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REVIEW

Intersection of GPCR trafficking and cAMP signaling at endomembranes

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GPCRs comprise the largest family of signaling receptors and control essentially every physiological process. Many biochemical reactions underlying GPCR signaling are now elucidated to atomic resolution in cell-free preparations, but how elemental signaling reactions are organized in intact cells remains less clear. Significant progress has been made toward bridging this knowledge gap by leveraging new tools and methodologies enabling the experimental detection, localization, and manipulation of defined signaling reactions in living cells. Here, we chronicle advances at this rapidly moving frontier of molecular and cell biology, focusing on GPCR-initiated signaling through the classical cAMP pathway as an example. We begin with a brief review of established concepts. We then discuss the still-evolving understanding that ligand-induced GPCR signaling occurs from endomembranes as well as the plasmalemma, and that this enables cells to flexibly sculpt downstream signaling responses in both space and time. Finally, we note some key limitations of the present understanding and propose some promising directions for future investigation.

Introduction

G protein–coupled receptors (GPCRs) are integral membrane proteins that sense biologically salient cues and trigger appropriate responses through stimulus-dependent, allosteric interactions with intracellular transducer proteins (Hilger et al., 2018; Liu et al., 2024). Critical transducers of GPCR signaling are a family of heterotrimeric guanine nucleotide-binding proteins (G proteins). G proteins are activated by a "coupling" reaction in which the activated GPCR transiently binds the inactive G protein and promotes guanine nucleotide exchange on its α-subunit, replacing the bound GDP with GTP. GTP binding drives dissociation of the heterotrimer into an active, GTP-bound α-subunit and "free" βγ heterodimeric subcomplex, each regulating specific enzymes and ion channels that constitute the downstream effector network of cellular GPCR signaling. G protein activity is terminated by GTP hydrolysis on the α-subunit that drives reassembly of an inactive, GDP-bound heterotrimer (Sunahara and Insel, 2016; Hilger et al., 2018).

G protein subclasses are defined by differences in the downstream effectors that they bind to and regulate, and GPCRs differ in their selectivity for coupling among distinct G protein subclasses. Together, these constraints establish a first layer of diversity and specificity in functional GPCR signaling, based on biochemical selectivity of the underlying protein interactions (Bourne, 2006). Productive signaling also requires active G proteins to bind effectors, and GTP hydrolysis intrinsically restricts the G protein active-state lifetime to seconds or less. Thus, a second layer of specificity in physiological signaling is based on the subcellular localization of relevant proteins over time.

A general model for the spatiotemporal organization of ligand-dependent GPCR signaling emerged through studies of a limited number of examples, most notably the β2-adrenergic receptor (β2AR) (Fig. 1). Agonist ligands that activate the β2AR include two endogenously produced catecholamines, epinephrine and norepinephrine, as well as many clinically beneficial drugs. Agonist-induced β2AR activation produces diverse effects on cardiovascular, respiratory, endocrine, immune, and neural physiology. An example of a beneficial agonist drug effect is bronchial smooth muscle relaxation used in the treatment of asthma (Liu et al., 2024).

β2ARs initiate signaling from the plasmalemma by coupling to the stimulatory Gs subclass that stimulates cyclic AMP (cAMP) production (Sunahara and Insel, 2016). β2AR signaling is attenuated by receptor phosphorylation mediated by GPCR kinases (GRKs) and subsequent binding to β-arrestins. β-Arrestins are so named for their ability to "arrest" β2AR coupling to G proteins, causing net desensitization of cellular ligand responsiveness over seconds to minutes (Lohse et al.,

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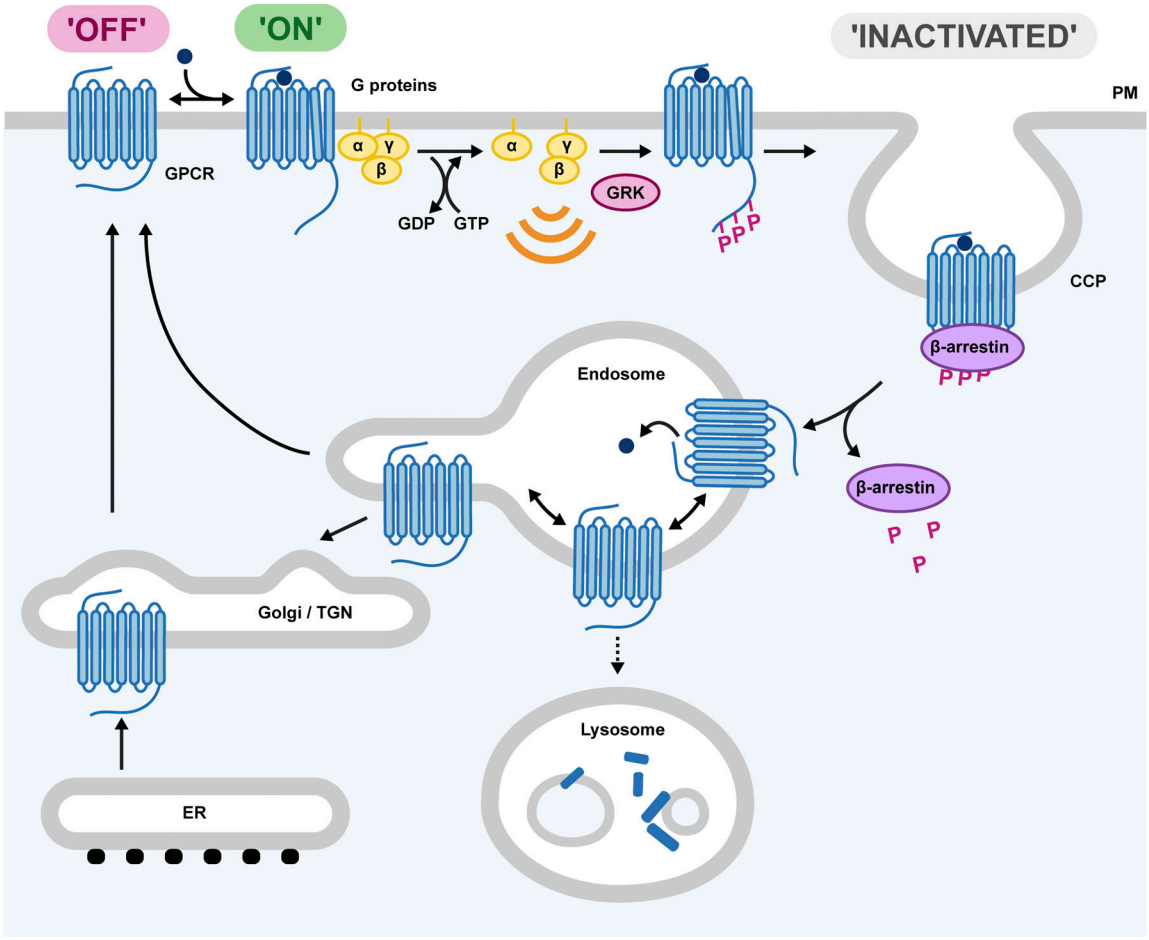

Figure 1. **Classical model of GPCR signaling and trafficking.** GPCRs are supplied to the PM through the biosynthetic pathway. Agonist binding allosterically activates the GPCR and promotes its transient coupling to a cognate heterotrimeric G protein that triggers nucleotide exchange on the G protein α-subunit, with GDP dissociating and GTP binding. The GTP-bound Gα dissociates from the complex to produce an "active" Gα monomer and free Gβγ heterodimer, each of which transduces signaling. Activated GPCRs are phosphorylated by GRKs and bind β-arrestin. β-Arrestin binding inactivates the GPCR to desensitize signaling, and it promotes receptor clustering in CCPs to stimulate GPCR endocytosis. β-Arrestin dissociates after endocytosis, and the GPCR is dephosphorylated. The receptor undergoes molecular sorting in endosomes between divergent pathways that mediate nondestructive recycling to the PM or delivery to lysosomes for degradation, thereby determining whether cellular GPCR signaling is "resensitized" or "down-regulated," respectively, after prolonged or repeated activation. Abbreviations: ER, endoplasmic reticulum; PM, plasmalemma.

1990; Lohse and Hofmann, 2015). β-Arrestins have additional cellular functions, such as stimulating β2AR endocytosis by promoting receptor clustering in clathrin-coated pits (CCPs) through interactions with clathrin, AP-2, and PIP_2 (Tian et al., 2014).

Upon β-arrestin binding, β2ARs rapidly internalize (beginning within about a minute) and β-arrestin dissociates during or immediately after β2AR-containing endocytic vesicles are formed (Puthenveedu and von Zastrow, 2006; Santini et al., 2002). β2ARs are then delivered to the limiting membrane of early endosomes, where they engage additional sorting proteins associated with the endosome limiting membrane. These interactions influence how long receptors remain in the endocytic network and determine whether receptors ultimately undergo recycling to the plasmalemma or traffic to lysosomes for proteolytic degradation. β2AR trafficking through the endocytic network thus exerts important and diverse effects on the long-term control of cellular signaling, ranging from restoring cellular ligand responsiveness (resensitization) to durably suppressing it (down-regulation) pending replenishment of receptors from the biosynthetic pathway (Hanyaloglu and von Zastrow, 2008; von Zastrow and Sorkin, 2021; Puthenveedu et al., 2010; Tian et al., 2016).

According to this traditional model, GPCRs are available for agonist-induced activation only in the plasmalemma and do not have any signaling activity in endomembrane compartments (Fig. 1) (Hanyaloglu and von Zastrow, 2008; Miller and Lefkowitz, 2001; Tan et al., 2004; von Zastrow, 2003; Drake et al., 2006). However, there is now considerable evidence supporting an expanded view that includes agonist-induced activation of receptors in endomembranes, as well as the plasmalemma, enabling GPCRs to leverage the spatiotemporal pattern of subcellular activation to control downstream signaling. We will discuss this still-evolving view by focusing on several GPCRs that couple to Gs and signal through the canonical cAMP pathway. The field is presently moving at a rapid pace, and our

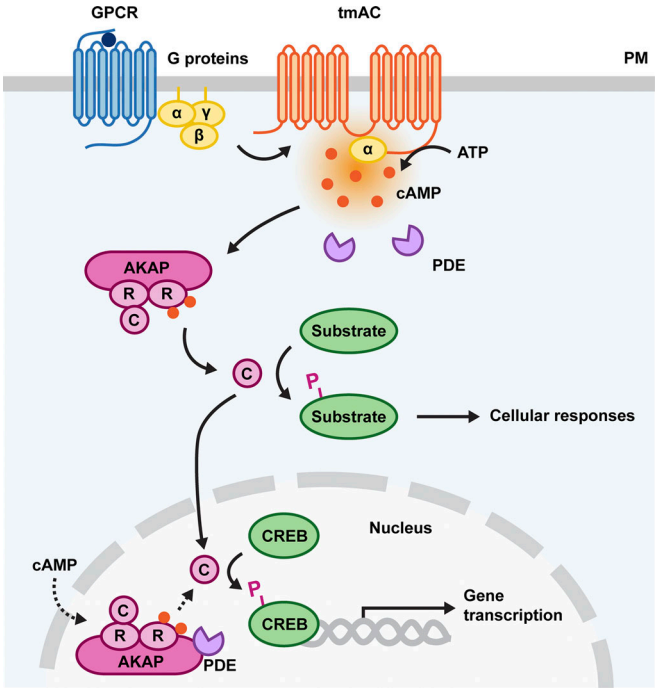

Figure 2. **cAMP/PKA signaling pathway.** GPCRs stimulate cAMP production by tmACs, and cAMP is degraded by PDEs. Gs stimulates tmACs to produce cAMP from ATP. cAMP binds PKA regulatory subunits (R) that are often scaffolded in the cytoplasm by binding to AKAPs, triggering the release of active catalytic subunits (C) from the inactive complex. Active catalytic subunits then phosphorylate substrates in the cytoplasm and also enter the nucleus by diffusing through nuclear pore complexes, where they phosphorylate nuclear substrates such as the transcription factor CREB that regulates the expression of related genes. Diffusion of active catalytic subunits into the nucleus is a major route for signaling to the nucleus in most cells. Some cells harbor an additional pool of PKA that is scaffolded in the nucleus by AKAPs and activated by diffusion of cAMP from the cytoplasm through nuclear pores. This enables a second, cell type–specific signaling route that has a higher activation threshold due to AKAP-mediated scaffolding of nuclear PKA with PDEs. Abbreviations: PM, plasmalemma.

goal here is not an exhaustive review for the specialist. Rather, we will discuss some key elements in a broader cell biological context.

Why location matters: Local effects of cAMP signaling

We will first highlight basic features of the cAMP signaling pathway that are important to our discussion and recommend authoritative reviews from others for additional information and perspective (Fimia and Sassone-Corsi, 2001; Taylor et al., 2013; Zaccolo et al., 2021). Gs-coupled GPCRs signal by stimulating transmembrane adenylyl cyclase enzymes (tmACs) to produce cAMP. cAMP then functions as an intracellular signaling mediator by binding to, and regulating the activity of, specific effectors that include cyclic nucleotide–gated ion channels, guanine nucleotide exchange proteins activated by cAMP, and cAMP-dependent protein kinase (PKA). PKA is a key cAMP-regulated effector protein with a plethora of substrates and downstream functions, and we will focus primarily on this effector node (Fig. 2).

PKA is present at a high concentration (in the micromolar range) in the cytoplasm. When cytoplasmic cAMP concentration is low, PKA exists as a heteromeric complex with its catalytic subunits inactivated by regulatory subunits. cAMP binding to regulatory subunits destabilizes the complex, releasing catalytic subunits in two active forms: fully dissociated catalytic subunit monomers and partially dissociated catalytic subunits (Smith et al., 2017; Taylor et al., 2005). Dissociated catalytic subunits can diffuse freely through the cytoplasm and phosphorylate substrates, while partially dissociated subunits remain tethered, targeting substrates within their vicinity. Additionally, catalytic subunit monomers are sufficiently small to diffuse into the nucleus, where they phosphorylate nuclear targets such as cAMP response element–binding protein (CREB). CREB binds to cAMP response element sequences in cAMP-responsive genes and becomes a transcriptional activator upon phosphorylation by PKA (Kandel, 2012; Altarejos and Montminy, 2011). In some cells, a separate pool of heteromeric PKA resides in the nucleus that can be activated by cAMP diffusing through nuclear pores from the cytoplasm, but this nucleus-resident pool has a higher activation threshold than the cytoplasmic PKA pool (Sample et al., 2012). Nuclear PKA activity is limited by PKA-inhibitory protein binding to catalytic subunits, which suppresses kinase activity and promotes active nuclear export of catalytic subunits (Taylor et al., 2013). The cAMP elevation that initiates PKA activation is terminated by phosphodiesterase enzymes (PDEs), present in both the cytoplasm and the nucleus, that hydrolyze cAMP to restore cAMP concentrations to a basal level (Beavo and Brunton, 2002).

Distance scales of cellular cAMP signaling

cAMP was initially proposed to function as a long-range mediator of cellular signaling due to its ability to freely diffuse in aqueous solutions, founding the "second messenger" concept for signaling by hormones (Sutherland and Rall, 1958). It was hypothesized later that cAMP can also mediate local signaling effects by forming spatial cAMP gradients in the cytoplasm (Corbin et al., 1977; Buxton and Brunton, 1983; Hayes et al., 1979). The development of fluorescent cAMP biosensors supported this concept by providing evidence for spatial heterogeneity of cAMP concentration in intact, living cells (DiPilato et al., 2004; Zaccolo and Pozzan, 2002; Maiellaro et al., 2016; Terrin et al., 2012; Di Benedetto et al., 2008; Calebiro and Maiellaro, 2014; Nikolaev et al., 2004, 2006; Maiellaro et al., 2016, 2016; Bacskai et al., 1993). More recently, the "FluoSTEPs" technology, using fluorescent protein complementation to localize biosensors to cellular proteins endogenously tagged with a short polypeptide sequence, has resolved cAMP and PKA signaling domains around proteins expressed at native levels (Tenner et al., 2021).

The existence of spatial cAMP gradients is now supported down to a distance scale of nanometers. By targeting fluorescent cAMP biosensors between lateral domains of the plasmalemma (Averaimo et al., 2016; Agarwal et al., 2014) and to plasmalemmal, sarcoplasmic reticular, and myofilament locations in cardiac myocytes (Surdo et al., 2017), evidence was obtained for the existence of submicron spatial gradients of cAMP that are detectable under steady-state conditions. Direct fusion of a

cAMP biosensor to GPCRs using rigid linkers resolved 30–50-nm "receptor-associated independent nanodomains" (RAINs) of local cAMP elevation. Interestingly, RAINs were observed only at low agonist concentrations that activate receptors sparsely but not at higher concentrations. This suggests that the distance scale of cellular GPCR-mediated cAMP signaling can vary considerably, from nanometer-scale RAINs to longer range elevations, depending on physiological parameters such as receptor density and the concentration of agonists available to activate them (Anton et al., 2022).

Principles of local cAMP gradient formation

The present understanding of how localized cAMP gradients are generated is based on reaction–diffusion models involving three key processes: local cAMP production and destruction, buffered and restricted diffusion of cAMP, and molecular scaffolding of cAMP signaling complexes (Lohse et al., 2024; Zaccolo et al., 2021).

cAMP production and destruction. Nine homologous tmAC genes are conserved in mammals, each encoding a 12-transmembrane protein activated by Gs. Individual tmAC subtypes differ in their regulation by other biochemical inputs, such as additional G proteins and calcium, and in their interactions with other cellular components that determine their subcellular localization (Johnson and Leroux, 2010; Marsden and Dessauer, 2019; Halls and Cooper, 2017). cAMP-hydrolyzing PDEs comprise a diverse group of proteins that include multiple gene products and alternatively spliced isoforms that also differ in their biochemical regulation, protein interactors, and subcellular localization (Di Benedetto et al., 2008; Mika et al., 2012; Brescia and Zaccolo, 2016; Conti and Beavo, 2007; Conti et al., 2014). Mammalian cells typically coexpress multiple tmACs and PDEs (Stangherlin and Zaccolo, 2012; Terrin et al., 2006; Mongillo et al., 2004; Conti et al., 2014; Dessauer et al., 2017) that are thought to function as localized sources and sinks, respectively, for producing spatial cAMP gradients in the cytoplasm (Lohse et al., 2017; Mika et al., 2012; Conti et al., 2014).

Buffered and restricted cAMP diffusion. The rate of cAMP diffusion in aqueous solutions is very fast relative to known catalytic rates of relevant tmACs and PDEs. As such, the hypothesis that these enzymes can generate significant spatial gradients over a biologically relevant timescale (and particularly at the steady state) has been challenged on biophysical grounds (Bock et al., 2020). A proposed explanation is based on cAMP buffering through reversible binding to cytoplasmic proteins such as PKA regulatory subunits, whose concentration exceeds that of cAMP under physiological conditions (Beavo et al., 1974). Such buffering has now been shown to slow the effective diffusion rate of cAMP in the cytoplasm (Agarwal et al., 2016; Bock et al., 2020). In addition, a PKA regulatory subunit (RIα) has been shown to form phase-separated cytoplasmic droplets that define restricted, nanometer-scale domains of buffered cAMP diffusion (Zhang et al., 2020). It is presently proposed that distinct processes of buffered and restricted cAMP diffusion (Saucerman et al., 2014), together, enable tmACs and PDEs to produce significant spatial gradients at known catalytic rates (Lohse et al., 2024).

Molecular scaffolding of signaling proteins. A third key element that underlies local cAMP signaling is enforced proximity mediated by molecular scaffolding. A diverse group of such molecular scaffolds are A-kinase anchoring proteins (AKAPs), named for their shared ability to bind PKA (Wong and Scott, 2004). Most AKAPs also bind other cellular proteins, including various tmACs and PDEs, and cells typically coexpress multiple AKAPs (Di Benedetto et al., 2008). Some AKAPs additionally associate with membranes and can laterally partition among membrane domains (Johnstone et al., 2018). Together, these properties are thought to enable AKAPs to precisely organize nanoscale cAMP signaling complexes within cells (Omar and Scott, 2020).

Current models of GPCR signaling from endomembranes

Considering that cAMP is able to mediate local signaling effects, the subcellular location of activated GPCRs has the potential to precisely dictate downstream signal transduction. Signal initiation from endomembranes is a still-evolving concept for ligand-activated GPCRs but the general concept is long-established, as the light-activated GPCR rhodopsin was shown >40 years ago to initiate phototransduction by coupling to G proteins on intracellular membranes of photoreceptor cells (Kwok-Keung Fung and Stryer, 1980). However, endomembrane activation was believed to be a unique property of light-activated GPCRs, and ligand-activated receptors were considered either to be functionally inert in endomembranes or to produce effects only through G protein–independent mechanisms (Luttrell et al., 1999).

The ability of ligand-dependent GPCRs to initiate G protein signaling from endomembranes was proposed in 2006, when the cellular response triggered by a peptide-activated yeast GPCR, Ste2p, was shown to involve G protein–coupled activation of a lipid kinase (Vps34p) on the endosome or vacuolar membrane (Slessareva et al., 2006). Evidence in mammalian cells emerged in 2009 through studies of cAMP signaling (Mullershausen et al., 2009; Ferrandon et al., 2009; Calebiro et al., 2009). Most notably, the parathyroid hormone receptor-1 (PTH1R), a glycoprotein hormone–activated GPCR, and the thyroid-stimulating hormone receptor (TSHR), a polypeptide-activated GPCR, were reported to trigger a second phase of Gs-coupled cAMP elevation after endocytosis. These GPCRs produce a sustained cAMP elevation after pulsatile activation, and a key distinction noted in these studies was that cAMP production from endomembranes was sustained, whereas production from the plasmalemma was transient (Ferrandon et al., 2009; Calebiro et al., 2009). It was then reported that the D1-type dopamine receptor (D1R or DRD1) and β2AR, two catecholamine-activated GPCRs that rapidly cycle through endosomes and produce an exclusively transient cAMP elevation, can also initiate signaling after endocytosis (Kotowski et al., 2011; Irannejad et al., 2013; Lohse and Calebiro, 2013). Independently, and over a similar time period, a number of GPCRs were observed to trigger G protein–dependent signaling from endomembrane compartments of the biosynthetic pathway (Campden et al., 2015; Jong et al., 2009; Irannejad et al., 2017).

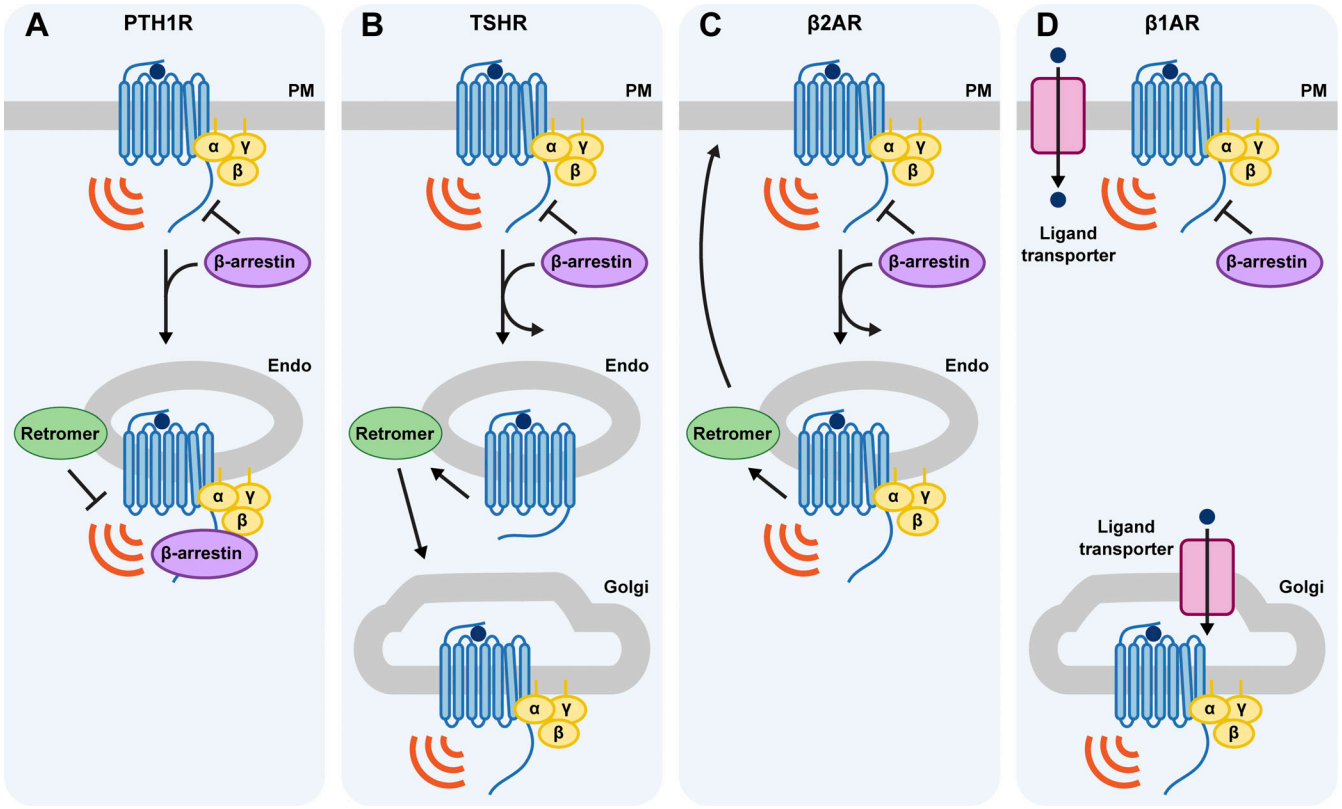

Figure 3. **Current models of GPCR signaling from endomembranes. (A)** Following activation at the PM, PTH1R undergoes β-arrestin–dependent inactivation and internalization. β-Arrestin remains bound to PTH1R at the endosome, where it promotes sustained signaling by scaffolding a signaling complex with the G protein. Signaling is terminated by retromer binding to PTH1R. **(B)** TSHR is inactivated and internalized following β-arrestin binding at the PM. β-Arrestin does not remain bound at the endosome; instead, TSHR engages retromer for retrograde transport to the TGN, where TSHR activates a second phase of signaling. **(C)** Like TSHR, the β2AR transiently engages β-arrestin at the PM to drive inactivation and internalization. Endosomal β2AR then activates a second phase of signaling before engaging retromer for rapid recycling back to the PM. **(D)** β1AR is localized to both the PM and Golgi membranes, and Golgi activation does not require the trafficking of receptors. Rather, Golgi β1AR is activated when ligands gain intracellular access via transporters. Abbreviations: Endo, endosome; PM, plasmalemma.

Following these early observations, a deluge of subsequent studies have supported the hypothesis that many ligand-activated GPCRs can initiate signaling through heterotrimeric G proteins from endomembranes and from the plasmalemma (Calebiro et al., 2025). The available evidence supports a diversity of cellular mechanisms. Below, we briefly summarize a few examples of current models derived from studies of signaling by Gs-coupled GPCRs through the cAMP pathway (Fig. 3), with the goal of orienting the reader to overall concepts and distinctions that will be discussed in more depth later.

PTH1R: Sustained cAMP production from endosomes by a receptor/β-arrestin supercomplex

PTH1R initiates signaling by coupling to Gs on the plasmalemma and then is desensitized and internalized after binding to β-arrestin. β-Arrestin remains bound after endocytosis and sequesters PTH1R in endosomes, where the PTH1R/β-arrestin complex initiates a sustained, second phase of cAMP production. β-Arrestin binding to PTH1R increases the strength and duration of Gs coupling on endosomes (Ferrandon et al., 2009), and this switch in β-arrestin's regulatory function, from desensitizing signaling at the plasmalemma to sustaining it at endosomes, occurs by remodeling of the PTH1R/β-arrestin complex after endocytosis to include Gs (Wehbi et al., 2013). The endosome signal is later terminated by dissociation of the complex and binding of PTH1R to retromer (Feinstein et al., 2011). Thus, PTH1R produces a sustained, second phase of cAMP production after endocytosis by stably sequestering receptors with β-arrestin and Gs in a signal-enhancing endosomal supercomplex.

TSHR: Sustained cAMP production after endocytic delivery to the Golgi apparatus

Like PTH1R, activated TSHR couples to Gs on the plasmalemma, is desensitized and internalized after binding to β-arrestin, and triggers a second phase of cAMP production after endocytosis that is sustained relative to signaling from the plasmalemma (Calebiro et al., 2009). β-Arrestin does not remain bound to TSHR after endocytosis (Frenzel et al., 2006), however, and the second signaling phase is not produced from endosomes. Rather, the second phase of TSHR-triggered Gs coupling occurs after (or during) TSHR delivery to the Golgi apparatus or the trans-Golgi network (TGN) through a retromer-dependent mechanism. The endomembrane TSHR signal is sustained because agonists wash

out from this compartment slowly after removal from the extracellular medium. In addition, the same membranes on which cAMP is generated during the second signaling phase are coated with PKA through binding to AKAPs. This increases the efficiency of PKA activation by the second phase of cAMP production through a proximity effect, based on cAMP production and PKA being situated "in cis" on the same membrane surface (Godbole et al., 2017). Thus, TSHR, like PTH1R, triggers a persistent signal after endocytosis, but it does so in a different way: TSHR triggers cAMP production from Golgi or TGN membranes, rather than from endosomes, and the second signaling phase is promoted, rather than attenuated, by retromer.

β2AR: Transient cAMP production from endosomes that efficiently activates PKA through membrane contacts

β2AR also internalizes by binding to β-arrestin in the plasmalemma, and then β-arrestin dissociates. Rather than being stably sequestered in an endomembrane compartment after endocytosis, β2AR iteratively cycles between the plasmalemma and endosomes in the presence of an agonist and couples to Gs transiently at both locations (Irannejad et al., 2013). The amount of cAMP produced from endosomes is limited by β2AR residence time in the endosome limiting membrane, and this is determined by how rapidly β2ARs partition into exit tubules coated with actin and retromer (Tian et al., 2016; Varandas et al., 2016; Temkin et al., 2011; Lauffer et al., 2010; Irannejad et al., 2013; Puthenveedu et al., 2010). Endosomes containing activated β2ARs exhibit microtubule-based motility and translocate from the cell periphery to the perinuclear region, where they directly contact Golgi membranes coated with PKA. This increases the efficiency of PKA activation by cAMP produced in the second phase through enforced proximity at sites of the endosome–Golgi membrane contact (Peng et al., 2021; Willette et al., 2024). Thus, β2AR couples transiently to Gs on endosomes to activate PKA on Golgi membranes "in trans."

β1-adrenergic receptor (β1AR): A non-endocytic endomembrane signaling mechanism with restricted agonist access

The β1AR is a catecholamine-activated GPCR closely homologous to β2AR, but it interacts with distinct cytoplasmic proteins that mediate the selective accumulation of an intracellular pool of β1AR in the Golgi apparatus and the TGN (Koliwer et al., 2015). This intracellular β1AR pool triggers agonist-induced cAMP production by locally coupling to Gs, but agonists required to trigger this signal cannot access the intracellular pool efficiently by endocytosis. Rather, agonist access to the intracellular receptor pool requires agonist ligands to cross membranes. Many drugs cross membranes passively, but physiological catecholamine agonists require cells to express specific facilitated transporter proteins (Irannejad et al., 2017). Thus, β1AR illustrates a non-endocytic route of endomembrane signaling with restricted agonist access.

Experimental tools and methods for investigating endomembrane GPCR signaling

Our present understanding of endomembrane GPCR signaling depends entirely on the quality and depth of the available experimental evidence. Early evidence for GPCR-mediated signaling through the cAMP pathway from endomembranes was limited to a simple correlation between the kinetics of receptor trafficking and cytoplasmic cAMP regulation (Mullershausen et al., 2009). Experimental precision and depth have increased greatly since that time, but many gaps remain. In this section, we describe major experimental approaches that are widely used in the field currently (Fig. 4), discuss examples of specific interpretations, and note some strengths and limitations of each approach.

Manipulations of membrane trafficking pathways, endomembrane localization, or composition

A common experimental strategy is to measure the signaling consequences of manipulating membrane trafficking pathways used by receptors. One approach involves inhibiting endocytosis by chemical or dominant-negative inhibition of dynamin, a GTPase required for endocytic vesicle formation from CCPs in animal cells (Mettlen et al., 2018). Early studies showed that endocytic inhibition shortened the duration of the cAMP elevation for PTH1R and TSHR, as discussed above, and based on these results, it was initially proposed that endomembrane GPCR signaling is restricted to a limited subset of receptors producing sustained cAMP elevations (Calebiro et al., 2009; Ferrandon et al., 2009). Later work demonstrated that endocytic inhibition also suppressed D1R and β2AR signaling, two Gs-coupled GPCRs that elevate cytoplasmic cAMP only transiently, with the most pronounced effects observed downstream of cAMP, such as PKA activation or later PKA-mediated effects (Tsvetanova and Von Zastrow, 2014; Kotowski et al., 2011; Peng et al., 2021; Irannejad et al., 2013).

There are major caveats to attributing the effects of manipulating general trafficking pathways to signaling by a specific GPCR. A more targeted approach involves depleting β-arrestin or mutating key phosphorylation sites on the receptor. However, such approaches often introduce complications. For example, mutations in a Ser/Thr cluster of the β2AR cytoplasmic tail, a major site of agonist-induced phosphorylation driving receptor endocytosis, impaired both internalization and desensitization at the plasmalemma. This resulted in excessive cAMP elevation, asking any potential contribution of cAMP production from endosomes (Hausdorff et al., 1991; Trester-Zedlitz et al., 2005).

Other experimental approaches in this category include manipulating the duration of receptor residence in the endosome membrane or perturbing the biochemical environment of endosomes. For example, depleting VPS35, which inhibits PTH1R recycling and thus increases its intracellular residence time (McGarvey et al., 2016), was found to further sustain the cytoplasmic cAMP elevation triggered by PTH1R activation (Feinstein et al., 2011). A similar result was obtained when bafilomycin was used to inhibit endosome acidification by the vacuolar ATPase (Gidon et al., 2014). Together, these results were interpreted as evidence that PTH1R-triggered cAMP production from endosomes is limited by both retromer and endosome acidification.

Another approach along this line is to specifically manipulate the subcellular location or signaling protein composition of relevant endomembranes. For example, in an elegant study using chemical recruitment of a kinesin to endosomes, acute

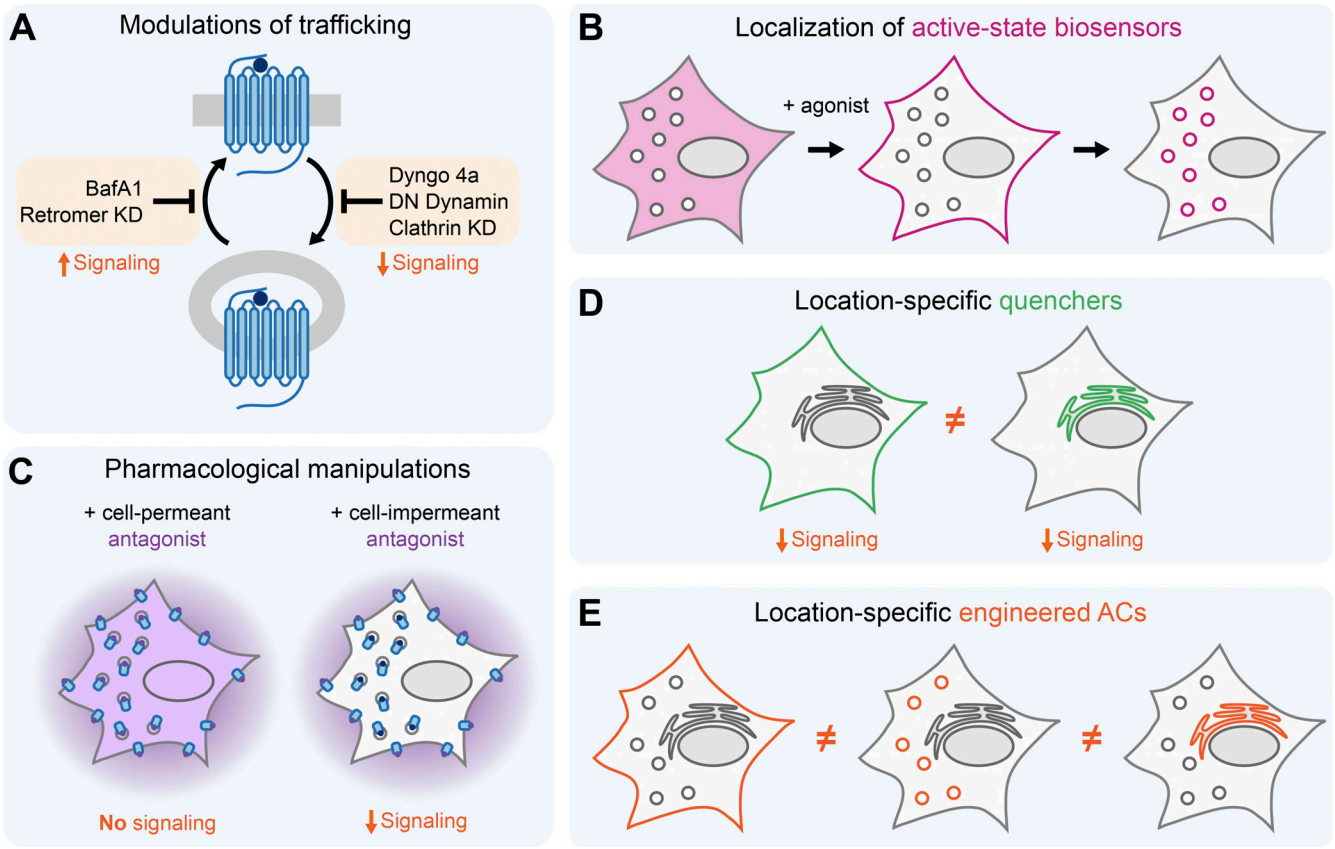

Figure 4. **Experimental evidence supporting GPCR-mediated signaling from internal membranes. (A)** Modulation of endocytic or recycling machinery decreases and increases signaling outputs, respectively. **(B)** Active-state biosensors, localized diffusely in the cytoplasm, are sequentially recruited to the plasmalemma and endocytic membranes upon agonist stimulation. **(C)** After the addition of an agonist, the application of a cell-permeant antagonist fully inhibits signaling, whereas the application of a cell-impermeant antagonist only partially inhibits signaling. **(D)** Recruitment of signaling quenchers to specific subcellular compartments partially inhibits signaling, and the effects differ depending on the subcellular compartment. **(E)** Engineered ACs that produce cAMP at specific subcellular compartments elicit unique downstream signaling responses. Abbreviations: BafA1, bafilomycin A1; DN, dominant negative; KD, knockdown.

redistribution of endosomes from the perinuclear region to the cell periphery was found to suppress the β2AR-triggered elevation of nuclear PKA activity and downstream transcription but not elevation of cytoplasmic cAMP (Willette et al., 2024). In a separate study, mutating a dileucine sequence in the AC9 N-terminal domain, which reduces the degree of AC9 concentration in endosomes, suppressed the D1R-triggered elevation of nuclear PKA activity without detectably changing the cytoplasmic cAMP elevation (Ripoll et al., 2024). Together, such results have been interpreted as support for "location-encoded" or "location-biased" signaling by cAMP from endomembranes, defined as differences in signaling that are determined specifically by trafficking-dependent changes in the subcellular location of cAMP production rather than its duration or overall amount (Peng et al., 2021; Willette et al., 2024; Ripoll et al., 2024; Tsvetanova and Von Zastrow, 2014).

Localizing G protein and receptor activation using conformational biosensors

Another experimental approach is to apply genetically encoded sensors to resolve the subcellular location of activated GPCRs in intact cells. Such sensors have so far been developed by repurposing tools originally developed to stabilize active-conformation receptors for structural studies. These include engineered G protein fragments (mini-G proteins [mG]) (Carpenter and Tate, 2016; Wan et al., 2018) and single-chain antibody fragments (nanobodies [Nb]) (Manglik et al., 2017), both of which selectively bind active-conformation GPCRs. A GFP-labeled version of Nb80, an active-state β2AR binder, redistributes from the cytoplasm to membranes upon agonist-induced activation of the β2AR, and sequential recruitment to the plasmalemma followed by endosomes revealed two distinct phases of β2AR activation (Steyaert and Kobilka, 2011; Rasmussen et al., 2011; Irannejad et al., 2013). Nb80 has also been used to detect intracellular activation of the closely similar β1AR (Irannejad et al., 2017), and a modified version of this Nb, Nb6B9, was used to detect agonist-induced activation of D1R in endomembranes (Ring et al., 2013; Puri et al., 2022).

A caveat of the conformational biosensor approach is that active-state binders inherently alter receptor conformation and can interfere with receptor signaling or trafficking. Such effects have been well described for both mG-based (Manchanda et al., 2024) and Nb-based conformational biosensors. For example, at high concentrations, Nb80 binds β2AR in the absence of an

agonist and suppresses β2AR-induced cAMP elevation (Staus et al., 2016). Using such tools to detect, rather than perturb, conformational activation of receptors thus requires careful control of biosensor expression levels (Irannejad et al., 2013).

Another Nb, Nb37, has been applied to detect GPCR coupling to Gs by selectively binding to a nucleotide-free form of the Gs α-subunit that is transiently produced during the GPCR-mediated coupling reaction (Westfield et al., 2011). When expressed at low levels, Nb37-GFP is recruited sequentially to the plasmalemma and endosomes following β2AR activation, providing evidence for sequential G protein activation phases from these locations (Irannejad et al., 2013). Again, care must be taken in designing and interpreting such experiments, as Nb37 can artificially drive Gs activation when present at high concentrations, emphasizing the need for careful calibration (Kwon et al., 2022). Additional engineered proteins or peptides have since been described that detect Gα subunits in an active, GTP-bound conformation (Avet et al., 2022; Janicot et al., 2024). These new biosensors require similar precautions but will surely prove to be powerful additional tools for cell biology.

Pharmacological strategies for spatial dissection

A third general approach takes advantage of differences in the ability of chemically distinct agonists or antagonists to access receptors present in the plasmalemma relative to endomembranes. For instance, a sustained component of cytoplasmic cAMP elevation triggered by the vasopressin receptor 2 (V2R) was rapidly inhibited by a membrane-permeant V2R antagonist but not by a membrane-impermeant antagonist; this was interpreted as evidence that V2R activation in endomembranes is required for sustained cytoplasmic cAMP production (Thomsen et al., 2016). Another example is chemical inhibition of solute transporters required for agonist access to a Golgi-localized pool of β1AR and D1R. This manipulation suppressed some but not all downstream signaling effects, which was interpreted as evidence for endomembrane activation driving specific downstream responses (Irannejad et al., 2017; Puri et al., 2022; Lin et al., 2024). Still another strategy is to compare signaling effects of agonists that differ in their regulation of GPCR trafficking. A natural PTH1R agonist (called PTH^{WT} by the investigators) strongly promotes PTH1R endocytosis, but an engineered derivative of this agonist (PTH^{7d}) activates the receptor without promoting endocytosis. While PTH^{WT} and PTH^{7d} produced a comparable level of overall cytoplasmic cAMP elevation, the cAMP elevation produced by PTH^{WT} was sustained after agonist washout, whereas that produced by PTH^{7d} was not. These findings were interpreted as evidence that PTH1R activation in endosomes selectively mediates the production of a sustained component of the cytoplasmic cAMP elevation impacting transcription (White et al., 2021). Caveats of such pharmacological strategies include the possibility that other differences among ligands, such as in binding affinity or off-rate, may complicate interpretations. This can be addressed by cross-validating results using manipulations not dependent on pharmacology (e.g., Jang et al., 2022).

Subcellular signal quenching

The signaling activity of receptors at defined locations has also been assessed by localizing quenchers of receptor coupling or downstream signaling. We previously noted that Nb80 can block β2AR coupling to Gs and suppress the cellular cAMP response when present at sufficiently high concentrations (Staus et al., 2016). The same is true for β1AR, which is structurally very similar to β2AR and also binds Nb80 essentially as a G protein mimic (Warne et al., 2019). Acutely concentrating Nb80 on the plasmalemma or on Golgi membranes only partially suppressed the overall cAMP elevation triggered by β1AR agonists. This was interpreted as evidence that β1AR activation at both locations contributes to the full cellular cAMP response naturally elicited by β1AR activation (Irannejad et al., 2017). Tethering a cAMP binding–defective mutant of a PKA regulatory subunit that has dominant-negative activity to different subcellular locations provided evidence that different local cAMP pools are selectively biased toward driving PKA-mediated phosphorylation of different downstream substrates (Lin et al., 2024).

Local cAMP production mediated by engineered adenylyl cyclases (ACs)

To further probe location-specific effects of cAMP production independently of GPCR or G protein activation, a handful of studies have employed ACs that can be localized to particular subcellular compartments and orthogonally activated by bicarbonate or light (Sample et al., 2012; Tsvetanova and Von Zastrow, 2014; O'Banion et al., 2019; Tsvetanova et al., 2021; Lin et al., 2024). These approaches are useful for experimentally isolating the effect of the subcellular location of cAMP production on downstream cAMP-mediated signaling. For example, optogenetic stimulation of cAMP production from endosomes was shown to preferentially promote signaling via PKA to the nucleus, relative to stimulated cAMP production from the plasmalemma. The location-dependent effect on downstream signaling was reduced in cells exposed to a broad-spectrum PDE inhibitor, which was interpreted as evidence that PDEs play an essential role in supporting location-biased cAMP signal discrimination (Sample et al., 2012; Tsvetanova and Von Zastrow, 2014; O'Banion et al., 2019).

Physiological significance and therapeutic implications of endomembrane GPCR signaling

We have emphasized studies using non-native cell models because they offer advantages for mechanistic elucidation. However, GPCR signaling from endomembranes was first recognized through investigations of physiological signaling processes (Slessareva et al., 2006; Calebiro et al., 2009; Ferrandon et al., 2009; Mullershausen et al., 2009), and the field continues to build on this foundation through subsequent work from many groups. We briefly note a few examples below, each with interesting physiological or therapeutic implications.

A study of physiological signaling mediated by the luteinizing hormone receptor (LHR) in ovarian explants provides an interesting example of spatiotemporal GPCR signaling through cAMP in an intact tissue. LHR expressed specifically on ovarian follicle cells was found to internalize and trigger a sustained

elevation of cytoplasmic cAMP. This endosomal signaling phase was essential for driving cAMP diffusion through gap junctions into the cytoplasm of the LHR-lacking oocyte enclosed in the ovarian follicle cell layer. Such transcellular diffusion of cAMP controls cell cycle progression in the oocyte, which is essential for later fertilization (Lyga et al., 2016). This study illustrates the value of studying signaling in intact tissues, and it suggests that transcellular communication might be a widespread function of endomembrane signaling.

A synthetic variant of PTH, PTH^{7d} as discussed above, was used to restrict cAMP production to the plasmalemma. This spatial bias supported homeostatic regulation of plasma calcium and phosphate concentrations in vivo. However, it did not support PTH1R-dependent transcriptional responses required to induce vitamin D biosynthesis, resulting in reduced circulating vitamin D levels and reduced bone formation in mice. These results emphasize the importance of spatial and temporal dimensions of endosomal cAMP signaling and suggest a strategy for improving the therapeutics of metabolic bone disorders through manipulation of signaling from endomembranes relative to the plasmalemma (White et al., 2021).

A recent study of cAMP signaling initiated by the β1AR in cardiac muscle cells further emphasizes the spatial cAMP signaling dimension in a different physiological context in vivo. β1AR activation in Golgi membranes of cardiac muscle cells was shown to produce different downstream effects than activation in the plasmalemma. In particular, cAMP generation from the plasmalemma increased muscle contractility, while cAMP generation from Golgi membranes promoted muscle relaxation (Lin et al., 2024). This example also carries interesting therapeutic implications, as it highlights new targets for manipulating GPCR effects, based on the Golgi-localized β1AR pool requiring membrane transporter proteins not required for plasmalemma signaling (Irannejad et al., 2017).

Experimental limitations, open questions, and future directions

Our understanding of the spatiotemporal organization of cellular GPCR signaling has evolved considerably over the past several years, but many caveats, knowledge gaps, and open questions remain. We highlight some of these below.

Subcellular organization of transducer and effector proteins

Much of what is presently known about the spatiotemporal organization of cellular GPCR signaling is focused on the receptors that initiate signaling. Productive signaling depends on proximity to transducer and effector proteins, and less is known about their subcellular localization or dynamics.

G proteins. Heterotrimeric G proteins have a complex intracellular itinerary (Wedegaertner, 2012; Tennakoon et al., 2021; Saini et al., 2009; Jang et al., 2024; Martin and Lambert, 2016). Briefly summarized, G proteins are associated with the inner leaflet of cellular membranes by lipidation, with inactive G protein heterotrimers typically enriched on the plasmalemma. G protein βγ subcomplexes are relatively broadly distributed on the plasmalemma and endomembranes at the steady state, and there is evidence that they dynamically traffic between the plasmalemma and various internal membranes both constitutively and in response to GPCR activation (Masuho et al., 2021; Jang et al., 2024; Michaelson et al., 2002). In the plasmalemma, GPCRs and G proteins diffuse separately and couple after collision at actin-associated surface domains or "hotspots" (Calebiro and Jobin, 2019). Less is known about lateral organization of G protein coupling on endomembranes. For some GPCRs, β-arrestin scaffolds a receptor–G protein complex at the endosome (Wehbi et al., 2013; Nguyen et al., 2019; Thomsen et al., 2016). However, it remains unclear how G protein coupling on endomembranes is organized for GPCRs that do not form stable complexes with β-arrestin. For example, active β2AR is distributed broadly on endosomes, whereas G protein activation appears to be distributed in a nonuniform pattern, suggesting the possible existence of Gs coupling hotspots also on endosomes (Varandas et al., 2016; Irannejad et al., 2013).

ACs. Despite early evidence that AC activity is present both on intracellular membranes and on the plasmalemma (Cheng and Farquhar, 1976), a significant gap remains in our understanding of tmAC localization and its functional effects. Several studies have reported the presence or activation of specific tmAC subtypes in endosomes and Golgi membranes (Kotowski et al., 2011; Calebiro et al., 2009; Cancino et al., 2014; Lazar et al., 2020; Ripoll et al., 2024; Ferrandon et al., 2009), and others have functionally linked specific tmACs to endomembrane cAMP signaling (Cancino et al., 2014; Jean-Alphonse et al., 2014; Lazar et al., 2020; Ripoll et al., 2024). For example, among the main three tmACs expressed in striatal neurons, AC9 is uniquely enriched in endosomes and appears to be specifically required for efficient regulation of nuclear PKA activity in response to D1R activation in endosomes (Ripoll et al., 2024). In addition to tmACs, there is evidence that a distinct form of AC that lacks transmembrane helices and is not directly stimulated by Gs ("soluble" AC) can also contribute to GPCR-initiated cAMP production from endosomes through an indirect mechanism involving receptor-triggered regulation of cytoplasmic free calcium levels (Caldieri and Sigismund, 2016). We anticipate that proximity labeling proteomics will be useful in future studies to elucidate local protein networks engaged by tmACs and other signaling relevant membrane proteins (Lobingier et al., 2017; Zhong et al., 2024) and note that a bimolecular fluorescence complementation method has already been shown to detect specific protein interactions with tmACs in intact cells (Doyle et al., 2019).

PKA. Three isoforms of PKA catalytic subunit (Cα, Cβ, and Cγ) are conserved in mammals and capable of binding four distinct isoforms of regulatory subunits (RIα, RIβ, RIIα, and RIIβ) (Taylor et al., 2012). PKAs are localized in cells through regulatory subunit binding of AKAPs, as discussed above. Many AKAPs bind to RII isoforms (Omar and Scott, 2020), mediating their concentration on Golgi membranes (Nigg et al., 1985) and on other subcellular structures (Omar and Scott, 2020; Diviani and Scott, 2001). RI isoforms were traditionally believed to be diffusely distributed in the cytoplasm, and their localization had received less attention. However, several studies have reported RIα localization to internal membranes such as autophagosomes, late endosomes, and multivesicular bodies (Day et al., 2011;

Mavrakis et al., 2006), as well as nanoscale molecular condensates as noted above (Zhang et al., 2020). We further note that particular PKA isoforms differ considerably in their expression across neuronal cell types, suggesting that there may exist significant cell type–specific differences in cAMP pathway organization (Ilouz et al., 2017).

Accordingly, an important goal moving forward is to understand which PKA isoform(s) mediate(s) signaling effects triggered from particular subcellular locations. PKA Cα and RIIβ subunits have been shown to concentrate in the Golgi and perinuclear region, with evidence indicating their close proximity to endosomes (Peng et al., 2021; Willette et al., 2024; Ripoll et al., 2024; Godbole et al., 2017). Considering the remarkable diversity of cellular proteins whose phosphorylation by PKA is dependent on the subcellular location of cAMP production (Tsvetanova et al., 2021), it seems likely that there is much more to be learned about the spatiotemporal organization of PKA localization relative to local sources of GPCR-regulated cAMP and its impact on regulatory phosphorylation of relevant substrates.

Role of membrane contact sites in GPCR signaling from endomembranes

Membrane contact sites are increasingly recognized to have important and diverse cellular functions (Scorrano et al., 2019). Membrane contacts between the plasmalemma and endoplasmic reticulum have been well described to function in calcium signaling. At such contacts, there is also evidence for a link to cAMP signaling effects through local activation of calcium-stimulated tmAC subtypes (Crul and Maléth, 2021; Motiani et al., 2018). Studies of endosomal signaling triggered by β2AR and D1R provide evidence supporting the existence of a different type of membrane contact, between endosomes and PKA-associated Golgi membranes, that is important for promoting location-biased signaling by the cAMP pathway when triggered from endosomes (Ripoll et al., 2024; Peng et al., 2021). Clearly, there is much more to learn, and we anticipate that membrane contact sites may prove important for supporting location-biased signaling by GPCRs more broadly.

Diversity in GPCR trafficking/signaling relationships

Our present understanding of GPCR-mediated intracellular signaling stems from studies of a limited subset of examples when compared to the number of distinct GPCR types that are encoded by the human genome (~800). As evidence for endomembrane signaling by additional ligand-dependent GPCRs continues to accumulate, the question of diversity and specificity in GPCR signaling and trafficking becomes increasingly important to address. Here, we briefly note several aspects that we believe are of particular interest presently.

GPCR/β-arrestin complexes. There has been evidence for many years supporting the existence of receptor-specific and location-specific differences in GPCR/β-arrestin complexes whose functional significance remains to be fully elucidated (Oakley et al., 2000). One key to this diversity is conformational heterogeneity among GPCR/β-arrestin complexes (Underwood et al., 2024; Maharana et al., 2024). β-Arrestin is a flexible protein that can engage GPCRs in multiple conformations, including but not limited to a traditional core-engaged complex that sterically blocks G protein coupling (Kang et al., 2016) and a tail-engaged complex that leaves the receptor core available for coupling (Kang et al., 2016; Nguyen et al., 2019; Thomsen et al., 2016). The existence of distinct core-engaged and tail-engaged conformations of GPCR/β-arrestin complex formation has been argued convincingly in studies of signaling by V2R, where a core-engaged complex suppresses signaling at the plasmalemma and a tail-engaged complex enhances or sustains signaling from endosomes (Shukla et al., 2014; Thomsen et al., 2016). Studies of the vasoactive intestinal peptide receptor 1 (VIPR1) similarly support a core-engaged inhibitory complex at the plasmalemma, but the proposed tail-engaged complex present on endosomes is evidently different from the endosomal complex formed with V2R (and PTH1R) because it does not detectably modulate receptor coupling to Gs on endosomes (Blythe and Von Zastrow, 2023). There is also evidence that different GPCR/β-arrestin conformations can exist in the same cellular membrane, determined through differences in the pattern of multisite receptor phosphorylation (Drube et al., 2022). Moreover, there is emerging evidence for the existence of another GPCR/β-arrestin complex that lacks Gs α-subunits and functions in trafficking G protein βγ-subcomplexes to endosomes (Sokrat et al., 2024).

Structural and biophysical approaches have provided significant insight into heterogeneity among GPCR/β-arrestin complexes, but clearly much more remains to be learned. What complexes actually form in vivo, and how are complexes remodeled during trafficking? Recent progress in our understanding regarding how GPCR/β-arrestin interactions can be experimentally manipulated (Barsi-Rhyne et al., 2022; Smith et al., 2021) and detected (Haider et al., 2022) in intact cells suggests a promising path toward bridging structural information to physiological signaling as it occurs in intact cells.

β-Arrestin–independent GPCR endocytosis. Until recently, it was widely believed that endosomal GPCR signaling required β-arrestin, even for receptors that do not engage β-arrestin after endocytosis, as β-arrestin was thought to be essential for their endocytosis. This view recently expanded after the demonstration that VIPR1 can efficiently internalize and trigger a second phase of G protein–mediated cAMP production from endosomes in cells lacking β-arrestins (Blythe and Von Zastrow, 2023). An essential starting point for understanding this additional diversity in GPCR cell biology is to elucidate the mechanistic basis of β-arrestin–independent GPCR endocytosis.

Regulated endocytosis of the glucagon-like peptide-1 receptor (GLP-1R) does not require β-arrestin, yet it is clathrin-dependent and regulated by GRK-mediated phosphorylation (McNeill et al., 2024). Similarly, endocytosis of the chemokine receptor CXCR4 is also clathrin- and GRK-dependent but requires sorting nexin 9 as an alternate endocytic adaptor, which promotes association of CXCR4 receptors with CCPs separately from β-arrestin (Robleto et al., 2024). The D2-dopamine receptor argues for yet more mechanistic diversity, as its dynamin-dependent endocytosis is promoted through interaction with myosin VI, independent of β-arrestin and receptor phosphorylation (Patel et al., 2024). How do different endocytic mechanisms or pathways impact GPCR signaling, and what signaling role(s)

do β-arrestins serve for GPCRs that internalize in a β-arrestin–independent manner? It is intriguing that neither GLP-1R nor VIPR1 requires β-arrestins to internalize, yet each recruits β-arrestin to both the plasmalemma and endosomes (Girada et al., 2017; Blythe and Von Zastrow, 2023). Biased agonists that have reduced ability to promote GLP-1R association with β-arrestin increase the duration of Gs-coupled cAMP production measured in cells after prolonged periods of ligand exposure (Jones et al., 2021). The significance of such interactions remains to be determined, but one possibility is that β-arrestin contributes to G protein–independent signaling processes on endomembranes. β-arrestin–mediated signaling from endomembranes was proposed before G protein–mediated signaling from endomembranes was discovered and may have important additional therapeutic implications (Luttrell et al., 1999; Ramirez-Garcia et al., 2023; DeFea et al., 2000).

GPCR trafficking itineraries. While many GPCRs localize primarily in the plasmalemma and rapidly internalize after agonist-induced activation, some receptors remain in the plasmalemma even after activation, and others exhibit constitutive internalization (Moo et al., 2021; Walker et al., 2024). Remarkable trafficking diversity is also observed in the biosynthetic pathway, with some GPCRs accumulating a reserve pool in the Golgi apparatus, which is then delivered to the plasmalemma in response to physiological regulation (Shiwarski et al., 2017). Among GPCRs that internalize and signal from endomembranes, there is evidence for still more diversity in behavior. An interesting example noted above is TSHR, which couples to Gs after endocytosis on a Golgi-related membrane distinct from endosomes (Godbole et al., 2017). Another interesting example is signaling triggered by LHR after endocytosis, which was shown to be initiated from endosomes marked by APPL1 rather than from EEA1-marked endosomes on which other GPCRs like β2AR engage G proteins. Mutations of LHR that redirect trafficking through EEA1-marked endosomes changed the kinetics of downstream signaling (Jean-Alphonse et al., 2014). Still another interesting aspect meriting further investigation is the regulation of membrane traffic at the ER–Golgi interface by local cAMP signaling (Cancino et al., 2014). Further study of the lateral organization of GPCR signaling complexes is also warranted. We have already mentioned long-standing evidence for lateral organization of GPCR signaling in the membrane bilayer by molecular scaffolding and lipid phase partitioning, and we note emerging evidence that higher order GPCR signaling complexes can also form in the bilayer through transient, low-affinity protein interactions (Zhang and MacKinnon, 2025).

GPCR posttranslational modification. Phosphorylation of many GPCRs impacts desensitization and endocytosis by promoting binding to β-arrestin, as we have discussed, but it can also have effects after endocytosis. For example, agonist-induced phosphorylation of a specific Ser residue in the distal cytoplasmic tail of β2AR is not needed for β2AR desensitization or endocytosis but biases postendocytic trafficking to lysosomes by destabilizing receptor association with retromer (Cao et al., 1999; Lauffer et al., 2010; Temkin et al., 2011). Agonist-induced ubiquitination of GPCRs can also bias trafficking to lysosomes by promoting receptor engagement of ESCRT (Henne et al., 2013; Hanyaloglu and von Zastrow, 2008; Marchese et al., 2003), but there are some exceptions to this behavior. For example, the μ-opioid receptor uses agonist-induced ubiquitination to engage ESCRT but does not require ubiquitination for delivery to lysosomes (Hislop et al., 2011), and GLP-1R is constitutively ubiquitinated and undergoes agonist-induced deubiquitination (Bitsi et al., 2023). Many (but not all) GPCRs are palmitoylated, and this modification has been linked to a variety of effects on GPCR function and localization (Chalhoub and McCormick, 2022). For example, palmitoylation of GLP-1R promotes receptor clustering in nanodomains of the plasmalemma that impact both receptor signaling and internalization (Buenaventura et al., 2019).

Toward an integrated understanding of physiological GPCR signaling

As mechanistic understanding continues to advance, key future directions are to investigate the cell biology of GPCR signaling in more complex tissue preparations and, ultimately, in intact animals. Significant progress has been made on this front with the development of genetically encoded biosensors for detecting the release of endogenous GPCR agonists (Patriarchi et al., 2019; Sun et al., 2018), and the use of genetically encoded biosensors to monitor cAMP and PKA activity levels in intact tissues (Zhang et al., 2021, 2025). Such tools are already having a huge impact on neuroscience by revealing, at cellular resolution, GPCR-mediated cAMP signaling processes that control complex behaviors (Singh Alvarado et al., 2024; Zhang et al., 2025). We anticipate that it will soon be feasible to extend such analysis to a level of subcellular resolution. Another important future direction is to scale such studies to a higher level of integration to investigate interactions between GPCR-triggered signaling pathways in more complex systems; this is beginning to be approached in the brain through the development of new multiplexed imaging and analysis methodologies (Chen et al., 2024).

Conclusion

In closing, we have provided a brief update of current knowledge regarding the spatiotemporal organization of ligand-dependent GPCR signaling and regulation in mammalian cells, beginning with the traditional schema depicted in Fig. 1 and chronicling key conceptual and methodological advances that have led the field since. Our current models, depicted in Fig. 3, remain "works in progress" with significant limitations and caveats. Surely, there is much more to be learned at this dynamic and important frontier of modern cell biology.

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