



Published in final edited form as:

*Trends Pharmacol Sci.* 2018 February ; 39(2): 200–208. doi:10.1016/j.tips.2017.11.009.

## Subcellular organization of GPCR signaling

Kelsie Eichel<sup>1</sup> and Mark von Zastrow<sup>2,3</sup>

<sup>1</sup>Program in Biochemistry and Molecular Biology, University of California, San Francisco School of Medicine, San Francisco CA 94158, USA

<sup>2</sup>Department of Psychiatry, University of California, San Francisco School of Medicine, San Francisco CA 94158, USA

<sup>3</sup>Department of Cellular and Molecular Pharmacology, University of California, San Francisco School of Medicine, San Francisco CA 94158, USA

### Abstract

G protein-coupled receptors (GPCRs) comprise a large and diverse class of signal-transducing receptors that undergo dynamic and isoform-specific membrane trafficking. GPCRs thus have an inherent potential to initiate or regulate signaling reactions from multiple membrane locations. The present review discusses emerging insight to the subcellular organization of GPCR function in mammalian cells, focusing on signaling transduced by heterotrimeric G proteins and  $\beta$ -arrestins. We summarize recent evidence indicating that GPCR-mediated activation of G proteins occurs not only from the plasma membrane but also from endosomes and Golgi membranes, and that  $\beta$ -arrestin-dependent signaling can be transduced from the plasma membrane by  $\beta$ -arrestin trafficking to clathrin-coated pits after dissociating from a ligand-activated GPCR.

### Keywords

Signaling; GPCR; G protein; arrestin; endosome; Golgi

### A spatial dimension of cellular GPCR signaling

GPCRs mediate cellular signaling by stimulus-triggered coupling to cytoplasmic transducer proteins. Previously, the subcellular location of ligand-dependent GPCR signaling was of limited interest because it was assumed that activation by ligand is restricted to the plasma membrane (PM). Recently, additional sites of ligand-induced activation have been recognized, and interest has grown in the functional consequences of localized activation. Progress in this area has been facilitated by improved understanding of GPCR trafficking

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Correspondence: mark.vonzastrow@ucsf.edu.

#### Conflict of Interest

The authors declare no conflict of interest.

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mechanisms and by the development of new approaches for achieving direct detection and spatiotemporal resolution of signaling reactions in living cells (e.g., [1–4]).

The present review focuses on spatial aspects of GPCR signal initiation through interactions with heterotrimeric G proteins and arrestins in mammalian cells. We first briefly outline early concepts regarding the location of GPCR-mediated signal initiation. We then review accumulating recent evidence supporting the current concept that ligand-dependent GPCRs activate G proteins and arrestin transducers at both PM and internal membrane locations. Finally, we discuss the evolving functional understanding of spatially organized GPCR signaling.

## GPCR-mediated activation of heterotrimeric G proteins

GPCRs initiate canonical (G protein-dependent) signaling by stimulus-dependent rearrangements of the seven-transmembrane helical bundle that are allosterically coupled to a reduced affinity of guanine nucleotide binding to the Ras-like  $\alpha$  subunit of the heterotrimeric G protein complex. This promotes release of bound GDP from the inactive  $G\alpha$  subunit and its replacement with GTP present in excess in the cytoplasm. GTP binding, in turn, stabilizes an activating conformational change in the  $G\alpha$  subunit that, depending on the G protein, promotes rearrangement or dissociation of the  $\beta\gamma$  subcomplex [5–8]. Both  $G\alpha$  and  $G\beta\gamma$  regulate downstream effectors, with activity terminated by hydrolysis of GTP to GDP on  $G\alpha$  that promotes reformation of the inactive complex [9]. Accordingly, GPCRs trigger G protein signaling catalytically through an intrinsic ligand-dependent guanine nucleotide exchange factor (GEF) activity [10].

Recent studies make clear that considerably more remains to be learned about the molecular dynamics of this fundamental activation mechanism and its operation in living cells. Single molecule measurements of Förster resonance energy transfer between beta-2 adrenergic receptors ( $\beta$ 2ARs) and the  $G_s$  heterotrimeric G protein *in vitro* revealed evidence for multiple conformational states depending on the bound ligand and identified a transient nucleotide-bound  $\beta$ 2AR- $G_s$  species that is distinct from presently known structures [11]. A recent study of single molecule trajectories in the PM of living cells revealed hot spots at which both the  $\beta$ 2AR and  $G_s$  are transiently immobilized and proposed to couple [12].

## Role of arrestins in terminating G protein activation

Many GPCR responses transduced by G proteins adapt over time with prolonged or repeated ligand exposure, exhibiting the phenomenon of desensitization. A well established mechanism of rapid desensitization was first recognized through studies of inactivation of the light-activated GPCR, rhodopsin, in retinal photoreceptor cells. Visual arrestin was shown to bind specifically to rhodopsin after its light-induced conformational activation and phosphorylation by rhodopsin kinase (GRK1). This interaction was found to produce a high-affinity ( $K_d \sim 50$  nM) GPCR-arrestin complex that terminates signaling by physical ‘arrest’ of the nucleotide exchange factor activity of the GPCR [13], [14].

Visual arrestin was then found to also inhibit the GEF activity of the  $\beta$ 2AR *in vitro*. Maximal inhibition required ligand-induced phosphorylation of the  $\beta$ 2AR by an enzyme that

is homologous to rhodopsin kinase and expressed outside of photoreceptor cells (beta-adrenergic receptor kinase or GRK2). However, a relatively high concentration of visual arrestin was required to achieve inhibition of  $\beta$ 2AR activity [15] and an arrestin homologue with higher apparent affinity for adrenergic receptors, called beta-arrestin ( $\beta$ -arrestin), was subsequently identified [16,17]. This mechanism for inactivating ligand-dependent GEF activity using GRKs and arrestins has proven to be relevant to many GPCRs, with two major  $\beta$ -arrestin isoforms ( $\beta$ -arrestin-1 and -2, also called Arrestin 2 and 3) and multiple GRK isoforms identified that are broadly expressed in mammalian tissues [17].

### Additional trafficking and signaling functions of $\beta$ -arrestins

In addition to terminating GEF activity by binding to GPCRs,  $\beta$ -arrestins were shown to function as endocytic adapters by interacting with phosphoinositides, clathrin, and AP-2 [18–22]. Through these interactions,  $\beta$ -arrestins promote the ligand-dependent concentration of GPCRs into clathrin-coated pits (CCPs) that form in the PM and pinch off by a dynamin-dependent membrane scission mechanism to produce receptor-containing endocytic vesicles [23–25].

Still more diversity of  $\beta$ -arrestin function was recognized by the discovery that  $\beta$ -arrestins can bind to over 20 different protein kinases [26–29]. These interactions are thought to support ‘non-canonical’ (G protein-independent) GPCR signaling by molecular scaffolding. In particular,  $\beta$ -arrestins have been shown to promote signaling by mitogen-activated protein kinases (MAPKs), serine/threonine kinases including ERK1/2, p38 kinases, and the c-Jun N-terminal kinases that are activated through phosphorylation mediated by an upstream protein kinase [26].

### $\beta$ -arrestin signaling from endosomes

Early evidence suggesting that ligand-dependent GPCR signaling can also occur from an internal membrane location emerged from investigations of the signal transducer function of  $\beta$ -arrestin. A correlation was noted between endocytosis and  $\beta$ -arrestin-dependent ERK activation triggered by ligand-dependent activation of several GPCRs [30,31], and dominant-negative versions of dynamin and  $\beta$ -arrestin-1 that inhibit  $\beta$ 2AR internalization were shown to also inhibit ligand-dependent activation of ERK [32]. More support for activation at endosomes came from the demonstration that c-SRC recruitment to  $\beta$ 2AR through binding to  $\beta$ -arrestin can promote ERK activation, and that ERK activation by the  $\beta$ 2AR can be blocked by over-expression of a mutant version of  $\beta$ -arrestin-1 (V53D) that binds c-SRC but does not promote  $\beta$ 2AR internalization [29]. Protease-activated receptor 2 was shown to form a complex after activation that includes  $\beta$ -arrestin-1, Raf-1, and phosphorylated ERK [33]. Ligand-activated AT<sub>1A</sub>Rs were found to form a complex including  $\beta$ -arrestin-2, Raf-1, MEK1, and ERK1/2 [34]. Moreover, ERK and  $\beta$ -arrestins were found to colocalize on endocytic vesicles together with several GPCRs [34,35]. Together, such findings led to the concept that endosomes act as signalosomes specific to  $\beta$ -arrestin [36].

Endosomal activation of  $\beta$ -arrestin signaling is also consistent with kinetic data. A G protein-dependent component of ERK activation mediated by AT<sub>1A</sub>Rs was found to peak ~2

minutes after the application of agonist ligand, corresponding to a time at which most receptors are still present in the PM. A  $\beta$ -arrestin-dependent component of the response became evident after 10 – 30 minutes, in line with the time required for AT<sub>1A</sub>Rs to internalize [37]. Together, these observations solidified the view that GPCR-mediated activation of G proteins is restricted to the PM and that signaling transduced by  $\beta$ -arrestin requires GPCR- $\beta$ -arrestin complex formation and trafficking of the complex to endosomes [38].

## G protein signaling from internal membrane compartments

Rhodopsin activates transducin primarily from internal membrane compartments in vertebrate photoreceptor cells [39] but intracellular G protein activation by ligand-dependent GPCRs was doubted for many years due to a number of assumptions, as discussed previously [40]. This view began to change with the work of several groups [41–47]. Briefly summarized, these studies provided (1) colocalization and co-fractionation data indicating that G proteins and adenylyl cyclase are present in internal membrane compartments containing ligand-internalized GPCRs and (2) inhibitor data demonstrating that acute endocytic blockade attenuates some G protein-dependent cellular responses.

The development of conformational biosensors, a class of genetically encoded fluorescent detectors of specific GPCR and G protein conformational states, allowed the subcellular location of  $\beta$ 2AR and Gs activation to be resolved dynamically in living cells. Application of agonist ligand produced conformational activation of both proteins at two locations sequentially, first the PM and then endosomes [48–50]. Some GPCRs, such as the  $\beta$ 2AR and D1 dopamine receptor, produce transient Gs activation at endosomes [41,48]. Other GPCRs, such as the PTH and V2 vasopressin receptors, produce more prolonged activation at endosomes and appear to do so by forming an alternate activation complex containing the G protein together with  $\beta$ -arrestin [51,52].

Conformational biosensors subsequently resolved the Golgi apparatus and trans-Golgi network as additional locations of ligand-dependent GPCR and Gs activation. Two cellular mechanisms for Golgi-associated signal activation have been proposed. Activation of beta-1 adrenergic receptors ( $\beta$ 1ARs) and Gs in the Golgi apparatus uses a preexisting receptor pool rather than receptors delivered from the cell surface and requires ligands to access the Golgi-localized receptor pool separately. Catecholamines gain access the Golgi-localized  $\beta$ 1AR pool by facilitated transmembrane transport involving the organic cation transporter 3 whereas more hydrophobic drugs appear to access the Golgi pool by passive diffusion [53]. Activation of Gs by TSH receptors in the trans-Golgi network utilizes receptors internalized from the PM together with ligand, similar to activation of Gs at endosomes by other GPCRs such as the  $\beta$ 2AR [54].

## $\beta$ -arrestin signaling from the PM

As noted above, there is considerable evidence that  $\beta$ -arrestin-dependent signaling can be initiated from endosomes. The possibility that  $\beta$ -arrestin can signal also from the PM was first suggested by a study demonstrating that engineered chemical recruitment of  $\beta$ -arrestin

to the PM is sufficient to activate ERK in the absence of activated receptors [55]. It remained unknown for some time if  $\beta$ -arrestin signaling from the PM is triggered by ligand-induced activation of a GPCR. Suggesting that this might be the case, several studies showed that endocytic blockade imposed by chemical inhibition of dynamin enhances rather than inhibits  $\beta$ -arrestin-dependent activation of ERK by some GPCRs [56–58].

Live cell fluorescence microscopy provided insight into this initially puzzling result by revealing that chemical inhibition of dynamin stalls CCPs in the PM while leaving  $\beta$ -arrestin associated with them. Further, it was found that blockade of endocytosis by RNAi-mediated depletion of clathrin heavy chain – a distinct manipulation that prevents CCP formation altogether rather than stalling them in the PM – inhibits rather than enhances the MAPK response [57]. An additional property of GPCRs that promote  $\beta$ -arrestin-dependent ERK signaling from the PM is that their activation can prolong the lifetime of arrestin-associated CCPs at the cell surface before dissociation of the coat structure that occurs shortly after endocytic vesicle formation. Interestingly, this natural prolongation of CCP lifetime – produced by receptor activation rather than by chemical inhibition of dynamin – was similarly correlated with enhancement of the  $\beta$ -arrestin-dependent MAPK response. Together these observations revealed a discrete cellular mechanism of  $\beta$ -arrestin-dependent activation of MAPK that is initiated at the PM and uses CCPs as dynamically regulated PM signaling stations.

### A discrete cellular mechanism of $\beta$ -arrestin activation

Additional insight to  $\beta$ -arrestin activation at the PM emerged from efforts to resolve another biological conundrum. The  $\beta$ 1AR was long recognized to promote  $\beta$ -arrestin signaling *in vivo* [59] but this GPCR, in contrast to the  $\beta$ 2AR, typically internalizes poorly via CCPs. Consistent with this, live cell imaging demonstrated that  $\beta$ 1ARs do not detectably accumulate in CCPs after ligand-induced activation. Nevertheless,  $\beta$ 1AR activation was found to robustly promote the accumulation of  $\beta$ -arrestin in CCPs apparently without an associated receptor. Further,  $\beta$ 1ARs were able to trigger ligand-dependent trafficking of  $\beta$ -arrestin to CCPs even under conditions in which the activating GPCR was laterally immobilized to assure its inability to move into CCPs [57]. These observations indicate that  $\beta$ -arrestin can operate as an autonomous transducer of GPCR activation, after dissociating from its upstream activating GPCR and trafficking to CCPs, and suggest that GPCRs can trigger  $\beta$ -arrestin signaling in a catalytic manner that does not require the GPCR and  $\beta$ -arrestin to remain stably bound.

In parallel with this emerging cell biological understanding, a confluence of recent structural and biophysical observations support additional flexibility of cellular  $\beta$ -arrestin function. Single particle electron microscopy has described two GPCR-arrestin complexes, a canonical complex in which both the GPCR tail and transmembrane core are engaged and another in which only the GPCR tail is engaged [60]. These discrete complexes may have functional relevance because mutations that selectively destabilize the GPCR transmembrane core interaction prevent functional desensitization of G protein signaling while maintaining receptor internalization and arrestin-dependent signaling [61–63]. Experiments using Förster resonance energy transfer have provided independent support for

the hypothesis that  $\beta$ -arrestin can remain in an activated conformation after dissociation from a GPCR [64], and suggest that more than one conformational state of  $\beta$ -arrestin can be generated in living cells depending on the GPCR [65].

## Concluding remarks and future directions

It is increasingly clear that ligand-dependent GPCRs can functionally couple to G protein and  $\beta$ -arrestin transducers at multiple subcellular membrane locations (summarized schematically in Figure 1). The PM, long recognized to be a major location of G protein activation, is also a site of  $\beta$ -arrestin-dependent signaling using CCPs. Endosomes, once thought to function only as a repository or sorting station for inactive or desensitized GPCRs, can also support G protein as well as  $\beta$ -arrestin-mediated signal initiation. In addition, some GPCRs mediate G protein activation at Golgi-associated membrane compartments. Together, these developments support a model in which GPCR signal initiation in mammalian cells is spatially and temporally organized across multiple membrane locations. This represents a significant change from the previous view and raises many new questions that remain to be investigated (see Outstanding Questions Box). Several of these are further elaborated below, placed in context of presently available data.

### Outstanding questions box

- How is signaling from internal membranes regulated and terminated?
- What are the functional consequences of localized G protein and  $\beta$ -arrestin activation?
- How much diversity and specificity exists in localized GPCR signaling?
- What is the spatiotemporal organization of cellular GPCR signaling in native tissues?

One important direction for future study is to determine how G protein and  $\beta$ -arrestin activities initiated from distinct membrane locations are terminated. As a general principle, cellular GPCR signaling is exquisitely regulated. Molecular mechanisms that terminate G protein signaling from the PM are now well understood, but much less is known about the control of G protein signal initiation from internal membranes. Endosome signaling may be terminated by acidification, receptor engagement of arrestins or arrestin-related proteins, or engagement of multi-protein regulatory complexes associated with the endosome membrane. In particular, an actin and sorting nexin 27-linked version of the conserved retromer complex (ASRT) is interesting because it contains an arrestin-related protein (Vps26) and exerts receptor-specific control of the strength of endosomal G protein activation by determining the residence time of activated GPCRs in the endosome membrane [45,66–68]. GPCR - G protein activation at the Golgi apparatus is acutely inhibited by corticosterone through control of facilitated ligand transport activity [53], but clearly much more remains to be learned about termination of signaling from Golgi-associated membranes. Endosome signaling by  $\beta$ -arrestin depends on stability of the GPCR- $\beta$ -arrestin complex and signaling from the PM is limited by the surface lifetime of arrestin-associated CCPs [57], but clearly

much more remains to be learned also about the regulation and termination of arrestin-dependent signaling.

A second proposed future direction is to delineate the functional significance of location-based GPCR signal initiation. Endosome-initiated signals are delayed in onset and often more prolonged than signals initiated from the PM. Accordingly, endocytosis inherently affects the timing of cellular signal initiation as well as its location. In some cases location appears to be a primary determinant of downstream signaling selectivity. For example, location of  $\beta$ 2AR-Gs activation in endosomes has been explicitly shown to be important for promoting cAMP-mediated transcriptional responses [69]. In other cases the primacy of one dimension over another is less clear, and it appears likely that both dimensions are salient. For example, TSH receptors drive a PKA-dependent phosphorylation of CREB and gene transcription in response to TSH that is delayed relative to PKA activation at the PM and occurs during, or upon, receptor relocalization from endosomes to the trans-Golgi network [54]. Endosomal activation of LH receptors in ovarian follicle cells sustains the cellular cAMP response elicited by the mid-cycle LH surge and also positions the site of cAMP generation near gap junctions; both effects are thought to be important for lifting meiotic arrest in the nucleus of the enclosed oocyte to drive oocyte maturation [70]. Clearly there is a great deal more to be learned about how spatial and temporal dimensions of GPCR-G protein signal initiation are interpreted functionally in living cells.

It is presently unclear if similar or different considerations apply to location-dependent signaling by  $\beta$ -arrestins.  $\beta$ -arrestin activation in endosomes is typically delayed in onset compared to activation in the PM, similar to what has been observed for endosomal G protein activation. However,  $\beta$ -arrestin-dependent activation of ERK from endosomes appears to restrict the activated kinase to the cytoplasm and prevent signaling to the nucleus [33,71,72]. This is opposite to endosomal Gs activation, which promotes nuclear responses. Thus, while endocytosis appears to affect the location and timing of  $\beta$ -arrestin activation in a similar manner as G protein activation, there may be significant differences in how location is interpreted to modify cellular function (Figure 2).

A third future direction is to determine how broadly the presently recognized principles apply across the large GPCR family. Even closely related GPCR subtypes, such as the  $\beta$ 1AR and  $\beta$ 2AR, are already known to differ in membrane trafficking properties that impact signaling. This is likely only the beginning of diversity and specificity in the GPCR family. For example, LH receptors internalize and recycle in a generally similar manner as the  $\beta$ 2AR but they recycle through a different population of endosomes that have been shown to change both the location and timing of downstream ERK activation [73].  $\beta$ 2AR-mediated activation of Gs in endosomes is terminated by receptor engagement with retromer [68], similar to termination of endosomal PTH receptor signaling [45], but retromer promotes rather than inhibits TSH receptor-mediated signaling transduced by Gs from the trans-Golgi network [54].

We also note that most of what is presently known about G protein activation from internal membranes is limited to activation of Gs. Other G protein isoforms have been observed at internal membranes but whether they undergo intracellular activation by GPCRs remains a

largely open question. Much also remains to be learned about diversity of  $\beta$ -arrestin engagement by GPCRs and its functional significance, particularly with accumulating evidence that the subcellular location of  $\beta$ -arrestin-dependent signal initiation can differ even among closely related GPCRs such as the CB1 and CB2 cannabinoid receptors [74].

A fourth proposed direction is to elucidate the spatial organization of GPCR signaling in a native cellular environment and at endogenous protein expression levels. Our present understanding is derived largely from model cell systems in which at least one component of the cascade is overexpressed. Live cell imaging of fluorescently tagged proteins, although a powerful tool for direct examination of subcellular location, has oftentimes required overexpression of components due to technical limitations in gene transfer methodologies or detection sensitivity. Improved genetic tools and *in situ* fluorescent labeling methodologies are now being used to overcome this barrier. For example, a recent study using CRISPR-based gene editing and an improved version of split GFP was used to achieve real-time imaging of cellular proteins at endogenous levels in living cells, including several that function in GPCR signaling [75]. Laminated optical sheet microscopy was used to visualize the presence of endogenous TSH receptors in the Golgi region of primary mouse thyroid cells and activation of endogenous Gs at this location [54]. It was also previously shown that internal TSH receptor activation after endocytosis is evident in some cell types but not others [46]. Improved transgenic animal models, such as a cAMP biosensor-expressing mouse that has been used successfully to study endosomal signaling in thyroid cells as well as intact ovarian follicles [70], offer the potential to examine location-based signaling by GPCRs in a near-native tissue environment. A recent study that applied Talen and CRISPR -based gene editing to investigate  $\beta$ 2AR-mediated ERK activation in an established cellular model system [76] indicates that such methods are also able to provide new mechanistic insight.

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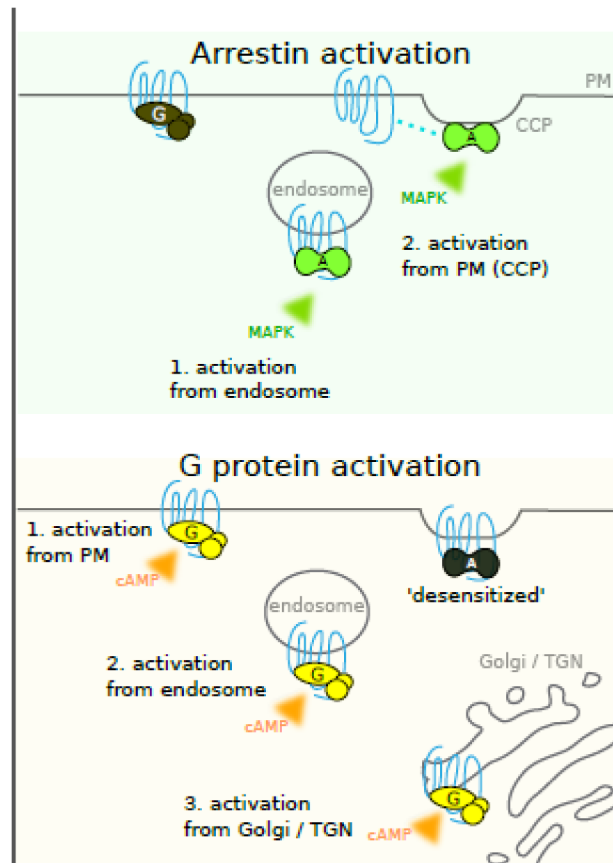
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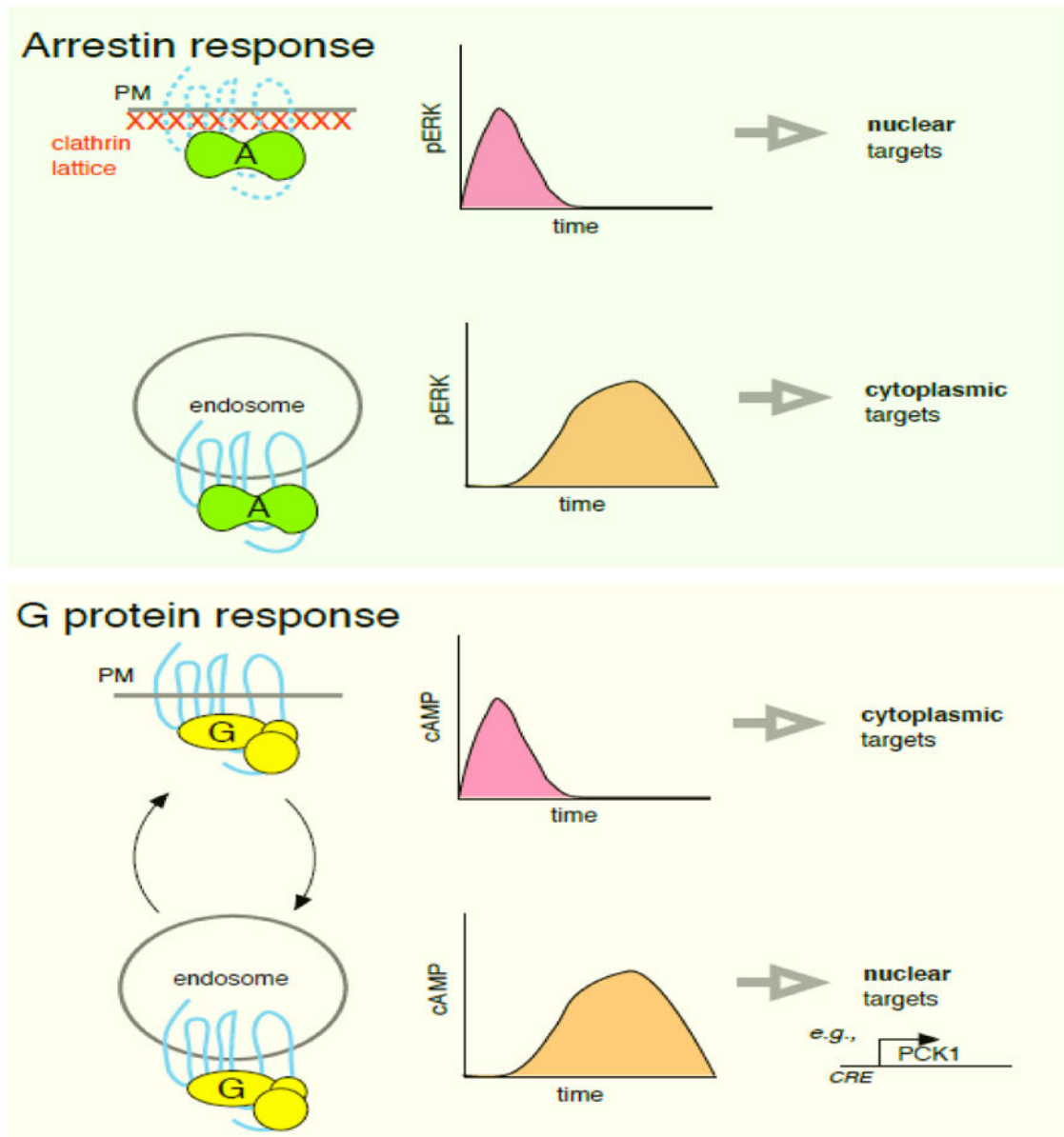
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**Trends box**

- active signal initiation by GPCRs is not restricted to the plasma membrane
- GPCR-mediated activation of Gs can occur in endosomes and the Golgi apparatus
- $\beta$ -arrestin-mediated activation of MAP kinase can occur from the plasma membrane
- $\beta$ -arrestin can mediate signaling after dissociating from its activating GPCR
- clathrin-coated pits function as  $\beta$ -arrestin signaling stations in the plasma membrane



**Figure 1.** Schematic summarizing subcellular locations of GPCR-mediated activation of arrestins and heterotrimeric G proteins supported by data discussed in the text. *Top panel:* Arrestin ( $\beta$ -arrestins -1 and -2) are thought to be subject to activation both in endosomes and the PM. *Bottom panel:* Gs activation by various GPCRs (see text for details) has been described in the PM, endosomes, Golgi apparatus and *trans*-Golgi network.



**Figure 2.** Schematic contrasting current view of functional impact of localized signaling transduced by  $\beta$ -arrestins and Gs. *Top panel:* GPCR-mediated activation of  $\beta$ -arrestins in the PM is thought to produce a rapid ERK response that can access nuclear targets. Activation in endosomes produces delayed ERK activation and sequesters ERK in the cytoplasm, thereby preventing access to nuclear targets. *Bottom panel:* GPCR-mediated activation of Gs in the PM produces a rapid and transient response that activates cAMP-dependent targets in the PM and cytoplasm. Activation in endosomes produces a delayed response that preferentially accesses nuclear targets (induction of the cAMP-responsive gene PCK1 is shown as an example. For GPCRs that are capable of efficient recycling, PM and endosome-initiated

activation waves can occur repeatedly in the prolonged presence of agonist through multiple rounds of endocytosis and recycling (depicted as curved arrows).

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