MINIREVIEW—EXPLORING THE BIOLOGY OF GPCRs: FROM IN VITRO TO IN VIVO

Spatial and Temporal Aspects of Signaling by G-Protein–Coupled Receptors

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ABSTRACT
Signaling by G-protein–coupled receptors is often considered a uniform process, whereby a homogeneously activated proportion of randomly distributed receptors are activated under equilibrium conditions and produce homogeneous, steady-state intracellular signals. While this may be the case in some biologic systems, the example of rhodopsin with its strictly local single-quantum mode of function shows that homogeneity in space and time cannot be a general property of G-protein–coupled systems. Recent work has now revealed many other systems where such simplicity does not prevail. Instead, a plethora of mechanisms allows much more complex patterns of receptor activation and signaling: different mechanisms of protein–protein interaction; temporal changes under nonequilibrium conditions; localized receptor activation; and localized second messenger generation and degradation—all of which shape receptor-generated signals and permit the creation of multiple signal types. Here, we review the evidence for such pleiotropic receptor signaling in space and time.

Introduction
One of the most puzzling aspects of G-protein–coupled receptor (GPCR) signaling is the fact that the large number of ligands and receptors appears to converge to a limited number of G-proteins and downstream signaling pathways. Hundreds of receptors signal to G-proteins, which are essentially limited to a few groups of Gα-subunits (given that the Gβγ-subunits display little specificity), despite the multitude of theoretical α-, β-, and γ-subunit combinations, and which define the downstream signaling pathways. However, this apparent lack of complexity of downstream signaling may be due to a lack of appreciation of information, which may be encoded not in the downstream signal per se (i.e., in the sequence and/or structure of the participating molecular units) but in its spatial and temporal patterns. This aspect of GPCR signaling has traditionally been neglected, and signaling has been conceptually reduced to plain and uniform increases or decreases of second messenger concentrations, such as cAMP, cGMP, and inositol trisphosphate, even though evidence for complex patterns of signaling has long been in existence. For example, it has been found in many signaling systems that constant receptor stimulation can result in temporally complex downstream signaling patterns, in particular in oscillations that have often been observed in intracellular Ca²⁺ signaling (Tsien and Tsien, 1990; Berridge, 1993; Parekh, 2011). Moreover, the kinetics of the initial signaling steps—i.e., receptor activation, G-protein coupling, and G-protein activation—have been elucidated in some detail and allow an understanding of signal transmission and its temporal integration by the entire signaling machinery (Lohse et al., 2008; Hofmann et al., 2009; Jensen et al., 2009).

A special and extremely well studied case is the light receptor of the retinal rod cell, which has over several decades led research into GPCRs and their signals. Rhodopsin can be activated by flashes of light with defined duration and intensity, making it a preferred subject of studies on the kinetics of receptor activation and interactions with signaling proteins, the G-protein transducin (Gt*), and the visual arrestin-1 (Sommer et al., 2015). Each light-activated rhodopsin, R*, on its trajectory through the disc membrane sequentially contacts multiple transducin (Gt) molecules, catalyzing their activation to (Gt*) via exchange of bound GDP for cytoplasmic GTP. Two Gt* molecules can bind to each phosphodiesterase (PDE) unit, which activates PDE to Gt*-PDE-Gt*. The activated Gt*-PDE-Gt* hydrolyzes cGMP, and the reduced cytoplasmic concentration of cGMP causes closure of cGMP-gated channels in the plasma membrane and an increase (with negative polarity) of receptor current. This so-called visual cascade is considered one of

ABBREVIATIONS: GPCR, G-protein–coupled receptor; PDE, phosphodiesterase.
the simplest models of signal transduction. Nevertheless, it should be noted that it takes a whole functional module (Hofmann et al., 2006), i.e., dozens of proteins and their spatially/temporally well ordered interaction during ~200 milliseconds, to shape the uniform rise and fall of the rod's single quantum response.

Receptor signaling is stopped by a mechanism common to most GPCRs: the C-terminal tail of the active receptor is phosphorylated by a receptor kinase. Arrestin then binds the active, phosphorylated receptor and thereby sterically blocks further interaction with the G-protein (Lohse and Hoffmann, 2014; Szczepak et al., 2014); in the case of nonrhodopsin GPCRs, this triggers a series of nonclassical signals (Shenoy and Lefkowitz, 2011), which follow the classic G-protein-mediated signaling wave (Lohse and Calebiro, 2013).

Temporal Aspects—Kinetics along the Signaling Chain

Receptor Activation and Interactions: Rhodopsin Case. Although the initial triggering event in GPCR activation is a distinct process for each specific receptor, the structural patterns and helix rearrangements are remarkably similar. Rhodopsin is a seeming exception in that the all-trans-retinal agonist forms by cis/trans isomerization of the covalently bound retinal and a series of irreversible, short-lived intermediates. Conformational changes begin around the retinal binding site and result after microseconds in breaking a constraining central ionic lock between the protonated Schiff base and its counter-ion Glu-113 (for a review, see Hofmann et al., 2009). However, once this has happened, the protein states that follow resemble those formed in other GPCRs; in the case of rhodopsin, the so-called Meta states all contain the all-trans-retinal agonist bound by a deprotonated retinal Schiff base, but are distinguished by specific arrangements of crucial amino acids and their connecting hydrogen bonds. Corresponding R and R° conformational states can be delineated for other GPCRs and strongly suggest the presence of multiple active forms of GPCRs (Zürn et al., 2009; Nygaard et al., 2013; Manglik et al., 2015).

These diverse active conformations may also differ in their signaling specificities, laying the basis of specific and biased signaling. Specifically, in rhodopsin the Meta IIa form is inactive both toward Gt and arrestin (Hofmann et al., 2009; Szczepak et al., 2014). It takes the prominent outward tilt of TM6, which leads to Meta Ib, and the proton uptake into the open binding cavity in Meta II H⁺ to generate the conformation that interacts tightly with the C-terminal key binding site of the G-protein and with the finger loop of arrestin (Knierim et al., 2007; Altenbach et al., 2008; Szczepak et al., 2014). The observation that in flexible detergents light-activated rhodopsin completely populates the Meta Ib/MIIbH⁺ conformations (Knierim et al., 2007) has led to the notion that the active state in rhodopsin is one homogeneous conformation (Manglik et al., 2015). However, in the native membrane and under physiologic conditions, all Meta states are present to a significant extent (Mahalingam et al., 2008; Elgeti et al., 2013). They interconvert on a submillisecond-to-millisecond timescale, illustrating a dynamic situation where time and timing play essential roles.

A picture of how such timing may govern the coupling of active rhodopsin with G-protein or arrestin is just emerging. The C-terminal binding fragment of the G-protein (GoCT) has, in the free GDP-bound Gt, properties of an intrinsically unstructured domain, but forms an α-helix when bound in R⁺ (Scheerer et al., 2008). Recent work with synthetic peptides from this fragment suggests that, in the course of coupling between the proteins, not only GoCT but also the receptor cavity gain (mostly α-helical) structure. Only the final state of interaction has properties of the Meta IIb/MIIbH⁺ states and of the X-ray structure (Elgeti et al., 2013). This mutual structuring, although still hypothetical, would be an example of a binding funnel, which is thought to speed up the interaction between proteins (Csermely et al., 2010).

In the course of structuring, manifest intermediates (so-called precomplexes) have now been identified for R⁺-Gt and R⁺-arrestin-I coupling. A structure of a precomplex with Gt is not known, but kinetic (Heck and Hofmann, 2001) and molecular dynamics (Scheerer et al., 2009) studies have led to the postulate of an R⁺-Gt-GDP intermediate in which GDP is still bound in its Gα-binding site. In R⁺-Gt-GDP, the GoCT fragment has already adopted α-helical structure but shows a rotation and tilt compared with the empty site X-ray structure (Scheerer et al., 2008). In the arrestin case, an X-ray structure of β-arrestin bound to a β₂-adrenergic receptor phosphopeptide has been obtained, in which the stabilizing C-terminal tail of arrestin is displaced (Shukla et al., 2013). The structure is similar to the one of a preactivated C-terminally truncated arrestin-1 (p44 protein) (Kim et al., 2013). Although a direct comparison between G-protein and arrestin precomplexes is not yet possible, a partial structuring of a key interaction site (GoCT fragment or Arr-FL finger loop) compared with the basal state appears to be a common property of the precomplexes.

Sequential changes of conformation thus appear to be essential for the activation process of rhodopsin. Although the linearity of the rhodopsin activation chain, with only one G-protein to interact with, has facilitated such analysis, it is possible that similar sequential schemes also apply to GPCRs, which are activated by diffusible ligands to turn on different G-proteins. Such generalization, which is also consistent with the involvement of highly conserved domains, would considerably extend the conformational repertoire of active GPCRs. Not only would they conformationally laterally adjust to different pathways (Rose et al., 2014; Manglik et al., 2015), they would also go through a sequence of conformations when they adapt to their signaling partners. For GPCRs, in general, such a scenario would open multiple ways of regulation via the timing of each of the interaction steps.

Nonrhodopsin GPCRs. Rapid dynamic transitions between different states are beginning to be observed for nonrhodopsin GPCRs, using a variety of methods including NMR and molecular dynamics simulations (Nygaard et al., 2013; Olofsson et al., 2014; Manglik et al., 2015). In contrast with this view, a recent study employing fluorescence correlation spectroscopy on soluble extracellular domains of a metabotropic glutamate receptor suggests that these receptors (or domains) may switch between only two conformations, termed open and closed (Olofsson et al., 2014). Overall, the transitions of GPCRs into truly active conformations appear to be incomplete under physiologic conditions, and stabilization by binding to a G-protein or to a (β-)arrestin appears
to be required in order to produce a fully active receptor (Elgeti et al., 2013; Manglik et al., 2015). Activation of nonrhodopsin receptors has been followed mostly using fluorescence resonance energy transfer–based sensors and rapid application of agonists by superfusion; this has led to reported activation times down to 10 milliseconds (Marcaggi et al., 2009), but mostly in the range of 30–80 milliseconds (Vilardaga et al., 2003; Lohse et al., 2008, 2012). Levitz et al. (2013) used a tethered, light-switchable agonist to activate a metabotropic glutamate receptor, but did not determine the speed of activation of the receptor. Using again various fluorescence resonance energy transfer–based sensors, Hlavackova et al. (2012) determined a sequence of activation events in a dimeric metabotropic glutamate receptor, beginning with movement of the two receptor moieties toward each other (≈30 milliseconds), followed by the conformational change in the transmembrane region thought to produce the active, G-protein coupling form (≈40 milliseconds). Surprisingly, however, the activation of G-proteins in these systems is much slower, taking ≈500 milliseconds for the GDP/GTP exchange that results in the active form of the G-protein, irrespective of its subtype (i.e., Gq, Gs, or G12) (Bünemann et al., 2003; Hein et al., 2005, 2006; Jensen et al., 2009; Hoffmann et al., 2012). This may indicate a slow step within the process of complex formation. In principle, any step within the sequence of conformational conversions described above could be affected and modulate the overall speed of G-protein activation. A possible scenario would be that one of the binding partners is held in a supramolecular structure, from or in which it is slowly made available for interaction (Schöneberg et al., 2014). This is reminiscent of recent work on rhodopsin, which has resulted in dramatically different simulated activation rates of Gαi, depending on the localization of the activating Rb within or outside rows of dimers of the receptor (Gunkel et al., 2015). Intriguingly, the high speed of G-protein activation in vitro (Heck and Hofmann, 2001) would only be true for a limited number of G-proteins, which are localized within the oligomer containing the Rb.

**Nonequilibrium GPCR Activation.** While continuous activation—such as a constant concentration of a hormone—may allow a GPCR system to reach equilibrium, many physiologic systems will often operate under nonequilibrium conditions. For example, this is true for synapses, where release and degradation and/or reuptake of neurotransmitters are very rapid and effect only pulsatile stimulation of a GPCR.

Nonequilibrium conditions significantly affect the responses of a GPCR system because they will result in an apparent loss of sensitivity and responsiveness. For example, over a time course of 60-millisecond to 3-second stimulation of the α2A-adrenergic receptor by norepinephrine, the sensitivity (half-maximal stimulation) changed from 26 to 2.5 μM, i.e., over a full log unit, and in the same experiments maximal activation by high agonist concentrations took at least 400 milliseconds (Ambrosio and Lohse, 2012). A similar pattern was observed for Gαi activation, where over a time ranging from 0.5 to 15 seconds the EC50 value for norepinephrine decreased from over 100 nM to less than 10 nM. Given the often short pulses of synaptic transmitter release, this indicates that GPCRs will often operate under nonequilibrium conditions, and hence far below their full sensitivity.

**Downstream Temporal Signaling Patterns.** Following formation of an active receptor and subsequent activation of a G-protein, a sequence of downstream signals is triggered that is specific, in its kinetic as well as spatial patterns, for a given receptor, cellular context, and often also for a given ligand. For many GPCRs there appear to be a series of distinct signaling waves (Lohse and Calebiro, 2013). A first, G-protein–dependent wave is triggered at the cell surface and results in the classical cAMP and inositol trisphosphate signals. This is followed by a β-arrestin–dependent wave initiated either at the cell surface from clathrin-coated pits and or from clathrin-coated vesicles and results in nonclassical signals such as ERK activation (Shenoy and Lefkowitz, 2011); ERK activation by GPCRs may, by the way, be achieved via multiple signaling pathways. A third wave has been described for an increasing number of GPCRs, which involves signaling at intracellular sites via G-proteins that appear to reside at these sites and results in intracellular cAMP signals, which may have specific physiologic outcomes (Calebiro et al., 2010, 2015; Vilardaga et al., 2014; Tsvetanova et al., 2015). This third wave is then terminated by dissociation of ligand and GPCRs in the vesicles, promoted by acidification of the vesicles, and ultimately the receptors either are degraded or recycle back to the cell surface (Vilardaga et al., 2014). The temporal sequence of these distinct signaling waves has been dissected mostly for simple cell lines, such as HEK cells, but also for some primary cells, notably thyroid follicles (Calebiro et al., 2015).

In addition to such sequential activation of different signaling pathways, it has long been known that intracellular signals may be fluctuating and that, in particular, intracellular calcium signals often show marked oscillations (Tsien and Tsien, 1990; Berridge, 1993). These intracellular oscillations, which may show widely differing amplitudes and frequencies, are generally thought to be triggered not at the receptor level but at subsequent steps of the signaling cascade. They may result from a variety of oscillators that involve Ca channels both at the cell membrane and on intracellular stores and Ca-dependent enzymes. Both the amplitude and the frequency, but also the spatial distribution (see **Spatial Aspects—Receptor Localization and Spatial Signaling Patterns**) of such calcium oscillations may encode a specific downstream response (Parekh, 2011). For example, it has recently been shown that different isoforms of the transcription factor NFAT are specifically activated by distinct patterns of Ca signals (Kar and Parekh, 2015).

More recently, it has become clear that oscillations may also occur in other intracellular signals, notably in cAMP, and that there are multiple links indicating how Ca and cAMP oscillations may be intertwined (Zaccolo and Pozzan, 2003). The basic principle here is that, directly or indirectly, the generating or degrading mechanisms are sensitive to the concentrations of Ca or cAMP, respectively. Ca-dependent adenyl cyclases and PDEs may cause synchronous and antisynchronous changes in cAMP, respectively, and both types of correlation have been observed in different, and sometimes even the same, cells. Depending on the specific isoform, Ca may both inhibit and stimulate cAMP production (Willoughby and Cooper, 2007; von Hayn et al., 2010). In the other direction, cAMP may both induce (e.g., Capiro et al., 1991) and modulate (e.g., Nuttle and Farley, 1996) Ca oscillations and downstream responses. Well examined examples
are the pancreatic β cells, where insulin secretion is controlled by oscillations in both cAMP and Ca (Harbeck et al., 2006; Dyachok et al., 2006). Oscillatory phosphorylation patterns have also been observed beyond the cAMP/PKA system, for example, for protein kinase C (Violin et al., 2003).

Temporal Signaling Patterns: Versatility and Precision. In visual transduction, the activation of both rhodopsin and the G-protein, transducin, are very rapid. In particular, they are fast compared with the lifetime of an active rhodopsin. This allows amplification of the signal by sequential activation of multiple (several hundred per second) (Heck and Hofmann, 2001) G, by a single R+. Overall, more than 50 proteins form a closed functional module, which is activated and deactivated within ∼200 milliseconds in mice (Hofmann et al., 2006). We have seen above how rhodopsin, by its funnel-like interaction process, can manage to interact with its signaling proteins both fast and precisely. The catalytic process lasts until R+ becomes deactivated, generating a surprisingly uniform single quantum response (see Azevedo et al., 2015). Current estimates of R+ lifetime in mouse rods are in the range of ∼40 milliseconds (Gross and Burns, 2010).

The fact that in nonrhodopsin systems GPCR activation and interaction with G-proteins occur at least 10 times faster than the activation of G-proteins presumably limits the amplification at this step. Although it is mechanically unclear how long the receptor needs to be in the active state and how long it needs to stay in contact with a G-protein to induce activation of the latter, the dissociation of receptors from G-proteins is slow (t1/2 of several seconds in various systems) (Hein et al., 2005, 2006). Thus, binding to a G-protein will prevent an active receptor from moving to and activating the next G-protein, and will thus limit signal amplification. It is unclear whether during the slow activation of a G-protein, a second receptor may interact with the same G-protein and modulate the activation by the first one; however, if we assume that the first one remains bound during the entire activation process, then it will prevent access of other receptors. This view is in line with the recently observed floppiness of receptors (Manglik et al., 2015). As we have seen, such conformational variability can be used to combine speed in the coupling process with precision in the final interaction, thus allowing precise interrogation of the receptor binding site. On the other hand, it can lead to surprising versatility of coupling, making signaling less reliable but much more versatile than in the rhodopsin system.

Temporal variations of signals apparently allow encoding of information into otherwise uniform signaling mechanisms. Such specific decoding has been demonstrated in several instances for temporal as well as spatial patterns of Ca oscillations (Parekh, 2011) (see Signaling in Space and Time and Compartmentation of Signals).

Spatial Aspects—Receptor Localization and Spatial Signaling Patterns

Moving Targets: GPCR Localization and Mobility at the Cell Surface. Photobleaching studies have been used for decades to assess the mobility of GPCRs in the cell membrane (Henis et al., 1982; Dorsch et al., 2009), and these have been more recently complemented by single molecule observations of fluorescently bound ligands (Hern et al., 2010; Kasai et al., 2011) or receptors themselves (Calebiro et al., 2013). From these data it is evident that GPCRs represent moving targets, although there is considerable variation in the reported fraction of mobile versus immobile receptors. This may depend on the type of cell, receptor, and activation state of the receptors. In several instances it has been reported that receptor mobility may be limited by fusing into small microdomains (Suzuki et al., 2005; Kasai et al., 2011), or for some receptors, such as the GABAA receptors, by attachment to the cytoskeleton (Calebiro et al., 2013). For the rod disc membrane, evidence for the native organization of rhodopsin in rows of dimers has now been obtained from cryoelectron tomography (Gunkel et al., 2015). The functional consequences of such supramolecular structures were already discussed previously in the section on nonrhodopsin GPCRs. Whether agonists change receptor mobility is controversial, but most recent studies find no or very little effect. The reported diffusion coefficients vary, but on average they are on the order of 0.1 μm²·s⁻¹ (Suzuki et al., 2005; Hern et al., 2010; Calebiro et al., 2013), although more rapid diffusion speeds have also been measured, particularly in photobleaching experiments (Henis et al., 1982; Carayon et al., 2014).

Single molecule mobility studies have also shown that receptors may dynamically form di- and oligomers in the cell membrane. In the course of their rapid movements on the cell surface, they may meet another receptor and stay together for various periods of time, thus creating a dimer or even a higher-order oligomer (Hern et al., 2010; Kasai et al., 2011; Calebiro et al., 2013). This type of dynamic association and dissociation of GPCRs is, obviously, compatible with this process being determined by their respective affinities; high affinities would cause predominance of di- and oligomers, compatible with quite static dimeric structures that have been reported in many studies on a large number of GPCRs. Dynamic di- and oligomerization of a GPCR has more recently also been suggested by spatial intensity distribution analysis of confocal images (Ward et al., 2015).

While the mobility may suggest that receptors are distributed evenly over the cell surface, there is ample evidence for specific localization to specific regions of a cell, most notably the synapse. Localization of GPCRs by PDZ proteins has recently been reviewed (Dunn and Ferguson, 2015) and will not be covered here. Functional evidence for even subtype-specific localization of GPCRs on the cell surface comes from cardiomyocytes, where β-adrenergic receptors appear to be ubiquitous, whereas the β2-subtype is specifically localized to t-tubules, where it triggers local rather than generalized increases in cAMP (Nikolaev et al., 2010).

β-Arrestin–Dependent Signaling. G-protein–mediated signaling from the cell surface is usually terminated by GRK-dependent receptor phosphorylation and binding of β-arrestins (Gross and Burns, 2010; Lohse and Hoffmann, 2014). This process occurs in a sequence of events because (β-)arrestin binding requires both the active structure of the receptor and phosphorylation by a GRK (Krasel et al., 2005). For nonvisual GPCRs, this process is generally linked to receptor assembly in clathrin-coated pits and subsequent internalization into clathrin-coated vesicles because β-arrestins serve as adapters between GPCRs and clathrin as well as β-adaptin, the β-subunit of the clathrin binding adapter AP2 (Goodman
et al., 1996; Tian et al., 2014). However, it should be noted that some GPCRs do not recruit β-arrestins, and that internalization may also occur in a β-arrestin–independent manner (Blaukat et al., 1996; Zhang et al., 1996; Pals-Rylaarsdam et al., 1997; van Koppen and Jakobs, 2004).

At this point, β-arrestins may interact with a plethora of further signaling proteins, which mediate nonclassical signals such as activation of multiple kinases including ERK and src (Shenoy and Lefkowitz, 2011). Spatially, this signaling is initiated either at the cell surface (from clathrin-coated pits) or during internalization (from clathrin-coated vesicles). This indicates that the second, β-arrestin–dependent wave of signaling is distinguished both temporally and spatially from the first, G-protein–dependent wave (Lohse and Calebiro, 2013). Biochemically, it is distinguished by the different pathways that are activated by G-proteins versus β-arrestins, i.e., effectors generating second messengers or mediating transmembrane ion conductance versus protein kinase signaling pathways. β-Arrestin–dependent signaling is terminated by the dissociation of the active complexes, notably of β-arrestins from the receptors, which may occur during or after formation of vesicles and their internalization and may be enhanced by acidification of the vesicle content (Vilardaga et al., 2014; Tsvetanova et al., 2015).

**Signaling from Internal Sites.** A new aspect of subcellular signaling was identified a few years ago, when several GPCRs were shown to signal via G-proteins after their internalization, i.e., from internal sites (Calebiro et al., 2010, 2015; Vilardaga et al., 2014; Tsvetanova et al., 2015). Thus, this type of signaling is not only temporally but also spatially distinct from the two preceding waves of signaling (Lohse and Calebiro, 2013). To permit such internal G-protein–dependent signaling, β-arrestins need to dissociate from the receptors (see above). It is believed that GPCRs internalize without their G-proteins, and that intracellularly they meet with G-proteins (plus adenyl cyclases) resident on intracellular vesicles. The exact nature of these vesicles, possibly a trans-Golgi network (Calebiro et al., 2010), remains to be established and may also differ between different receptors and between different cell types. So far, this process has been observed only for adenyl cyclase–coupled receptors, both Gs and Gi, and it remains to be seen whether other G-protein effectors can also be activated at intracellular sites.

The physiologic consequences of G-protein–mediated signaling from internal sites remain to be elucidated, but there are first hints for specific signaling outcomes. These specific downstream effects appear to include selective changes in gene expression, demonstrated for β-adrenergic receptors, and the control of thyroid hormone secretion in the case of the thyroid-stimulating hormone receptors (Calebiro et al., 2010; Tsvetanova et al., 2015). However, signaling from internal sites may also affect processes at the cell surface, as shown for dopamine D1-receptor–dependent neuronal excitability (Kotowski et al., 2011).

A further nonconventional signaling mechanism from intracellular sites has been described for cannabinoid CB1 receptors, which have been observed on mitochondria and have been suggested to regulate neuronal energy metabolism (Bénard et al., 2012). It remains to be seen whether this is an exception to GPCR-mediated signaling or whether other intracellular sites and functions can be discovered.

These findings suggest that GPCR-mediated signaling from internal sites represents a distinct and functionally relevant process, and a third wave of GPCR-mediated signaling, which is separated from the preceding two waves temporally, spatially, biochemically, and presumably also in terms of the downstream outcome. However, the details of this mechanism as well as its physiologic role remain to be further elucidated.

### Signaling in Space and Time and Compartmentation of Signals

If we consider temporal as well as spatial patterns of signaling, then it becomes clear that GPCRs trigger far more than the simple and uniform up and down of global second messenger concentrations. Instead, a picture emerges of a complex and well orchestrated spatiotemporal pattern of signaling waves, which use distinct biochemical pathways and involve local as well as generalized subcellular localization and temporal modulation and oscillation. The spatiotemporal pattern of distinct signaling waves (Lohse and Calebiro, 2013) has been described above and is in its principles understood, even though many details remain to be elucidated. The same is true for oscillations in second messengers, where biochemical circuits and feedback loops involving the messenger-generating and degrading proteins result in temporal signaling patterns.

The mechanisms of spatial compartmentation of signals, in contrast, appear to require much better explanations than are currently available. Numerous data suggest that second messenger signals may be spatially compartmentalized in many cell types, notably neurons and cardiomyocytes, and that, for example, receptors may cause localized versus generalized responses (Nikolaev et al., 2010; Mika et al., 2012) or that the submembrane compartment shows changes that differ from those in the cytosol (Rich et al., 2014). However, this is not compatible with the essentially free diffusion of second messengers in cells, which has also been measured repeatedly and seems to preclude the generation of intracellular concentration gradients (Bacskaï et al., 1993; Nikolaev et al., 2004).

Several factors have been proposed to contribute to the generation of spatial signal compartmentation: specific localization of receptors and other signaling proteins through anchoring proteins and clustering of these proteins to functional units, which may be shielded from the general cytosolic environment (Davare et al., 2001; Willoughby and Cooper, 2007; Nikolaev et al., 2010; Wachten et al., 2010). It has also been proposed that the speed of second messenger diffusion might be much lower than generally assumed, in order to permit the establishment of concentration gradients (Rich et al., 2014). This would suggest the presence of very significant obstacles to diffusion, and the biologic nature of these needs to be delineated. It is not clear whether proteins that degrade or bind second messengers (such as PDEs) have sufficient enzymatic or buffering capacity.

Spatial compartmentation of second messengers also has to be assumed if signaling from internal sites (see **Spatial Aspects—Receptor Localization and Spatial Signaling Patterns**) is to produce distinct functional effects. However, such distinct effects can be inferred from specific transcriptional...
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