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MINIREVIEW

G Protein-coupled Receptor (GPCR) Signaling via Heterotrimeric G Proteins from Endosomes*

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Some G protein-coupled receptors (GPCRs), in addition to activating heterotrimeric G proteins in the plasma membrane, appear to elicit a "second wave" of G protein activation after ligand-induced internalization. We briefly summarize evidence supporting this view and then discuss what is presently known about the functional significance of GPCR-G protein activation in endosomes. Endosomal activation can shape the cellular response temporally by prolonging its overall duration, and may shape the response spatially by moving the location of intracellular second messenger production relative to effectors.

It has been more than 20 years since regulated endocytosis of GPCRs[3] through ligand-dependent concentration in coated pits was established (1, 2). Much has been learned since that time about the large number of GPCRs that engage this cellular regulatory mechanism, its biochemical underpinnings, and later events determining receptor-specific trafficking itineraries. However, our understanding of how endocytosis impacts canonical G protein-dependent signaling has remained unchanged. Fundamental to this paradigm is the belief that receptor-mediated activation of cognate heterotrimeric G proteins is restricted to the plasma membrane, and that internalized receptors are effectively silent with regard to this transduction mechanism.

Evidence accumulated over the past several years is beginning to challenge this view. Here we summarize data supporting an alternative hypothesis, that endosomes represent dynamic sites of GPCR-G protein activation. We then focus on what is beginning to be learned about the functional significance of the endosome signal, limiting scope to the relatively few GPCRs for which relevant data are presently available. When one considers this limitation, together with the remarkable diversity of membrane trafficking properties that distinguish even very similar GPCR homologs (e.g. Ref. 3) and splice variants (e.g. Ref. 4), it seems likely that much more remains to be learned.

Pathways and Mechanisms of GPCR Endocytic Trafficking

Detailed investigations of several GPCR family members have provided a reasonably clear outline of major events in the regulated endocytic trafficking of receptors (Fig. 1A). Binding of an agonist ligand present in the extracellular milieu promotes GPCR-dependent activation of cognate heterotrimeric G proteins associated with the inner leaflet of the plasma membrane. Ligand-activated receptors are preferred substrates for phosphorylation by GPCR kinases (GRKs). Phosphorylation on multiple residues in a specific phospho-acceptor sequence favors the subsequent interaction of receptors with β-arrestins (or "non-visual" arrestins) by a mechanism involving conformational change in both the receptor and arrestin (5–8). GRK-arrestin engagement contributes to shutting off the receptor's enzymatic activity as a guanine nucleotide exchange factor (GEF), preventing subsequent activation of G proteins and contributing to functional desensitization of cellular ligand responsiveness. β-Arrestins have multiple additional functions (9), including binding to lipid and protein components of coated pits (also called clathrin-coated pits). In this way, β-arrestins act as endocytic adaptor proteins promoting GPCR concentration in clathrin-coated pits, which subsequently internalize by dynamin-dependent membrane scission and are delivered to endosomes (5, 10).

In endosomes, GPCRs engage additional molecular sorting machineries that determine receptor-specific downstream trafficking itinerary. Three "core" sorting machineries have been identified so far.

The first core sorting machinery is the ubiquitin-ESCRT machinery, so named for a multi-protein "endosome sorting complex required for transport" (ESCRT) (11) that is conserved in yeast and animal cells as well as (at least in part) in protists and plants (12). This machinery recognizes a wide range of membrane proteins in the endosome limiting membrane according to the presence of covalently attached ubiquitin, mediating transfer to vesicles formed within the endosome lumen (13, 14).

The second core sorting machinery is the GASP machinery, so named for a putative "GPCR-associated sorting protein" (GASP) that binds various GPCR cytoplasmic tails without requiring ubiquitination (15). GASP connects to several endosome-associating proteins, including components of the ESCRT (16–18). GASP itself appears restricted to mammals, but its interaction partners are more widely distributed. This machinery is thought to reduce the lateral mobility of selected receptors in the endosome limiting membrane, thereby kinetically restricting receptor access to relatively short-lived mem-
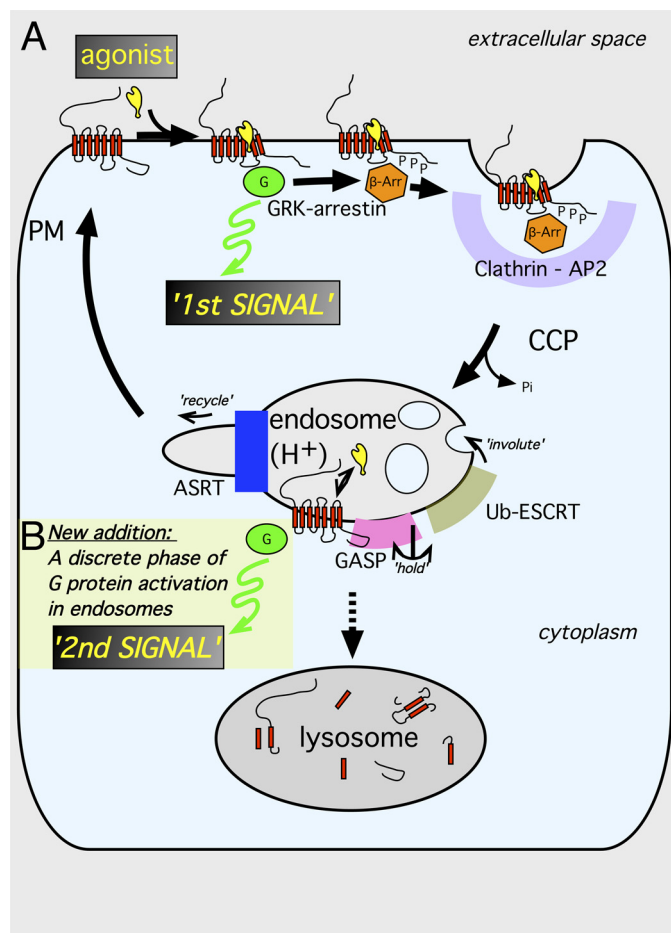
FIGURE 1. **GPCR endocytic trafficking relative to G protein-mediated signal initiation.** *A*, main steps of ligand-dependent activation of heterotrimeric G proteins, endocytosis and post-endocytic sorting of GPCRs in mammalian cells. *PM*, plasma membrane; *β-Arr*, β-arrestin; *Ub*, ubiquitin. *B*, the new element in this model is the existence of a discrete phase of GPCR-G protein activation in endosomes.

brane tubules that emanate from endosomes and mediate "bulk" membrane export and recycling (19).

The third core sorting machinery is the actin-sorting nexin 27-retromer tubule (ASRT) machinery, so named for three of its essential components but also associated with additional proteins (20, 21). This machinery is conserved in animals, and part (*e.g.* retromer subunits) but not all (*e.g.* sorting nexin 27) of it is found in yeast as well as in protists and plants (22). The ASRT machinery recognizes GPCRs (as well as a variety of other membrane proteins (21)) based on recognition of a C-terminal PDZ motif, driving selective exit from endosomes via a specialized population of membrane tubules for subsequent delivery either back to the plasma membrane (recycling pathway) or back to the Golgi apparatus (retrograde transport) (23, 24).

GPCR interaction with each of these core machineries is regulated by post-translational modification (25–28) as well as by non-covalent interactions with various (possibly many) other proteins including G proteins (*e.g.* Ref. 16) and arrestins (*e.g.* Refs. 29–31). The net result is that discrete *"involute," "hold,"* and *"recycle"* operations are executed at the endosome limiting membrane in a receptor-specific manner, subject to physiolog-

ical control through post-translational modification and non-covalent interactions, with each operation representing an elemental instruction in a conserved cellular program that flexibly and specifically determines receptor post-endocytic fate.

GPCR delivery to lysosomes (directed by engagement of ubiquitin-ESCRT and/or GASP machineries) promotes proteolysis and long-term down-regulation of cellular ligand responsiveness. Receptor recycling to the plasma membrane (directed by engagement of the ASRT machinery) promotes nondestructive return of internalized receptors to the plasma membrane, sustaining cellular ligand responsiveness in the prolonged presence of ligand or promoting the efficient recovery of responsiveness (resensitization) after ligand-induced desensitization.

The ability of endocytic membrane trafficking to adjust cellular GPCR responsiveness after prolonged or repeated activation is well established. What is new, and still controversial, is the idea that the endocytic network also contributes to the ligand-dependent signaling response itself by generating a discrete phase of GPCR and G protein activation in endosomes (Fig. 1*B*).

A Historical Bias against GPCR-G Protein Activation in Endosomes

The hypothesis that signaling can be initiated from endosomes long precedes even the recognition of regulated endocytosis of GPCRs (reviewed in Ref. 32). Over the ensuing years, GPCR endocytosis has been implicated in a wide variety of G protein-independent signaling mechanisms (reviewed in Ref. 33). However, GPCR signaling through activation of heterotrimeric G proteins was generally thought, or assumed, *not* to occur in endosomes. Fully tracing the historical development of this view is beyond the present scope. Here we briefly discuss three of the main current reservations, based on studies of the β_2 adrenergic receptor (β_2AR).

First, it is often assumed that internalized GPCRs are unable to couple to G proteins because they are highly phosphorylated in endosomes. Although phosphorylated β_2ARs indeed exist in both the plasma membrane and the endosomes of agonist-exposed cells (34, 35), β_2AR phosphorylation and dephosphorylation occur dynamically. Indeed, in early experiments using whole-cell metabolic labeling with [^{32}P]orthophosphate combined with subcellular fractionation, β_2ARs present in a light membrane fraction (likely endosomes) were found to be underphosphorylated relative to a faster-pelleting (likely plasma membrane) fraction (35). More recent data, derived from analysis of the native phosphorylation status of β_2ARs isolated from intact cells by mass spectrometry, revealed a remarkable degree of heterogeneity in receptor phosphorylation states under all conditions tested (36). Indeed, even in cells exposed to a saturating concentration of a full agonist ligand that drives maximal internalization, a considerable signal was detected corresponding to receptors fully unphosphorylated in the critical GRK acceptor sequence (26, 36, 37). Accordingly, there is presently no compelling evidence for extensive inactivation of the internalized β_2AR pool by phosphorylation.

Second, experimental manipulations reducing receptor engagement with the GRK-arrestin system, such as mutation of essential phosphorylation sites in the cytoplasmic acceptor sequence (37) or

depletion of relevant GRKs or arrestins (38), reduce β_2AR internalization but increase overall cytoplasmic cAMP accumulation. At first glance, this appears inconsistent with a significant endosome signal. A caveat is that such manipulations, by blocking the desensitization machinery, not only reduce endocytosis but aberrantly increase G protein activation in the plasma membrane. Indeed, as discussed further below, inhibiting endocytosis using more specific manipulations actually reduces net cAMP accumulation (39).

Third, endosome acidification has been proposed to preclude significant ligand-GPCR binding. A caveat to this belief is that the moderate acidity of early endocytic vesicles (pH ~6.5) is in a range compatible with high-affinity binding and ligand-dependent activation of β_2ARs *in vitro* (40), and titratable residue(s) in the β_2AR may themselves modulate receptor activity (41).

Evidence for a Non-canonical Mechanism of G Protein Activation in Endosomes

To our knowledge, the first positive evidence suggesting that G protein-linked signaling occurs from endosomes emerged through study of the mating response initiated by activation of the Ste2p GPCR in yeast. A screen of yeast knock-out strains identified a set of endosomal proteins supporting a late component of the mating response. Here it was concluded that a discrete signaling complex, including the G protein α subunit Gpa1 but devoid of conventional $\beta\gamma$ subunits, mediates this later signaling phase through Gpa1 activation in the endosome or vacuole membrane (42).

Early suggestions of endosomal G protein activation in mammalian cells emerged through investigation of the prolonged cellular effects of an anti-inflammatory drug on sphingosine-1 phosphate receptors (43), as well as the sustained actions of certain polypeptide ligands on receptors for thyroid-stimulating hormone (44) and parathyroid hormone (45). In these studies, G protein-linked signaling from endosomes appeared to be substantially delayed relative to the acute (presumably plasma membrane-derived) component, and it was poorly or slowly reversed after ligand removal from the culture medium. Together, these results support the occurrence of G protein activation from endosomes through a mechanism different from that occurring in the plasma membrane.

Explicit evidence for a distinct mechanism of sustained G protein activation in mammalian cells emerged from study of parathyroid hormone receptor signaling, in which a complex containing the GPCR together with arrestin and G protein $\beta\gamma$ subunits was identified (46). More recently, a similar complex has been linked to a sustained component of cAMP generation by vasopressin 2 receptors (47).

The sustained cAMP response that is ascribed to endosomal G protein activation also appears different in how it is turned off. Inactivation has been proposed to occur by receptor binding to the retromer complex, an essential component of the ASRT machinery discussed above in the context of GPCR endocytic sorting, perhaps via the retromer subunit VPS26 that is similar in tertiary structure to conventional arrestins (47, 48).

Early Evidence for Canonical GPCR-G Protein Activation in Endosomes

A study of mammalian D_1 dopamine receptors provided early evidence suggesting a rapid and reversible form of G protein activation in endosomes (49). Endocytic inhibitors reduced the magnitude of G_s-dependent cAMP accumulation elicited by receptor agonists in transfected fibroblastic cells and primary neuronal cultures. In both cell types, the cAMP response was rapid, and it reversed within minutes after agonist removal. Endocytic blockade also reduced a cAMP-dependent electrophysiological response elicited by endogenous D_1 receptor activation in brain slices. Again this effect was acute and rapidly reversible (49).

Direct Detection of GPCR and G Protein Activation in Endosomes Using Conformational Biosensors

A fundamental problem in interpreting all of the studies summarized above was their reliance on temporal correlation, together with possible complications of off-target or pleiotropic effects of endocytic inhibitors. Direct evidence for GPCR or G protein activation in endosomes was lacking, and alternative interpretations could not be ruled out (*e.g.* see discussions in Refs. 48 and 49).

Independent and arguably direct evidence emerged from experiments in which single-domain antibody fragments (nanobodies), developed initially as tools for structural investigations (50), were repurposed to function as genetically encoded "conformational biosensors" of discrete GPCR and G protein activation states in living cells (Fig. 2, *A–C*) (39).

The first conformational biosensor was developed from a nanobody raised against purified β_2ARs bound to an irreversible agonist. This nanobody, Nb80, appears to bind activated receptors selectively because it mimics the cognate G protein (G_s) α subunit in its nucleotide-free form (51, 52). The same nanobody, when present at a much lower concentration than in structural studies, can effectively detect the activated receptor conformation without forcing activation in the absence of agonist. By fusing Nb80 to the green fluorescent protein (Nb80-GFP), and expressing it as a genetically encoded intrabody at a suitably low concentration in the cytoplasm, ligand-dependent and reversible conformational activation of the β_2AR was successfully detected in living cells (39) (Fig. 2*B*).

Live-cell fluorescence imaging of Nb80-GFP localization revealed an interesting series of events. Agonist (isoproterenol) addition to cells first promoted Nb80-GFP recruitment to the plasma membrane, then β_2ARs clustered in coated pits apparently devoid of associated Nb80-GFP, and then Nb80-GFP was recruited to endosomes. Endosome recruitment of Nb80-GFP was visible several minutes after recruitment to the plasma membrane and occurred as a discrete second phase, after the delivery of receptors to endosomes devoid of bound nanobody (39).

Another biosensor was then generated, this one based on a distinct nanobody (Nb37) recognizing a helical region of the G_s α subunit that is not exposed in nucleotide-bound conformations, but which becomes mobile and exposed when the nucleotide binding pocket is empty (39). This is characteristic of α
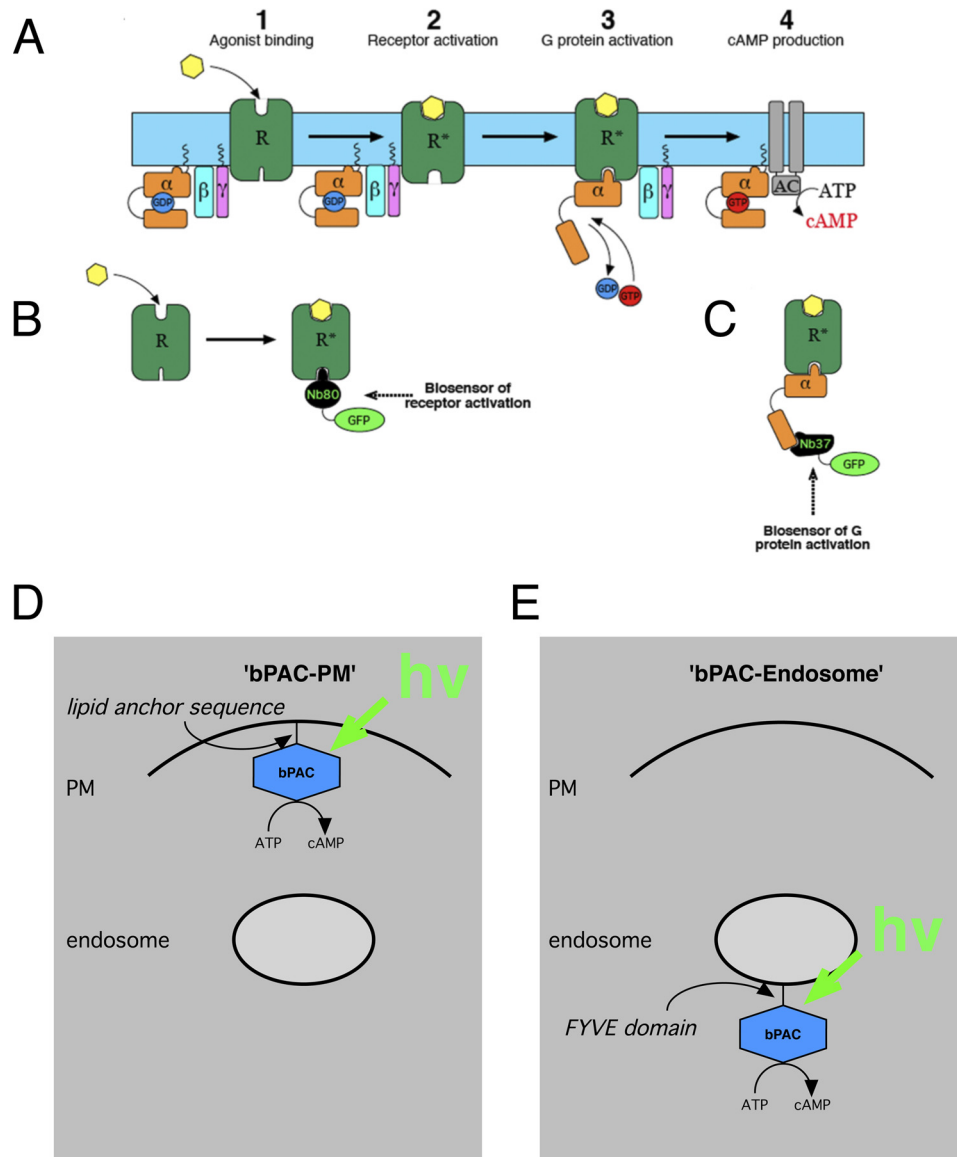
FIGURE 2. **New genetically encoded tools for probing and manipulating spatiotemporal signaling in living cells.** *A*, diagram depicting the canonical scheme of GPCR-mediated activation of adenylyl cyclase. *Steps 2* and *3* are the key steps of receptor and G protein activation probed by the biosensors. *B*, design of Nb80-GFP. *C*, design of Nb37-GFP. *D*, design of bPAC-PM for optogenetic activation of adenylyl cyclase activity on the plasma membrane (*PM*). *E*, design of bPAC-Endosome for optogenetic activation of adenylyl cyclase activity on the endosome limiting membrane.

subunit present in the canonical agonist-GPCR-G protein ternary complex, which is thought to represent the key catalytic intermediate in G protein activation (53).

Fusing Nb37 to the green fluorescent protein (Nb37-GFP) created another useful conformational biosensor (Fig. 2C). When expressed at a suitably low level, Nb37-GFP localized diffusely in the cytoplasm in the absence of β_2AR agonist. Agonist application initiated two phases of Nb37-GFP recruitment, first to the plasma membrane and then to endosomes ~1 min after receptor arrival. Both recruitment phases of Nb37-GFP, like those of Nb80-GFP, reversed rapidly and completely after agonist removal. Further, they were correlated with time-resolved components of β_2AR-mediated cAMP accumulation, with the second phase selectively sensitive to endocytic inhibitors (39).

Accordingly, it is now reasonably clear that activated GPCRs, and conformational activation of cognate G proteins, can

indeed occur in endosomes (Fig. 1B). For catecholamine receptors, this is rapid and reversible, as in the plasma membrane. Further, although some GPCRs associate with arrestins in endosomes, D_1 dopamine receptors and β_2ARs do so weakly or not at all (54). Moreover, nanobody-based biosensors suggest that similar protein conformational states accompany β_2AR and G_s activation in endosomes and the plasma membrane. The simplest interpretation of these findings, taken together, is that some GPCRs can activate G proteins in endosomes by a similar (or the same) mechanism as in the plasma membrane.

Evidence for Temporal and Spatial Consequences of Endosomal GPCR-G Protein Activation

If endosomes are indeed sites of *bona fide* GPCR-G protein activation, what is the functional significance of the endosome signal? This is a fascinating question that is only

beginning to be addressed. Evidence available so far supports two main effects.

First, endosome-based activation confers temporal control on the cellular response. GPCR-G protein activation in endosomes has been observed consistently to extend the duration of the response and, in some studies, has been reported to sustain the response after ligand removal. The downstream manifestations of these temporal effects remain largely unexplored but, considering how many physiological processes depend on signal timing, they are likely widespread (*e.g.* Refs. 44, 45, 55, and 56). Temporal effects may also be important to mediate the therapeutic or toxic actions of drugs, particularly high-affinity compounds that remain associated with target GPCRs for long periods of time (*e.g.* Ref. 43).

Second, emerging evidence suggests that endosome-based activation can confer a discrete type of spatial control on the cellular response. Endocytic inhibitors were found to reduce the magnitude of β_2AR-elicited induction of a large repertoire of cAMP-dependent genes, including for example PCK1, the gene encoding phosphoenolpyruvate carboxykinase 1 that determines the rate of gluconeogenesis. This effect did not correlate with changes in the overall level of cytoplasmic cAMP accumulation and was associated with reduced phosphorylation of the cAMP-response element-binding protein (CREB) that drives overall cAMP-dependent transcriptional induction. Together, these results suggest that GPCR-G protein activation in endosomes confers a discrete type of spatial control over the specificity of downstream signaling, likely by increasing the efficiency of cAMP-dependent phosphorylation of CREB through physical proximity (57).

An Optogenetic Strategy to Selectively Probe Spatial Effects of the Endosome Signal

Definitively testing the "spatial encoding" hypothesis was not possible using only endocytic blockade, for similar reasons that this approach was limited for initially detecting GPCR-G protein activation in endosomes. In addition, because endocytic inhibitors inherently alter temporal properties of the cellular signal, a primary spatial effect of endocytic blockade is difficult to resolve from secondary consequences of altering the signal in time. Thus an orthogonal approach was developed to test spatial effects directly.

To do so, a bacteria-derived photoactivated adenylyl cyclase (bPAC) (58) was engineered to localize either to the plasma membrane (Fig. 2*D*) or to the endosomes (Fig. 2*E*) using established targeting sequences. Under illumination conditions adjusted to produce similar elevations of overall cytoplasmic cAMP concentration from each location, recombinant adenylyl cyclase activated on endosomes was found to induce cAMP-dependent transcription much more efficiently than adenylyl cyclase activated on the plasma membrane. However, when cells were exposed to rolipram, a chemical inhibitor of phosphodiesterase-4 enzymes concentrated in the peripheral cytoplasm and associated with the plasma membrane (59), adenylyl cyclase localized to the plasma membrane strongly induced transcription (57).

These findings provide independent and arguably direct support for the hypothesis that cAMP generated from endosomes indeed confers a discrete spatial effect on the downstream cellular response. In essence, receptor-containing endosomes appear to function as flexible signal delivery vehicles that move the site of intracellular second messenger production in proximity to a relevant effector (such as CREB), with local phosphodiesterase activity setting the distance scale over which effective signal transduction can occur (Fig. 3).

Conclusion and Future Perspectives

There is now reasonably strong evidence indicating that some GPCRs, in addition to initiating ligand-dependent signal transduction by coupling to heterotrimeric G proteins in the plasma membrane, can also activate cognate G proteins after endocytosis. GPCR and G protein activation in endosomes appears, at first glance, to contradict long-held ideas regarding the cellular basis of GPCR desensitization. However, this emerging view is compatible with previous understanding if one recognizes selective downstream effector coupling of the endosome-derived signal.

Many interesting questions are posed by these developments. First, the overall functional significance of endosomal GPCR-G protein activation remains a critical question that has been only partially addressed, and very little is known about it in native cell types or tissues. Second, the range of G protein-linked transduction machinery that can be engaged from endosomes is presently unknown. In particular, the identity and subcellular distribution of specific adenylyl cyclase(s) responsible for the endocytosis-dependent signaling effects discussed in the present review remain undefined. Another open question is whether more than one mechanism of GPCR-G protein activation operates in endosomes. The present data support the existence of two mechanisms, differing in kinetics and reversibility, and mediated through the formation of biochemically distinct signaling complexes on the endosome limiting membrane. Do discrete mechanisms of G protein activation operate from the same or different endosomes, and are their functions redundant or distinct? Another fascinating question, which is only beginning to be explored, is how particular mechanism(s) of endosome-based signaling are terminated. Considering the long-recognized importance of GPCR-G protein signal termination at the plasma membrane (7, 8), this would seem a critical future direction. Yet another interesting direction is toward investigating broader implications of endomembrane G protein activation, such as its recently proposed role in homeostatic control of the biosynthetic pathway by the seven-transmembrane KDEL cargo receptor through receptor-mediated activation of G_s in the Golgi apparatus (60).

The present review has focused specifically on GPCR signaling via heterotrimeric G proteins. As noted briefly above, there is also considerable previous evidence suggesting that endosomes support various G protein-independent transduction mechanisms, particularly those initiated by GPCRs that (unlike D_1 dopamine receptors and β_2ARs) strongly associate with arrestins after internalization (reviewed in Refs. 9 and 33). It
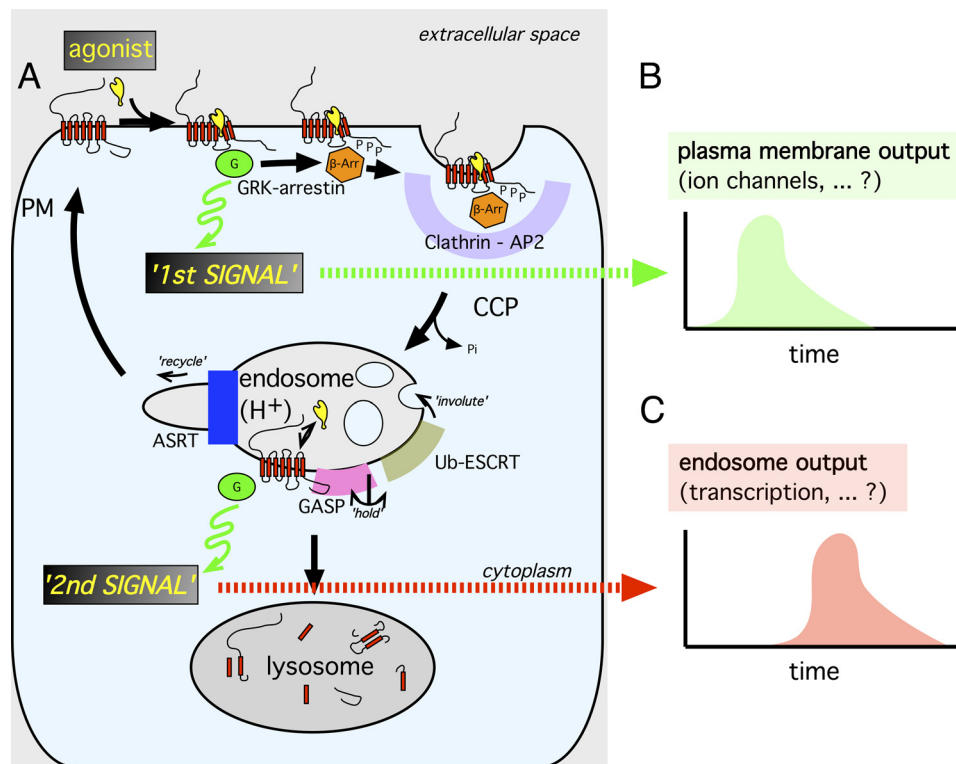
FIGURE 3. **Temporal and spatial consequences of signaling from endosomes.** *A*, endocytic trafficking map as shown in Fig. 1, with plasma membrane (*PM*)- and endosome-initiated signals extracted to the *right*. β-Arr, β-arrestin; CCP, clathrin-coated pit; *Ub*, ubiquitin. *B*, The plasma membrane signal is spatially coupled to effectors near or in the plasma membrane, such as ion channels. The graph depicts a time course of the acute signaling response. *C*, the endosome signal is preferentially coupled by spatial proximity to transcriptional control and perhaps other intracellular effectors not presently defined. The timing of this signal is delayed and, for some GPCRs, sustained relative to the plasma membrane signal.

appears, indeed, that much remains to be learned about how the endocytic network impacts cellular signaling mediated by the large and diverse GPCR family.

REFERENCES

1. von Zastrow, M., and Kobilka, B. K. (1992) Ligand-regulated internalization and recycling of human β$_2$-adrenergic receptors between the plasma membrane and endosomes containing transferrin receptors. *J. Biol. Chem.* **267,** 3530–3538
2. von Zastrow, M., and Kobilka, B. K. (1994) Antagonist-dependent and -independent steps in the mechanism of adrenergic receptor internalization. *J. Biol. Chem.* **269,** 18448–18452
3. von Zastrow, M., Link, R., Daunt, D., Barsh, G., and Kobilka, B. (1993) Subtype-specific differences in the intracellular sorting of G protein-coupled receptors. *J. Biol. Chem.* **268,** 763–766
4. Tanowitz, M., Hislop, J. N., and von Zastrow, M. (2008) Alternative splicing determines the post-endocytic sorting fate of G-protein-coupled receptors. *J. Biol. Chem.* **283,** 35614–35621
5. Goodman, O. B., Jr., Krupnick, J. G., Santini, F., Gurevich, V. V., Penn, R. B., Gagnon, A. W., Keen, J. H., and Benovic, J. L. (1998) Role of arrestins in G-protein-coupled receptor endocytosis. *Adv. Pharmacol.* **42,** 429–433
6. Shukla, A. K., Westfield, G. H., Xiao, K., Reis, R. I., Huang, L.-Y., Tripathi-Shukla, P., Qian, J., Li, S., Blanc, A., Oleskie, A. N., Dosey, A. M., Su, M., Liang, C.-R., Gu, L.-L., Shan, J.-M., Chen, X., Hanna, R., Choi, M., Yao, X. J., Klink, B. U., Kahsai, A. W., Sidhu, S. S., Koide, S., Penczek, P. A., Kossiakoff, A. A., Woods, V. L., Jr., Kobilka, B. K., Skiniotis, G., and Lefkowitz, R. J. (2014) Visualization of arrestin recruitment by a G-protein-coupled receptor. *Nature* **512,** 218–222
7. Lefkowitz, R. J., Pitcher, J., Krueger, K., and Daaka, Y. (1998) Mechanisms of β-adrenergic receptor desensitization and resensitization. *Adv. Pharmacol.* **42,** 416–420
8. Krupnick, J. G., and Benovic, J. L. (1998) The role of receptor kinases and arrestins in G protein-coupled receptor regulation. *Annu. Rev. Pharmacol. Toxicol.* **38,** 289–319
9. Shenoy, S. K., and Lefkowitz, R. J. (2011) β-Arrestin-mediated receptor trafficking and signal transduction. *Trends Pharmacol. Sci.* **32,** 521–533
10. Ferguson, S. S., Zhang, J., Barak, L. S., and Caron, M. G. (1998) Role of β-arrestins in the intracellular trafficking of G-protein-coupled receptors. *Adv. Pharmacol.* **42,** 420–424
11. Katzmann, D. J., Babst, M., and Emr, S. D. (2001) Ubiquitin-dependent sorting into the multivesicular body pathway requires the function of a conserved endosomal protein sorting complex, ESCRT-I. *Cell* **106,** 145–155
12. Schmidt, O., and Teis, D. (2012) The ESCRT machinery. *Curr. Biol.* **22,** R116–20
13. Henne, W. M., Buchkovich, N. J., and Emr, S. D. (2011) The ESCRT pathway. *Dev. Cell* **21,** 77–91
14. Raiborg, C., and Stenmark, H. (2009) The ESCRT machinery in endosomal sorting of ubiquitylated membrane proteins. *Nature* **458,** 445–452
15. Whistler, J. L., Enquist, J., Marley, A., Fong, J., Gladher, F., Tsuruda, P., Murray, S. R., and von Zastrow, M. (2002) Modulation of postendocytic

sorting of G protein-coupled receptors. *Science* **297,** 615–620

16. Rosciglione, S., Thériault, C., Boily, M.-O., Paquette, M., and Lavoie, C. (2014) Gα$_s$ regulates the post-endocytic sorting of G protein-coupled receptors. *Nat. Commun.* **5,** 4556

17. Marley, A., and von Zastrow, M. (2010) Dysbindin promotes the post-endocytic sorting of G protein-coupled receptors to lysosomes. *PLoS One* **5,** e9325

18. He, C., Wei, Y., Sun, K., Li, B., Dong, X., Zou, Z., Liu, Y., Kinch, L. N., Khan, S., Sinha, S., Xavier, R. J., Grishin, N. V., Xiao, G., Eskelinen, E.-L., Scherer, P. E., Whistler, J. L., and Levine, B. (2013) Beclin 2 functions in autophagy, degradation of G protein-coupled receptors, and metabolism. *Cell* **154,** 1085–1099

19. Puthenveedu, M. A., Lauffer, B., Temkin, P., Vistein, R., Carlton, P., Thorn, K., Taunton, J., Weiner, O. D., Parton, R. G., and von Zastrow, M. (2010) Sequence-dependent sorting of recycling proteins by actin-stabilized endosomal microdomains. *Cell* **143,** 761–773

20. Temkin, P., Lauffer, B., Jäger, S., Cimermancic, P., Krogan, N. J., and von Zastrow, M. (2011) SNX27 mediates retromer tubule entry and endosome-to-plasma membrane trafficking of signalling receptors. *Nat. Cell Biol.* **13,** 715–721

21. Steinberg, F., Gallon, M., Winfield, M., Thomas, E. C., Bell, A. J., Heesom, K. J., Tavaré, J. M., and Cullen, P. J. (2013) A global analysis of SNX27-retromer assembly and cargo specificity reveals a function in glucose and metal ion transport. *Nat. Cell Biol.* **15,** 461–471

22. Koumandou, V. L., Klute, M. J., Herman, E. K., Nunez-Miguel, R., Dacks, J. B., and Field, M. C. (2011) Evolutionary reconstruction of the retromer complex and its function in Trypanosoma brucei. *J. Cell Sci.* **124,** 1496–1509

23. Bonifacino, J. S., and Rojas, R. (2006) Retrograde transport from endosomes to the trans-Golgi network. *Nat. Rev. Mol. Cell Biol.* **7,** 568–579

24. Seaman, M. N. J. (2012) The retromer complex: endosomal protein recycling and beyond. *J. Cell Sci.* **125,** 4693–4702

25. Cao, T. T., Deacon, H. W., Reczek, D., Bretscher, A., and von Zastrow, M. (1999) A kinase-regulated PDZ-domain interaction controls endocytic sorting of the β$_2$-adrenergic receptor. *Nature* **401,** 286–290

26. Nobles, K. N., Xiao, K., Ahn, S., Shukla, A. K., Lam, C. M., Rajagopal, S., Strachan, R. T., Huang, T.-Y., Bressler, E. A, Hara, M. R., Shenoy, S. K., Gygi, S. P., and Lefkowitz, R. J. (2011) Distinct phosphorylation sites on the β$_2$-adrenergic receptor establish a barcode that encodes differential functions of β-arrestin. *Sci. Signal.* **4,** ra51

27. Vistein, R., and Puthenveedu, M. A. (2013) Reprogramming of G protein-coupled receptor recycling and signaling by a kinase switch. *Proc. Natl. Acad. Sci. U.S.A.* **110,** 15289–15294

28. Yudowski, G. A., Puthenveedu, M. A., Henry, A. G., and von Zastrow, M. (2009) Cargo-mediated regulation of a rapid Rab4-dependent recycling pathway. *Mol. Biol. Cell* **20,** 2774–2784

29. Han, S.-O., Kommaddi, R. P., and Shenoy, S. K. (2013) Distinct roles for β-arrestin2 and arrestin-domain-containing proteins in β$_2$ adrenergic receptor trafficking. *EMBO Rep.* **14,** 164–171

30. Malik, R., and Marchese, A. (2010) Arrestin-2 interacts with the endosomal sorting complex required for transport machinery to modulate endosomal sorting of CXCR4. *Mol. Biol. Cell* **21,** 2529–2541

31. Audet, N., Charfi, I., Mnie-Filali, O., Amraei, M., Chabot-Doré, A.-J., Millecamps, M., Stone, L. S., and Pineyro, G. (2012) Differential association of receptor-Gβγ complexes with β-arrestin2 determines recycling bias and potential for tolerance of δ opioid receptor agonists. *J. Neurosci.* **32,** 4827–4840

32. Posner, B. I., and Bergeron, J. J. M. (2014) Assessment of internalization and endosomal signaling: studies with insulin and EGF. *Methods Enzymol.* **535,** 293–307

33. Irannejad, R., and von Zastrow, M. (2014) GPCR signaling along the endocytic pathway. *Curr. Opin. Cell Biol.* **27,** 109–116

34. Tran, T. M., Friedman, J., Baameur, F., Knoll, B. J., Moore, R. H., and Clark, R. B. (2007) Characterization of β$_2$-adrenergic receptor dephosphorylation: comparison with the rate of resensitization. *Mol. Pharmacol.* **71,** 47–60

35. Sibley, D. R., Strasser, R. H., Benovic, J. L., Daniel, K., and Lefkowitz, R. J. (1986) Phosphorylation/dephosphorylation of the β-adrenergic receptor regulates its functional coupling to adenylate cyclase and subcellular distribution. *Proc. Natl. Acad. Sci. U.S.A.* **83,** 9408–9412

36. Trester-Zedlitz, M., Burlingame, A., Kobilka, B., and von Zastrow, M. (2005) Mass spectrometric analysis of agonist effects on posttranslational modifications of the β-2 adrenoceptor in mammalian cells. *Biochemistry* **44,** 6133–6143

37. Hausdorff, W. P., Campbell, P. T., Ostrowski, J., Yu, S. S., Caron, M. G., and Lefkowitz, R. J. (1991) A small region of the β-adrenergic receptor is selectively involved in its rapid regulation. *Proc. Natl. Acad. Sci. U.S.A.* **88,** 2979–2983

38. Violin, J. D., DiPilato, L. M., Yildirim, N., Elston, T. C., Zhang, J., and Lefkowitz, R. J. (2008) β$_2$-adrenergic receptor signaling and desensitization elucidated by quantitative modeling of real time cAMP dynamics. *J. Biol. Chem.* **283,** 2949–2961

39. Irannejad, R., Tomshine, J. C., Tomshine, J. R., Chevalier, M., Mahoney, J. P., Steyaert, J., Rasmussen, S. G. F., Sunahara, R. K., El-Samad, H., Huang, B., and von Zastrow, M. (2013) Conformational biosensors reveal GPCR signalling from endosomes. *Nature* **495,** 534–538

40. Ghanouni, P., Schambye, H., Seifert, R., Lee, T. W., Rasmussen, S. G., Gether, U., and Kobilka, B. K. (2000) The effect of pH on β$_2$ adrenoceptor function: evidence for protonation-dependent activation. *J. Biol. Chem.* **275,** 3121–3127

41. Dror, R. O., Arlow, D. H., Maragakis, P., Mildorf, T. J., Pan, A. C., Xu, H., Borhani, D. W., and Shaw, D. E. (2011) Activation mechanism of the β$_2$-adrenergic receptor. *Proc. Natl. Acad. Sci. U.S.A.* **108,** 18684–18689

42. Slessareva, J. E., Routt, S. M., Temple, B., Bankaitis, V. A, and Dohlman, H. G. (2006) Activation of the phosphatidylinositol 3-kinase Vps34 by a G protein α subunit at the endosome. *Cell* **126,** 191–203

43. Mullershausen, F., Zecri, F., Cetin, C., Billich, A., Guerini, D., and Seuwen, K. (2009) Persistent signaling induced by FTY720-phosphate is mediated by internalized S1P1 receptors. *Nat. Chem. Biol.* **5,** 428–434

44. Calebiro, D., Nikolaev, V. O., Gagliani, M. C., de Filippis, T., Dees, C., Tacchetti, C., Persani, L., and Lohse, M. J. (2009) Persistent cAMP-signals triggered by internalized G-protein-coupled receptors. *PLoS Biol.* **7,** e1000172

45. Ferrandon, S., Feinstein, T. N., Castro, M., Wang, B., Bouley, R., Potts, J. T., Gardella, T. J., and Vilardaga, J.-P. (2009) Sustained cyclic AMP production by parathyroid hormone receptor endocytosis. *Nat. Chem. Biol.* **5,** 734–742

46. Wehbi, V. L., Stevenson, H. P., Feinstein, T. N., Calero, G., Romero, G., and Vilardaga, J.-P. (2013) Noncanonical GPCR signaling arising from a PTH receptor-arrestin-Gβγ complex. *Proc. Natl. Acad. Sci. U.S.A.* **110,** 1530–1535

47. Feinstein, T. N., Yui, N., Webber, M. J., Wehbi, V. L., Stevenson, H. P., King, J. D., Jr., Hallows, K. R., Brown, D., Bouley, R., and Vilardaga, J.-P. (2013) Noncanonical control of vasopressin receptor type 2 signaling by retromer and arrestin. *J. Biol. Chem.* **288,** 27849–27860

48. Feinstein, T. N., Wehbi, V. L., Ardura, J. A., Wheeler, D. S., Ferrandon, S., Gardella, T. J., and Vilardaga, J. P. (2011) Retromer terminates the generation of cAMP by internalized PTH receptors. *Nat. Chem. Biol.* **7,** 278–284

49. Kotowski, S. J., Hopf, F. W., Seif, T., Bonci, A., and von Zastrow, M. (2011) Endocytosis promotes rapid dopaminergic signaling. *Neuron* **71,** 278–290

50. Steyaert, J., and Kobilka, B. K. (2011) Nanobody stabilization of G protein-coupled receptor conformational states. *Curr. Opin. Struct. Biol.* **21,** 567–572

51. Rasmussen, S. G. F., Choi, H.-J., Fung, J. J., Pardon, E., Casarosa, P., Chae, P. S., Devree, B. T., Rosenbaum, D. M., Thian, F. S., Kobilka, T. S., Schnapp, A., Konetzki, I., Sunahara, R. K., Gellman, S. H., Pautsch, A., Steyaert, J., Weis, W. I., and Kobilka, B. K. (2011) Structure of a nanobody-stabilized active state of the β$_2$ adrenoceptor. *Nature* **469,** 175–180

52. Rasmussen, S. G. F., DeVree, B. T., Zou, Y., Kruse, A. C., Chung, K. Y., Kobilka, T. S., Thian, F. S., Chae, P. S., Pardon, E., Calinski, D., Mathiesen, J. M., Shah, S. T. A., Lyons, J. A., Caffrey, M., Gellman, S. H., Steyaert, J., Skiniotis, G., Weis, W. I., Sunahara, R. K., and Kobilka, B. K. (2011) Crystal structure of the β$_2$ adrenergic receptor-G$_s$ protein complex. *Nature* **477,** 549–555

53. Westfield, G. H., Rasmussen, S. G. F., Su, M., Dutta, S., DeVree, B. T., Chung, K. Y., Calinski, D., Velez-Ruiz, G., Oleskie, A. N., Pardon, E., Chae, P. S., Liu, T., Li, S., Woods, V. L., Jr., Steyaert, J., Kobilka, B. K., Sunahara, R. K., and Skiniotis, G. (2011) Structural flexibility of the Gα_s α-helical domain in the β_2-adrenoceptor G$_s$ complex. *Proc. Natl. Acad. Sci. U.S.A.* **108,** 16086–16091

54. Oakley, R. H., Laporte, S. A., Holt, J. A., Caron, M. G., and Barak, L. S. (2000) Differential affinities of visual arrestin, βarrestin1, and βarrestin2 for G protein-coupled receptors delineate two major classes of receptors. *J. Biol. Chem.* **275,** 17201–17210

55. Vilardaga, J.-P., Gardella, T. J., Wehbi, V. L., and Feinstein, T. N. (2012) Non-canonical signaling of the PTH receptor. *Trends Pharmacol. Sci.* **33,** 423–431

56. Lohse, M. J., and Calebiro, D. (2013) Cell biology: Receptor signals come in waves. *Nature* **495,** 457–458

57. Tsvetanova, N. G., and von Zastrow, M. (2014) Spatial encoding of cyclic AMP signaling specificity by GPCR endocytosis. *Nat. Chem. Biol.* **10,** 1061–1065

58. Stierl, M., Stumpf, P., Udwari, D., Gueta, R., Hagedorn, R., Losi, A., Gärtner, W., Petereit, L., Efetova, M., Schwarzel, M., Oertner, T. G., Nagel, G., and Hegemann, P. (2011) Light modulation of cellular cAMP by a small bacterial photoactivated adenylyl cyclase, bPAC, of the soil bacterium *Beggiatoa. J. Biol. Chem.* **286,** 1181–1188

59. Conti, M., and Beavo, J. (2007) Biochemistry and physiology of cyclic nucleotide phosphodiesterases: essential components in cyclic nucleotide signaling. *Annu. Rev. Biochem.* **76,** 481–511

60. Cancino, J., Capalbo, A., Di Campli, A., Giannotta, M., Rizzo, R., Jung, J. E., Di Martino, R., Persico, M., Heinklein, P., Sallese, M., and Luini, A. (2014) Control systems of membrane transport at the interface between the endoplasmic reticulum and the Golgi. *Dev. Cell* **30,** 280–294