to explain the high specificity observed in GPCR signalling, despite the fact that all GPCRs converge onto a few common signalling pathways. One hypothesis was that of signal compartmentalisation. For instance, pioneering work done in the late 1970s had shown that β -adrenergic and prostaglandin E1 (PGE1) receptors produce dissimilar biological responses in cardiomyocytes while inducing similar cAMP increases (12, 13). To explain these findings, it was proposed that different receptors might induce signals in separate subcellular signalling compartments, thus leading to a differential activation of downstream effectors like protein kinase A (PKA) and, ultimately, distinct biological responses (14). However, directly proving this hypothesis with classical biochemical and pharmacological methods was challenging. A turning point was represented by the introduction of novel biophysical methods such as fluorescence resonance energy transfer (FRET), which allowed receptor signalling to be monitored directly in living cells (15, 16, 17, 18, 19). Importantly, studies done using this approach revealed that, although cAMP is a highly diffusible small molecule, cAMP/PKA signalling in living cells can be highly compartmentalized (20, 21, 22, 23, 24, 25, 26). Yet, how receptors would trigger those compartmentalized signals remained enigmatic.

In the meantime, there had been growing reports that GPCRs, which were initially believed to be monomeric receptors, were able to assemble into both homo- and heterodimers as well as larger oligomeric complexes (27). However, the stability and functional consequences of these receptor-receptor interactions were highly debated.

Furthermore, growing evidence indicated that GPCRs are often promiscuous in their G protein coupling and, thus, potentially activate multiple signalling cascades. It had also become apparent that agonists for the same receptor can preferentially activate one or more of these signalling pathways, a phenomenon known as biased signalling (28). Although it was suggested that biased

signalling might be linked to the ability of agonists to stabilize receptors in different conformations coupled to distinct downstream signalling pathways, the exact mechanisms were elusive.

Alterations of GPCR signalling in endocrine disease

Given the involvement of GPCRs in virtually all physiological processes, alterations of GPCR signalling are commonly encountered in human disease. This is particularly true when considering the endocrine system, where mutations in the genes encoding GPCRs and key elements of their downstream signalling pathways are responsible for both inherited and acquired disorders (4).

A first example is represented by mosaic, activating mutations in the gene coding for the G_{α_s} protein (*GNAS1*) (29). These mutations were originally identified in patients affected by the McCune-Albright syndrome, characterized by bone fibrous dysplasia, *café au-lait* skin spots and hypersecretion from different endocrine glands. A minor fraction of McCune–Albright patients develop Cushing's syndrome due to adrenal hyperplasia (30).

A second genetic defect involves the gene coding for the $R_{I\alpha}$ subunit of PKA (*PRKARIA*). *PRKARIA* mutations are responsible for Carney complex, another multiple endocrine neoplasia syndrome characterized by the presence of primary pigmented nodular adrenocortical disease (PPNAD), cutaneous and neuronal tumours, cardiac myxomas, as well as characteristic pigmented lesions of the skin and mucosae (31). These mutations cause a reduced expression of the $R_{I\alpha}$ subunit or impair its association with the C subunit, leading to constitutive PKA activation (32).

Moreover, somatic mutations affecting GPCR signalling have been found in both benign and malignant endocrine tumours (4). For instance, activating mutations in either the G_s protein or the TSH receptor are found in approximately 60% of autonomous thyroid adenomas (33, 34, 35).

My first contribution to the study of genetic alteration in GPCR signalling in endocrine disease was during my Specialization in Endocrine and Metabolic Diseases at the University of Milan with Luca Persani, Paolo Beck-Peccoz and Anna Spada. The Milan group had previously identified a new form of dominantly inherited TSH resistance caused by heterozygous loss-of-function TSH receptor mutations. Using FRET, we could show that the TSH receptor mutations present in these patients cause

the retention of the WT TSH receptor in the endoplasmic reticulum, thus explaining the resistance to TSH observed in these patients (36).

More recently, my group played an important role in the discovery that mutations in the catalytic α subunit of protein kinase A (PKA) cause cortisol-producing adrenocortical adenomas, which are responsible for Cushing's syndrome (37). Cushing's syndrome is an endocrine disorder caused by cortisol excess (38). The clinical picture of Cushing's syndrome includes centripetal obesity, proximal muscle weakness, moon face, *striae rubrae*, and hirsutism (39, 40). In addition, hypertension, osteoporosis and alterations of glucose and lipid metabolism are common (41, 42, 43). If untreated, Cushing's syndrome is associated with an increased incidence of cardiovascular events and mortality (40, 44). By performing a whole-exome sequencing study in sporadic cortisol-secreting adrenocortical adenomas (37), we found two mutations in the gene coding for the $C\alpha$ subunit of PKA (*PRKACA*) in about 30% of these tumours (37, 45). The by far more frequent of the two mutations (p.Leu206Arg) results in the substitution of a leucine residue at position 206 with arginine; the second mutation (Leu199_Cys200insTrp) causes insertion of a tryptophan residue between amino acids 199 and 200 (37). To investigate the functional consequences of these mutations, we performed FRET experiments in living cells expressing a FRET reporter of PKA activity. These experiments revealed that both mutations cause constitutive PKA activation, that is, PKA activation in the absence of cAMP (37). These findings were confirmed by three independent studies by other groups (46, 47, 48). However, the molecular mechanisms responsible for constitutive PKA activation in the presence of these mutations were unclear. To address this issue, we performed a detailed functional characterisation of both *PRKACA* mutations (p.Leu206Arg and Leu199_Cys200insTrp) identified in our initial study (49). *In vitro* experiments and real-time FRET measurements revealed that both mutations largely abolished the interaction with the R subunits. This was associated with high PKA activity irrespective of the cAMP concentration. Moreover, we could demonstrate that both mutations interfere with the formation of a stable PKA holoenzyme and caused the loss of regulation by cAMP also in intact cells (49). In addition, we could subsequently show that *PRKACA* mutations alter PKA substrate specificity leading to hyperphosphorylation of proteins like histone 1.4, which is implicated in the control of cell proliferation (50). Furthermore, it has been shown by NMR spectroscopy that the Leu206Arg

substitution changes the intramolecular allosteric network in PKA interfering with the normal cooperativity between cAMP and substrate binding (51). These findings provide a mechanistic explanation for the aberrant activation of PKA caused by *PRKACA* mutations, and, thus, for the development of cortisol-secreting adrenocortical adenomas in the presence of these mutations.

The discovery of GPCR signalling at intracellular sites

Until recently, GPCRs were considered to be strictly cell surface receptors, incapable of signalling from other cellular compartments. This picture began to change with the description of non-classical β -arrestin mediated signals that continued after receptor internalisation (52). However, classical, G protein-dependent signalling was believed to occur only at the plasma membrane.

Our group played an important role in challenging this classical model of GPCR signalling by showing that GPCRs are able to signal via G proteins at various intracellular sites (Fig. 1) (53, 54, 55, 56, 57, 58). In a first study investigating TSH receptor signalling in intact mouse thyroid follicles that express a FRET reporter for cAMP, we could show that upon prolonged TSH stimulation, TSH receptors internalize and mediate persistent cAMP signalling from an intracellular compartment (53). Similar results were independently obtained by the group of Jean-Pierre Vilardaga studying the PTH receptor (59). Persistent TSH receptor signalling occurs in primary thyroid cells but not in simple cell lines (55). Subsequently, classical signalling

at intracellular sites has been documented for a growing number of GPCRs by several groups (60, 61, 62, 63, 64, 65, 66), making it a hot topic in the field. Intriguingly, different receptors appear to signal in different subcellular compartments. A follow-up study by our group has shown that TSH receptors traffic retrogradely to the trans-Golgi network, where they meet a resident pool of G proteins and adenylyl cyclases. The ensuing local cAMP signals are required to efficiently activate PKA II on membranes of the Golgi/trans-Golgi network, which, in turn, induces nuclear CREB phosphorylation and gene transcription in response to TSH (57). A related, albeit mechanistically distinct, modality of GPCR signalling in the Golgi has been described for the β_1 -adrenergic receptor (61, 67). In contrast, other receptors such as the PTH and β_2 -adrenergic receptors have been shown to signal on membranes of early endosomes (59, 68). Moreover, there is growing evidence that GPCRs might signal in other subcellular compartments, including the nucleus (69, 70, 71, 72, 73), endoplasmic reticulum (74), mitochondria (75, 76, 77, 78, 79, 80, 81, 82) and lysosomes (83, 84). Importantly, similar signals generated from different compartments could produce profoundly different biological responses. For instance, TSH receptor signalling at the plasma membrane and in the Golgi/trans-Golgi network generate similar increases in cAMP levels. However, only the signals originating from the Golgi/trans-Golgi are able to efficiently stimulate CREB phosphorylation and gene transcription (57).

Whereas we are only beginning to understand the functional impact of GPCR signalling at intracellular sites and its role in biased signalling, growing evidence suggests that it may have important physiological consequences. For example, our group has shown that persistent cAMP signalling by internalised LH receptors in ovarian follicles is required for efficient oocyte meiosis resumption, a fundamental event in female reproduction that is triggered by the LH surge at mid-cycle (56). Additional studies have hinted to a critical role of GPCR signalling at intracellular sites in the regulation of a variety of physiological functions, including nociception (85, 86), insulin secretion (64, 87), renal water and sodium reuptake (63) and neuronal excitability (65).

Moreover, endosomal GPCR signalling might be involved in pathophysiological processes. An intriguing example is provided by adaptor protein-2 σ -subunit (AP2 σ) mutations that are responsible for familial hypocalcaemic hypercalcaemia (FHH). In a recent study, it has been shown that these mutations owe their effects to an impairment of calcium-sensing receptor internalisation and signalling at

Figure 1

Emerging picture of compartmentalised GPCR signalling. New evidence indicates that GPCRs signal in small nanodomains located both the cell surface (hot spots) and at intracellular sites. The ensuing local signals likely play an important role in the high diversity and specificity observed in GPCR signalling.

intracellular sites (88). Another example is catecholamine-induced cardiac hypertrophy, which has been suggested to be mediated by resident β_1 -adrenergic receptors signalling in the Golgi compartment of cardiomyocytes (67).

Finally, the concept of GPCRs signalling in distinct subcellular compartments might be exploited therapeutically to develop more effective and, potentially, more selective drugs. One possible approach is to attach to a GPCR agonist or antagonist a lipophilic moiety that causes its accumulation in endosomes (85). Another possibility is to incorporate the drug in pH-sensitive nanoparticles, which are taken up by cells and cause the controlled release of the drug once it reaches the acidic environment of the endosomal compartment (89).

New opportunities offered by single-molecule microscopy

The visionary idea of imaging and manipulating individual molecules was proposed by Richard Feynman in the 1960's (90). Thanks to key technological advances and the pioneering work of scientists like William E Moerner (91) and Eric Betzig (92), single molecule microscopy has now become a reality (93). Single molecule microscopy offers a number of important advances compared to ensemble methods, including the possibility of directly analysing complex mixtures of unsynchronized molecules. Moreover, it can achieve spatial and temporal resolutions of about 10 ms and 10 μ m, which is approximately 20-times better than with standard fluorescence microscopy. This allows investigating biological processes in living cells on the spatiotemporal scales on which key events like protein–protein interactions take place. This approach has been instrumental to elucidate the nanoscale organisation of the plasma membrane, leading to the formulation of the fence-and-picket model (94). According to this model, barriers provided by the cytoskeleton ('fences') and associated membrane proteins ('pickets') partition the plasma membrane into small nanodomains where other membrane proteins are transiently trapped, leading to the emergence of complex diffusion and interaction behaviours.

New insights on receptor dimerisation from single-molecule microscopy

Although originally believed to be monomeric receptors, new evidence suggested that GPCRs might form dimers

and possibly higher-order oligomers on the plasma membrane (95). It was hypothesised that these GPCR supramolecular complexes might play important roles in the modulation of GPCR function. However, apart from few notable exceptions such as Family C GPCRs (96), the nature, stability and functional relevance of GPCR di-/oligomerisation was a matter of intense debate. Single-molecule studies by our and other groups shed new light on this elusive phenomenon. Two initial studies used fluorescently ligands to investigate the muscarinic M1 acetylcholine receptor (97) and the N-formyl peptide receptor (98). Subsequently, our group compared three GPCRs, that is, β_1 -adrenergic, β_2 -adrenergic and GABA_B receptors, that were directly labelled with bright organic fluorophores via insertion of a SNAP tag (99). Altogether, these studies revealed that Family A GPCRs rapidly associate and dissociate on the plasma membrane, forming transient complexes. More studies appear required to further investigate the functional relevance of the highly dynamic interactions among receptors revealed by single-molecule microscopy.

Hotspots for receptor-G protein signalling

More recently, our group succeeded in observing individual receptors and G proteins as they interact and signal on the surface of living cells (100). Two prototypical GPCRs, that is, α_{2A} - and β_2 -adrenergic receptors, and their main interacting G proteins, G_i and G_s , were imaged and tracked simultaneously at high speed (~30 ms) and resolution (~20 ms), which allowed probing their diffusion and mutual interactions. This approach led to a number of important observations.

First, it allowed estimating the duration of receptor-G protein interactions, which was calculated to be in the range of 1–2 s for the investigated receptors (100). This clarified, among other aspects, the nature of receptor-G protein interactions, ruling out the presence of a relevant component of stable, preformed receptor-G protein complexes under the employed experimental conditions. However, since receptor-G protein interactions might be affected by several external factors, including the potential presence of accessory binding proteins, it will be important to further investigate whether their characteristics might vary depending on the cellular context.

Additionally, it revealed that agonists mainly control GPCR signalling by increasing the probability that a collision between a receptor and a G protein results in the formation of a productive interaction (100).

This probability was found to vary depending on the employed agonist, providing a new mechanistic basis to understand agonist efficacy. These findings have relevant pharmacological implications, as they might be exploited to more rationally design drugs for this important receptor family in the future.

Moreover, this study revealed that receptor-G protein interactions do not occur randomly on the cell surface, but, rather, at nanometre-sized 'hot spots', where receptors and G proteins are transiently trapped (Fig. 1) (100). These hot spots appear to at least partially emerge from a combination of the barriers provided by the actin cytoskeleton, in agreement with the fence-and-picket model of the plasma membrane (94), and the occurrence of basal interactions between unstimulated receptors and G proteins. These findings allows us to better understand two key aspects of GPCR signalling, that is, its high signal specificity and efficiency. By segregating different receptors and G proteins in time and space, the formation of hot spots causes GPCR signals to stay local. This may potentially allow receptors coupled to otherwise similar downstream signalling pathways to produce distinct biological responses in our cells. At the same time, by keeping receptors and G proteins close to each other after a previous interaction, we hypothesize that the formation of hot spots increases the speed and efficiency of GPCR signalling, allowing our cells to respond rapidly to extracellular stimuli. This might be particularly relevant in specialized signalling structures such as neuronal synapses, where a high spatial organisation of receptor signalling appears crucial to achieve rapid and coordinated responses (101). Future studies are needed to further investigate these mechanisms as well as to study their involvement in human physiology and their potential alterations in disease.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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