Molecular Pharmacology Fast Forward. Published on July 16, 2015 as DOI: 10.1124/mol.115.100248
This article has not been copyedited and formatted. The final version may differ from this version.

MOL #100248

Review for *Molecular Pharmacology*

2015

Spatial and temporal aspects of signaling by GPCRs

Martin J. Lohse and Klaus Peter Hofmann

Institute of Pharmacology and Toxicology, and Rudolf Virchow Center, and Comprehensive Heart Failure Center, University of Würzburg, 97078 Würzburg (MJL) and Institut für Medizinische Physik und Biophysik (CC2), Charité-Universitätsmedizin Berlin, Charitéplatz 1, 10117 Berlin, and Zentrum für Biophysik und Bioinformatik, Humboldt-Universität zu Berlin, 10115 Berlin, Germany (KPH)

Molecular Pharmacology Fast Forward. Published on July 16, 2015 as DOI: 10.1124/mol.115.100248
This article has not been copyedited and formatted. The final version may differ from this version.

Running title: Spatial and temporal aspects of signaling by GPCRs

Corresponding author:

Martin J. Lohse

lohse@toxi.uni-wuerzburg.de

Tel. ++49-931-31 48400 Fax ++49-931-201 48411

Institute of Pharmacology and Toxicology

Versbacher Str. 9, 97078 Würzburg, Germany

Document Statistics:

Text pages – 32

References – 80

Abstract - 147

Introduction - 549

Molecular Pharmacology Fast Forward. Published on July 16, 2015 as DOI: 10.1124/mol.115.100248
This article has not been copyedited and formatted. The final version may differ from this version.

Abstract

Signaling by G-protein-coupled receptors (GPCRs) 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 biological 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 non-equilibrium conditions, localized receptor activation, and localized 2nd messenger generation and degradation, all 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 in time.

Molecular Pharmacology Fast Forward. Published on July 16, 2015 as DOI: 10.1124/mol.115.100248
This article has not been copyedited and formatted. The final version may differ from this version.

1. 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, despite the multitude of theoretical α-, β- and γ-subunit combinations are essentially limited to a few groups of Gα-subunits (given that the Gβγ-subunits display little specificity), 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 cyclic AMP (cAMP), cyclic GMP (cGMP), and inositol trisphosphate (IP_3), even though evidence for complex patterns of signaling has long been existing.

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 which have often been observed in intracellular Ca^{2+} 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).

Molecular Pharmacology Fast Forward. Published on July 16, 2015 as DOI: 10.1124/mol.115.100248
This article has not been copyedited and formatted. The final version may differ from this version.

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 (G_t) 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 (G_t) molecules, catalyzing their activation to (G_t *) via exchange of bound GDP for cytoplasmic GTP. Two G_t *’s can bind to each phosphodiesterase unit, which activates PDE to G_t *-PDE- G_t *. The activated G_t *-PDE-G_t * 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 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 spatio-temporally well-ordered interaction during ca. 200 ms, 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; Szczepek *et al.* 2014); in the case of non-rhodopsin GPCRs, this triggers a series of “non-classical” signals (Shenoy and Lefkowitz 2011), which follow the classical G-protein-mediated signaling “wave” (Lohse and Calebiro 2013).

Molecular Pharmacology Fast Forward. Published on July 16, 2015 as DOI: 10.1124/mol.115.100248
This article has not been copyedited and formatted. The final version may differ from this version.

2. Temporal aspects – kinetics along the signaling chain

2a. Receptor activation and interactions: the rhodopsin case

Although the initial triggering event in GPCR activation is a distinct process for each specific receptor, the structural patterns and helix re-arrangements 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 of irreversible, short-lived intermediates. Conformational changes begin around the retinal binding site and result after μs 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). But 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 towards G_t and arrestin (Hofmann *et al*., 2009; Szczepek *et al.* 2014). It takes the prominent outward tilt of TM6 that leads to Meta IIb and the proton uptake into the open binding cavity in Meta II H^+ to generate the conformation that interacts tightly

Molecular Pharmacology Fast Forward. Published on July 16, 2015 as DOI: 10.1124/mol.115.100248
This article has not been copyedited and formatted. The final version may differ from this version.

with the C-terminal key binding site of the G-protein and with the "finger loop" of arrestin (Szczepek *et al*., 2014; Altenbach et al, 2008; Knierim *et al*. 2007). The observation that in flexible detergents, light-activated rhodopsin completely populates the Meta IIb/ $MIIbH^+$ conformations (Knieriem 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 physiological conditions, all Meta states are present to a significant extent (Mahalingam *et al*. 2008; Elgeti *et al*. 2013). They interconvert on a sub-millisecond to millisecond timescale, illustrating a dynamic situation where time and timing play essential roles.

A picture, 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 (GαCT) 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 GαCT 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 pre-complexes) have now been identified for R*-Gt and R*-arrestin-1 coupling. A structure of a pre-complex with G_t 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

Molecular Pharmacology Fast Forward. Published on July 16, 2015 as DOI: 10.1124/mol.115.100248
This article has not been copyedited and formatted. The final version may differ from this version.

which GDP is still bound in its Gα-binding site. In R*-Gt-GDP, the GαCT fragment has already adopted α-helical structure but shows a rotation and tilt, as compared to the empty site X-ray structure (Scheerer et al., 2008). In the arrestin case, an X-ray structure of β-arrestin bound to a β_2-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 pre-activated C-terminally truncated arrestin-1 (p44 protein) (Kim *et al*. 2013). Although a direct comparison between G protein and arrestin pre-complexes is not yet possible, a partial structuring of a key interaction site (GαCT fragment or Arr-FL finger loop), as compared to the basal state appears to be a common property of the pre-complexes.

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 well 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 (Manglik *et al*. 2015; Rose *et al*. 2014), 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.

2b: Non-rhodopsin GPCRs

Molecular Pharmacology Fast Forward. Published on July 16, 2015 as DOI: 10.1124/mol.115.100248
This article has not been copyedited and formatted. The final version may differ from this version.

Rapid dynamic transitions between different states are beginning to be observed for non-rhodopsin 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 physiological conditions and stabilization by binding to a G-protein or to a (β-)arrestin appears required to produce a fully active receptor (Elgeti *et al*. 2013, Manglik *et al*. 2015). Activation of non-rhodopsin receptors has been followed mostly using FRET-based sensors and rapid application of agonists by superfusion; this has led to reported activation times down to 10 ms (Marcaggi *et al.* 2009), but mostly in the range of 30-80 ms (Vilardaga *et al*. 2003; Lohse *et al.* 2008, 2012). Levitz *et al.* (2013) have used a tethered, light-switchable agonist to activate a metabotropic glutamate receptor, but have not determined the speed of activation of the receptor. Using again various FRET-based sensors, Hlavackova *et al.* (2012) determined a sequence of activation events in a dimeric metabotropic glutamate receptor, beginning with a movement of the two receptor moieties towards each other (≈30 ms), followed by the conformational change in the transmembrane region thought to produce the active, G-protein coupling form (≈40 ms}. Surprisingly, however, the activation of G-proteins in these systems is much slower, taking ≈500 ms for the GDP/GTP exchange that results in the active form of the G-protein, irrespective of its subtype (i.e. G_i, G_s or G_q; Bünemann *et al*. 2003; Hein *et al.* 2005, 2006; Jensen *et al*. 2009; Hoffmann *et al*. 2012). This may

Molecular Pharmacology Fast Forward. Published on July 16, 2015 as DOI: 10.1124/mol.115.100248
This article has not been copyedited and formatted. The final version may differ from this version.

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 G_t, depending on the localization of the activating R* 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 R*.

2c. Non-equilibrium GPCR activation

While continuous activation – such as a constant concentration of a hormone – may allow a GPCR system to reach equilibrium, many physiological systems will often operate under non-equilibrium conditions. This is, for example, true for synapses, where release and degradation or re-uptake of neurotransmitters are very rapid and effect only pulsatile stimulation of a GPCR.

Non-equilibrium conditions significantly affect the responses of a GPCR system, since they will result in an apparent loss of sensitivity and responsiveness. For example, over a time course of 60 ms to 3 s stimulation of the α_{2A}-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 ms (Ambrosio *et al*. 2012). A similar pattern was observed for Gi-activation,

Molecular Pharmacology Fast Forward. Published on July 16, 2015 as DOI: 10.1124/mol.115.100248
This article has not been copyedited and formatted. The final version may differ from this version.

where over a time ranging from 0.5 to 15 s the EC_{50}-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 non-equilibrium conditions and, hence, far below their full sensitivity,

2d. 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 spatial patterns, for a given receptor, cellular context and often also 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 IP_3 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 "non-classical" signals such as ERK activation (Shenoy and Lefkowitz 2011); ERK activation 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 physiological 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

Molecular Pharmacology Fast Forward. Published on July 16, 2015 as DOI: 10.1124/mol.115.100248
This article has not been copyedited and formatted. The final version may differ from this version.

distinct signaling waves has been dissected mostly for simple cell lines, such as HEK cells, but also 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 below) 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, 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 adenylyl cyclases and phosphodiesterases may cause synchronous or anti-synchronous 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 or stimulate cAMP production (Willoughby and Cooper 2007; von Hayn *et al.* 2010). In the other

Molecular Pharmacology Fast Forward. Published on July 16, 2015 as DOI: 10.1124/mol.115.100248
This article has not been copyedited and formatted. The final version may differ from this version.

direction, cAMP may both induce (e.g. Capiod *et al.* 1991) or modulate (e.g. Nuttle and Farley 1996) Ca-oscillations and downstream responses. A well examined example are the pancreatic beta cells, where insulin secretion is controlled by oscillations in both cAMP and Ca (Harbeck *et al.* 2005; Dyachok *et al.* 2006). Oscillatory phosphorylation patterns have also been obserevd beyond the cAMP/PKA system, for example for protein kinase C (Violin *et al.* 2003).

2e. 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 to the life-time of an active rhodopsin. This allows amplification of the signal by sequential activation of multiple (several hundred per second (Heck and Hofmann 2001)) G_t by a single R*. Overall, more than 50 proteins form a closed functional module, which is activated and deactivated within ca. 200 ms 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 ms (Gross and Burns 2010).

The fact that in non-rhodopsin 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 mechanistically 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

Molecular Pharmacology Fast Forward. Published on July 16, 2015 as DOI: 10.1124/mol.115.100248
This article has not been copyedited and formatted. The final version may differ from this version.

induce activation of the latter, the dissociation of receptors from G-proteins is slow ($t_{1/2}$ 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. It can, on the other hand, lead to a 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 below).

3. Spatial aspects – receptor localization and spatial signaling patterns

3a. 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

Molecular Pharmacology Fast Forward. Published on July 16, 2015 as DOI: 10.1124/mol.115.100248
This article has not been copyedited and formatted. The final version may differ from this version.

these data it is evident that GPCRs represent “moving targets”, although there is considerable variation in the reported fraction of mobile vs. immobile receptors. This may depend on the type of cell, receptor and actvation state of the receptors. In several instances it has been reported that receptor mobility may be limited by “fencing” into small microdomains (Suzuki *et al*. 2005; Kasai *et al.* 2011) or, for some receptors like the $GABA_B$ 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 in section 2b. Whether or not 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 $\mu m^2 \cdot s^{-1}$ (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, bind to it for various periods of time and thus create a di- or also 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 being a process 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

Molecular Pharmacology Fast Forward. Published on July 16, 2015 as DOI: 10.1124/mol.115.100248
This article has not been copyedited and formatted. The final version may differ from this version.

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_1-adrenergic receptors appear to be ubiquitous, whereas the β_2-subtype is specifically localized to t-tubules, where it triggers local rather than generalized increases in cAMP (Nikolaev *et al.* 2010).

3b. 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, since (β-)arrestin binding requires both the active structure of the receptor and the phosphorylation by a GRK (Krasel *et al*. 2005).

For non-visual 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*. 2006; Tian *et al*. 2014). However, it should be noted that some GPCRs do not recruit β-arrestins, and that

Molecular Pharmacology Fast Forward. Published on July 16, 2015 as DOI: 10.1124/mol.115.100248
This article has not been copyedited and formatted. The final version may differ from this version.

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 "non-classical" 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 vs. β-arrestins, i.e. effectors generating second messengers or mediating transmembrane ion conductance, vs. 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).

3c. 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). This type of signaling is, thus, not only temporally but also spatially distinct from the two preceding waves of signaling (Lohse and Calebiro 2013). In order to permit such internal

Molecular Pharmacology Fast Forward. Published on July 16, 2015 as DOI: 10.1124/mol.115.100248
This article has not been copyedited and formatted. The final version may differ from this version.

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 adenylyl cyclases) resident on intracellular vesicles. The exact nature of these vesicles, possibly *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 adenylyl cyclase-coupled receptors, both G_s and G_i, and it remains to be seen whether other G-protein effectors can also be activated at intracellular sites.

The physiological 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 effect appears to include selective changes in gene expression, demonstrated for β-adrenergic receptors, and the control of thyroid hormone secretion in the case of the TSH receptors on thyroid cells (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 D_1-receptor dependent neuronal excitability (Kotowski *et al*. 2011).

A further non-conventional signaling mechanism from intracellular sites has been described for cannbinoid CB_1 receptors, which have been observed on mitochondria and 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

Molecular Pharmacology Fast Forward. Published on July 16, 2015 as DOI: 10.1124/mol.115.100248
This article has not been copyedited and formatted. The final version may differ from this version.

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 physiological role remain to be further elucidated.

4. 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 spatio-temporal pattern of signaling waves, which use distinct biochemical pathways and involves local as well as generalized subcellular localization and temporal modulation and oscillation. The spatio-temporal 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 feed-back loops involving the messenger-generating and degrading proteins result in temporal signaling patterns.

The mechanisms of spatial compartementation of signals, in contrast, appear to require much better explanations than are currently available. A lot of 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 vs. generalized responses (Nikolaev *et al*. 2010; Mika *et al*. 2012;) or that the sub-membrane 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

Molecular Pharmacology Fast Forward. Published on July 16, 2015 as DOI: 10.1124/mol.115.100248
This article has not been copyedited and formatted. The final version may differ from this version.

in cells, which has also been measured repeatedly and seems to preclude the generation of intracellular concentration gradients (Bacskai *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 biological 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 above) is to produce distinct functional effects. Such distinct effects can, however, be inferred from specific transcriptional activation (in the case of β-adrenergic receptors; Tsvetanova *et al.* 2015), but also in the regulation of hormone secretion (Calebiro *et al*. 2010). Specific mechanisms of decoding spatially and temporally distinct second messenger signals (Parekh 2011; Kar and Parekh 2015) can thus be translated into specific biological functions. Interestingly, compartmentation of cAMP may be altered in disease, as suggested particularly in the case of heart failure (Nikolaev *et al.* 2010; Sprenger *et al.* 2015).

Molecular Pharmacology Fast Forward. Published on July 16, 2015 as DOI: 10.1124/mol.115.100248
This article has not been copyedited and formatted. The final version may differ from this version.

Taken together, several kinds of mechanisms appear to contribute to the generation of temporal and spatial patterns of second messengers. Such patterns may be specifically decoded by cells, such that in spite of the low number of second messengers, a plethora of distinct responses may be triggered by GPCR activation, and the disruption of such patterns may be related to diseases.

Authorship Contributions:

Wrote or contributed to the writing of the manuscript – Lohse and Hofmann

Molecular Pharmacology Fast Forward. Published on July 16, 2015 as DOI: 10.1124/mol.115.100248
This article has not been copyedited and formatted. The final version may differ from this version.

References

Altenbach C, Kusnetzow AK, Ernst OP, Hofmann KP, Hubbell WL (2008) High-resolution distance mapping in rhodopsin reveals the pattern of helix movement due to activation. *Proc Natl Acad Sci USA* **105**: 7439-7444

Ambrosio M, Lohse MJ (2012) Non-equilibrium activation of a G-protein-coupled receptor. *Mol Pharmacol*. **81**: 770-777

Azevedo AW, Doan T, Moaven H, Sokal I, Baameur F, Vishnivetskiy SA, Homan KT, Tesmer JJ, Gurevich VV, Chen J, Rieke F (2015) C-terminal threonines and serines play distinct roles in the desensitization of rhodopsin, a G protein-coupled receptor. *eLife* **4**: e05981

Bacskai BJ, Hochner B, Mahaut-Smith M, Adams SR, Kaang BK, Kandel ER, Tsien RY (1993) Spatially resolved dynamics of cAMP and protein kinase A subunits in Aplysia sensory neurons. *Science* **260**: 222-226

Bénard G, Massa F, Puente N, Lourenço J, Bellocchio L, Soria-Gómez E, Matias I, Delamarre A, Metna-Laurent M, Cannich A, Hebert-Chatelain E, Mulle C, Ortega-Gutiérrez S, Martín-Fontecha M, Klugmann M, Guggenhuber S, Lutz B, Gertsch J, Chaouloff F, López-Rodríguez ML, Grandes P, Rossignol R, Marsicano G (2012) Mitochondrial CB_1 receptors regulate neuronal energy metabolism. *Nat Neurosci*. **15**: 558-564

Berridge MJ (1993) Inositol trisphosphate and calcium signalling. *Nature* **361**: 315-325

Blaukat A, Alla SA, Lohse MJ, Müller-Esterl W (1996) Ligand-induced phosphorylation/ dephosphorylation of the endogenous bradykinin B2 receptor from human fibroblasts. *J Biol Chem*. **271**: 32366-32374

Bünemann M, Frank M, Lohse MJ (2003) Gi protein activation in intact cells involves subunit rearrangement rather than dissociation. *Proc. Natl. Acad. Sci. USA* **100**: 16077-16082

Calebiro D, Nikolaev VO, Persani L, Lohse MJ (2010) Signaling by internalized G-protein-coupled receptors. *Trends Pharmacol Sci*. **31**: 221-228

Calebiro D, Rieken F, Wagner J, Sungkaworn T, Zabel U, Borzi A, Cocucci E, Zürn A, Lohse MJ (2013) Single-molecule analysis of fluorescently labeled GPCRs reveals receptor-specific complexes with distinct dynamics and organization. *Proc Natl Acad Sci USA* **110**, 743–748

Calebiro D, Godbole A, Lyga S, Lohse MJ (2015) Trafficking and function of GPCRs in the endosomal compartment. *Methods Mol Biol*. **1234**: 197-211

Capiod T, Noel J, Combettes L, Claret M (1991) Cyclic AMP-evoked oscillations of intracellular [Ca^{2+}] in guinea-pig hepatocytes. *Biochem J*. **275**: 277-280

Carayon K, Moulédous L, Combedazou A, Mazères S, Haanappel E, Salomé L, Mollereau C (2014) Heterologous regulation of Mu-opioid (MOP) receptor mobility in the membrane of SH-SY5Y cells. *J Biol Chem*. **289**: 28697-28706

Csermely P, Palotai R, Nussinov R (2010) Induced fit, conformational selection and independent dynamic segments: an extended view of binding events. *Trends Biochem Sci*. **35**: 539-546

Davare MA, Avdonin V, Hall DD, Peden EM, Burette A, Weinberg RJ, Horne MC, Hoshi T, Hell JW (2001) A β_2 adrenergic receptor signaling complex assembled with the Ca^{2+} channel Cav1.2. *Science* **293**: 98-101

Dorsch S, Klotz KN, Engelhardt S, Lohse MJ, Bünemann M (2009) Analysis of receptor oligomerization by FRAP microscopy. *Nat Meth***. 6**: 225-230

Dunn HA, Ferguson SS (2015) PDZ protein regulation of GPCR trafficking and signaling pathways. Mol Pharmacol. pii: mol.115.098509. [Epub ahead of print]

Dyachok O, Isakov Y, Sågetorp J, Tengholm A (2006) Oscillations of cyclic AMP in hormone-stimulated insulin-secreting beta-cells. *Nature* **439**: 349-352

Elgeti M, Rose AS, Bartl FJ, Hildebrand PW, Hofmann KP, Heck M (2013) Precision vs flexibility in GPCR signaling. *J Am Chem Soc*. **135**: 12305-12312

Goodman OB Jr, Krupnick JG, Santini F, Gurevich VV, Penn RB, Gagnon AW, Keen JH, Benovic JL (1996) β-Arrestin acts as a clathrin adapter in endocytosis of the β_2-adrenergic receptor. *Nature* **383**: 447-450

Gross OP, Burns ME (2010) Control of rhodopsin's active lifetime by arrestin-1 expression in mammalian rods. *J Neurosci* **30**: 3450-3457.

Gunkel M, Schöneberg J, Alkhaldi W, Irsen S, Noé F, Kaupp UB, Al-Amoudi A (2015) Higher-order architecture of rhodopsin in intact photoreceptors and its implication for phototransduction kinetics. *Structure* **23**: 628-638

Harbeck MC, Chepurny O, Nikolaev VO, Lohse MJ, Holz GG, Roe MW (2006) Simultaneous optical measurements of cytosolic Ca^{2++} and cAMP in single cells. Science STKE **353**: pl6

Heck M, Hofmann KP (2001) Maximal rate and nucleotide dependence of rhodopsin-catalyzed transducin activation: initial rate analysis based on a double displacement mechanism. *J Biol Chem*. **276**: 10000-10009

Hein P, Frank M, Hoffmann C, Lohse MJ, Bünemann M (2005) Dynamics of receptor/G protein coupling in living cells. *EMBO J*. **24**: 4106-4114

Hein P, Rochais F, Hoffmann C, Dorsch S, Nikolaev VO, Engelhardt S, Berlot CH, Lohse MJ, Bünemann M (2006) Gs Activation is time-limiting in initiating receptor-mediated signaling. *J Biol Chem*. **281**: 33345-33351

Henis YI, Hekman M, Elson EL, Helmreich EJM (1982) Lateral motion of ß receptors in membranes of cultured liver cells. *Proc. Natl. Acad. Sci. USA* **79**: 2907-2911

Hern JA, Baig AH, Mashanov GI, Birdsall B, Corrie JE, Lazareno S, Molloy JE, Birdsall NJ (2010) Formation and dissociation of M_1 muscarinic receptor dimers seen by total internal reflection fluorescence imaging of single molecules. *Proc Natl Acad Sci USA* **107**: 2693-2698

Hlavackova V, Zabel U, Frankova D, Bätz J, Hoffmann C, Prezeau L, Pin J-P, Blahos J, Lohse MJ (2012) Sequential inter- and intra-subunit rearrangements during asymmetric activation of dimeric metabotropic glutamate receptor 1. *Science Sign*. **5**: ra59

Hoffmann C, Nuber S, Zabel U, Ziegler N, Winkler C, Hein P, Berlot CH, Bünemann M, Lohse MJ (2012) Comparison of the activation kinetics of the M3-ACh-receptor and a constitutively active mutant receptor in living cells. *Mol Pharmacol*. **82**: 236-245

Hofmann KP, Spahn CM, Heinrich R, Heinemann U (2006) Building functional modules from molecular interactions. *Trends Biochem Sci*. **31**: 497-508

Hofmann KP, Scheerer P, Hildebrand PW, Choe HW, Park JH, Heck M, Ernst OP (2009) A G protein-coupled receptor at work: the rhodopsin model. *Trends Biochem Sci* **34**: 540-552

Jensen JB, Lyssand JS, Hague C, Hille B (2009) Fluorescence changes reveal kinetic steps of muscarinic receptor-mediated modulation of phosphoinositides and Kv7.2/7.3 K^+ channels. *J Gen Physiol* **133**: 347-359

Kar P, Parekh AB (2015) Distinct spatial Ca^{2+} signatures selectively activate different NFAT transcription factor isoforms. Mol Cell. **58**: 232-243

Kasai RS, Suzuki KG, Prossnitz ER, Koyama-Honda I, Nakada C, Fujiwara TK, Kusumi A (2011) Full characterization of GPCR monomer-dimer dynamic equilibrium by single molecule imaging. *J Cell Biol*. **192**: 463-480

Kim YJ, Hofmann KP, Ernst OP, Scheerer P, Choe HW, Sommer ME (2013) Crystal structure of pre-activated arrestin p44. Nature **497**: 142-146

Knierim B, Hofmann KP, Ernst OP, Hubbell WL (2007) Sequence of late molecular events in the activation of rhodopsin. Proc Natl Acad Sci USA **104**:20290-20295

Kotowski SJ, Hopf FW, Seif T, Bonci A, von Zastrow M (2011) Endocytosis promotes rapid dopaminergic signaling. *Neuron* **71**: 278-290

Krasel C, Bünemann M, Lorenz K, Lohse MJ (2005) β-Arrestin binding to the β_2-adrenergic receptor requires both receptor phosphorylation and receptor activation. *J Biol Chem*. **280**: 9528-9535

Levitz J, Pantoja C, Gaub B, Janovjak H, Reiner A, Hoagland A, Schoppik D, Kane B, Stawski P, Schier AF, Trauner D, Isacoff EY (2013) Optical control of metabotropic glutamate receptors. *Nat Neurosci*. 16: 507-516

Lohse MJ, Calebiro D (2013) Receptor signals come in waves. *Nature* **495**: 457-458

Lohse MJ, Hoffmann C (2014) Interaction between arrestins and receptors. *Handb. Exp. Pharmacol*. **219**: 15-56.

Lohse MJ, Nikolaev VO, Hein P, Hoffmann C, Vilardaga JP, Bünemann M (2008) Optical techniques to analyze real-time activation and signaling of G-protein-coupled receptors. Trends Pharmacol. Sci. **29**: 159-165

Lohse MJ, Nuber S, Hoffmann C (2012) Fluorescence/bioluminescence resonance energy transfer techniques to study G-protein-coupled receptor activation and signaling. *Pharm Rev*. **64**: 299–336

Mahalingam M, Martínez-Mayorga K, Brown MF, Vogel R (2008) Two protonation switches control rhodopsin activation in membranes. *Proc Natl Acad Sci USA*. **105**: 17795-800

Manglik A, Kim TH, Masureel M, Altenbach C, Yang Z, Hilger D, Lerch MT, Kobilka TS , Thian FS, Hubbell WL, Prosser RS, Kobilka BK (2015) Structural insights into the dynamic process of β_2-adrenergic receptor signaling. *Cell* (in press)

Marcaggi P, Mutoh H, Dimitrov D, Beato M, Knöpfel T (2009) Optical measurement of mGluR1 conformational changes reveals fast activation, slow deactivation, and sensitization. *Proc Natl Acad Sci USA* **106**: 11388-11393

Mika D, Leroy J, Vandecasteele G, Fischmeister R (2012) PDEs create local domains of cAMP signaling. *J Mol Cell Cardiol*. **52**: 323-329

Nikolaev VO, Bünemann M, Hein L, Hannawacker A, Lohse MJ (2004) Novel single chain cAMP sensors for receptor-induced signal propagation. *J Biol Chem*. **279**: 37215-37218.

Nikolaev VO, Moshkov A, Lyon AR, Miragoli M, Novak P, Paur H, Lohse MJ, Korchev YE, Harding SE, Gorelik J (2010) β2-Adrenergic receptor redistribution in heart failure changes cAMP compartmentation. *Science* **327**: 1653-1657

Nuttle LC, Farley JM (1996) Frequency modulation of acetylcholine-induced oscillations in Ca^{++} and Ca^{++}-activated Cl^{-} current by cAMP in tracheal smooth muscle. *J Pharmacol Exp Ther*. **277**: 753-760

Nygaard R, Zou Y, Dror RO, Mildorf TJ, Arlow DH, Manglik A, Pan AC, Liu CW, Fung JJ, Bokoch MP, Thian FS, Kobilka TS, Shaw DE, Mueller L, Prosser RS, Kobilka BK (2013) The dynamic process of β_2-adrenergic receptor activation. *Cell* **152**: 532-542

Olofsson L, Felekyan S, Doumazane E, Scholler P, Fabre L, Zwier JM, Rondard P, Seidel CA, Pin JP, Margeat E (2014) Fine tuning of sub-millisecond conformational dynamics controls metabotropic glutamate receptors agonist efficacy. *Nat Commun*. **5**: 5206.

Pals-Rylaarsdam R, Gurevich VV, Lee KB, Ptasienski JA, Benovic JL, Hosey MM (1997) Internalization of the m2 muscarinic acetylcholine receptor. Arrestin-independent and -dependent pathways. *J Biol Chem*. **272**: 23682-23689

Parekh AB (2011) Decoding cytosolic Ca^{2+} oscillations. *Trends Biochem Sci*. **36**: 78-87

Rich TC, Webb KJ, Leavesley SJ (2014) Can we decipher the information content contained within cyclic nucleotide signals? *J Gen Physiol*. **143**: 17-27

Rose AS, Elgeti M, Zachariae U, Grubmüller H, Hofmann KP, Scheerer P, Hildebrand PW (2014) Position of transmembrane helix 6 determines receptor G protein coupling specificity. *J Am Chem Soc*. **136**: 11244-11247

Scheerer P, Park JH, Hildebrand PW, Kim YJ, Krauss N, Choe HW, Hofmann KP, Ernst OP (2008) Crystal structure of opsin in its G-protein-interacting conformation. *Nature* **455**: 497-502

Scheerer P, Heck M, Goede A, Park JH, Choe HW, Ernst OP, Hofmann KP, Hildebrand PW (2009) Structural and kinetic modeling of an activating helix switch in the rhodopsin-transducin interface. *Proc Natl Acad Sci USA*. **106**: 10660-10665

Schöneberg J, Heck M, Hofmann KP, Noé F (2014) Explicit spatiotemporal simulation of receptor-G protein coupling in rod cell disk membranes. *Biophys J*. **107**: 1042-1053

Shenoy SK, Lefkowitz RJ (2011) β-Arrestin-mediated receptor trafficking and signal transduction. *Trends Pharmacol Sci* **32**: 521-533

Shukla AK, Manglik A, Kruse AC, Xiao K, Reis RI, Tseng WC, Staus DP, Hilger D, Uysal S, Huang LY, Paduch M, Tripathi-Shukla P, Koide A, Koide S, Weis WI, Kossiakoff AA, Kobilka BK, Lefkowitz RJ (2013) Structure of active β-arrestin-1 bound to a G-protein-coupled receptor phosphopeptide. *Nature* **497**:137-141

Sommer ME, Elgeti M, Hildebrand PW, Szczepek M, Hofmann KP, Scheerer P (2015) Structure-based biophysical analysis of the interaction of rhodopsin with G protein and arrestin. *Methods Enzymol*. **556**:563-608

Sprenger JU, Perera RK, Steinbrecher JH, Lehnart SE, Maier LS, Hasenfuss G, Nikolaev VO (2015) In vivo model with targeted cAMP biosensor reveals changes in receptor-microdomain communication in cardiac disease. *Nat Commun*. **6**: 6965

Suzuki K, Ritchie K, Kajikawa E, Fujiwara T, Kusumi A (2005) Rapid hop diffusion of a G-protein-coupled receptor in the plasma membrane as revealed by single-molecule techniques. *Biophys J*. **88**: 3659–3680

Szczepek M, Beyrière F, Hofmann KP, Elgeti M, Kazmin R, Rose A, Bartl FJ, von Stetten D, Heck M, Sommer ME, Hildebrand PW, Scheerer P (2014) Crystal structure of a common GPCR-binding interface for G protein and arrestin. *Nat Commun*. **5**: 4801

Tian X, Kang DS, Benovic JL (2014) β-arrestins and G protein-coupled receptor trafficking. *Handb Exp Pharmacol*. **219**: 173-186

Tsvetanova NG, Irannejad R, von Zastrow M (2015) G protein-coupled receptor (GPCR) signaling via heterotrimeric G proteins from endosomes. *J Biol Chem*. **290**: 6689-6696

Tsien RW, Tsien RY (1990) Calcium channels, stores, and oscillations. Annu Rev Cell Biol. **6**: 715-760

Vilardaga JP, Bünemann M, Krasel C, Castro M, Lohse MJ (2003) Measurement of the millisecond activation switch of G-protein-coupled receptors in living cells. *Nature Biotechnology* **21**: 807-812.

Vilardaga JP, Jean-Alphonse FG, Gardella TJ (2014) Endosomal generation of cAMP in GPCR signaling. *Nat Chem Biol*. **10**: 700-706

Violin JD, Zhang J, Tsien RY, Newton AC (2003) A genetically encoded fluorescent reporter reveals oscillatory phosphorylation by protein kinase C. *J. Cell Biol*. **161**: 899–909

van Koppen CJ, Jakobs KH (2004) Arrestin-independent internalization of G protein-coupled receptors. *Mol Pharmacol*. **66**: 365-367.

Von Hayn K, Werthmann RC, Nikolaev VO, Hommers LG, Lohse MJ, Bünemann M (2010) G_q-mediated Ca^{2+} signals inhibit adenylyl cyclases 5/6 in vascular smooth muscle cells. *Am J Physiol Cell Physiol*. **298**: C324-C332

Wachten S, Masada N, Ayling LJ, Ciruela A, Nikolaev VO, Lohse MJ, Cooper DM (2010) Distinct pools of cAMP centre on different isoforms of adenylyl cyclase in pituitary-derived GH3B6 cells. *J Cell Sci*. **123**: 95-106

Ward RJ, Pediani JD, Godin AG, Milligan G (2015) Regulation of oligomeric organization of the serotonin 5-hydroxytryptamine 2C ($5\text{-}HT_{2C}$) receptor observed by spatial intensity distribution analysis. *J Biol Chem*. **290**: 12844-12857

Willoughby D, Cooper DM (2007) Organization and Ca^{2+} regulation of adenylyl cyclases in cAMP microdomains. *Physiol Rev*. **87**: 965-1010

Zaccolo M, Pozzan T (2003) cAMP and Ca^{2+} interplay: a matter of oscillation patterns. *Trends Neurosci*. **26**: 53-55

Zhang J, Ferguson SS, Barak LS, Ménard L, Caron MG (1996) Dynamin and b-arrestin reveal distinct mechanisms for G protein-coupled receptor internalization. *J Biol Chem*. **271**: 18302-18305

Zürn A, Zabel U, Vilardaga JP, Schindelin H, Lohse MJ, Hoffmann C (2009) Fluorescence resonance energy transfer analysis of α_{2A}-adrenergic receptor activation reveals distinct agonist-specific conformational changes. *Mol Pharmacol*. **75**: 534-541