Spatial resolution of cAMP signaling by soluble adenylyl cyclase

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G protein–coupled receptor signaling starts at the plasma membrane and continues at endosomal stations. In this issue, Inda et al. (2016. *J. Cell Biol.* http://dx.doi.org/10.1083/jcb.201512075) show that different forms of adenylyl cyclase are activated at the plasma membrane versus endosomes, providing a rationale for the spatial encoding of cAMP signaling.

The spatial encoding of receptor-mediated signaling is commonly achieved through the physical association of signaling molecules with different biomembranes along the endocytic pathway (Sigismund et al., 2012). Although endocytosis has long been connected with signaling attenuation, endosomes are emerging as heterogeneous and versatile signaling platforms that allow signals originated from the plasma membrane (PM) to be sustained or differentiated (Sigismund et al., 2012). Indeed, to achieve long-term activation and a productive downstream biological response, many signaling receptors require endosomal sorting and prolonged signaling from the endosomal compartment (Sigismund et al., 2008; Calebiro et al., 2009; Ferrandon et al., 2009; Brankatschk et al., 2012; Irannejad et al., 2013; Villaseñor et al., 2015). Thus, the same type of signaling that originated at the PM can be continued at the endosomal station.

Endosomes are also sites for the initiation of specific signaling. Certain signaling complexes are differentially assembled at the PM versus the endosomal membrane, allowing for the spatial diversification of signaling. Furthermore, different studies have highlighted the existence of distinct populations of endosomes that have receptor-specific sorting mechanisms that contribute to the generation of signal diversity (Jean-Alphonse et al., 2014; Ménard et al., 2014; Kalaidzidis et al., 2015).

An additional layer of regulation stems from the fact that signaling receptors, including receptor tyrosine kinases and G protein–coupled receptors (GPCRs), can directly regulate the endosomal population that they traffic to, in terms of endosome number and their molecular composition. In this way, receptors directly control the outcome of their signaling output in a feedback loop mechanism (Collinet et al., 2010). For instance, epidermal growth factor receptor (EGFR) signaling controls the endosomal system by directly acting on the fusion/fission machinery, thereby controlling the number of endosomes and, consequently, the packing of active receptors into the endosomal membrane. A constant EGFR/endosome ratio is thus maintained across a wide range of EGF/EGFR levels, providing robustness to EGFR signaling (Villaseñor et al., 2015).

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These concepts have recently been reevaluated in the case of GPCR signaling. Canonical GPCR signaling starts at the PM, where ligand binding activates GPCR to act as a guanine nucleotide exchange factor on the associated G protein complex. Once GTP is loaded, the stimulatory Gα subunit is released from the complex to activate adenylyl cyclase (AC). GPCRs then undergo phosphorylation and β-arrestin recruitment, followed by the uncoupling of trimeric G proteins and receptor endocytosis (Irannejad and von Zastrow, 2014). The acknowledged view was that endosome-localized GPCRs are signaling incompetent in terms of second messenger signaling. However, this view was challenged by several studies, which reported that the generation of cAMP from activated GPCRs can also be initiated at the endosomal station (Calebiro et al., 2009; Ferrandon et al., 2009; Kotowski et al., 2011; Irannejad et al., 2013). More recently, thanks to the use of conformational biosensors that allow activated GPCRs and trimeric G proteins to be detected, this issue was finally resolved. It was shown that the full transcriptional response triggered by β2-adernergic receptor activation is endocytosis dependent and therefore relies on the subcellular site of cAMP production by AC (Tsvetanova and von Zastrow, 2014). Indeed, cAMP produced at endosomal sites was shown to be specifically required for the efficient activation of the β2-adernergic receptor downstream transcriptional response (Tsvetanova and von Zastrow, 2014).

Additional complexity arises from the existence of diverse forms of ACs, transmembrane AC (tmAC) and soluble AC (sAC), which provide a potential basis for further signal diversification. There are nine known tmACs that could, in principle, contribute to the diversification of the signaling response from different GPCRs (Schmid et al., 2014). Moreover, membrane compartmentalization of tmACs (e.g., PM vs. endosomes) could be used by cells to spatially control cAMP production. In contrast, there is only one sAC gene, although by alternative splicing multiple isoforms can be generated with potentially distinct regulatory mechanisms (Schmid et al., 2014). Although GPCR-mediated cAMP production was deemed solely dependent on tmAC, a possible involvement of sAC in GPCR signaling has recently emerged (Ivonnet et al., 2015).

The work reported by Inda et al. in this issue integrates and expands our emerging knowledge of localized GPCR signaling at the endosomal level. In a previous work, these authors showed that Erk activation by corticotropin-releasing hormone receptor 1 (CRHR1) signaling is biphasic, with an early re-

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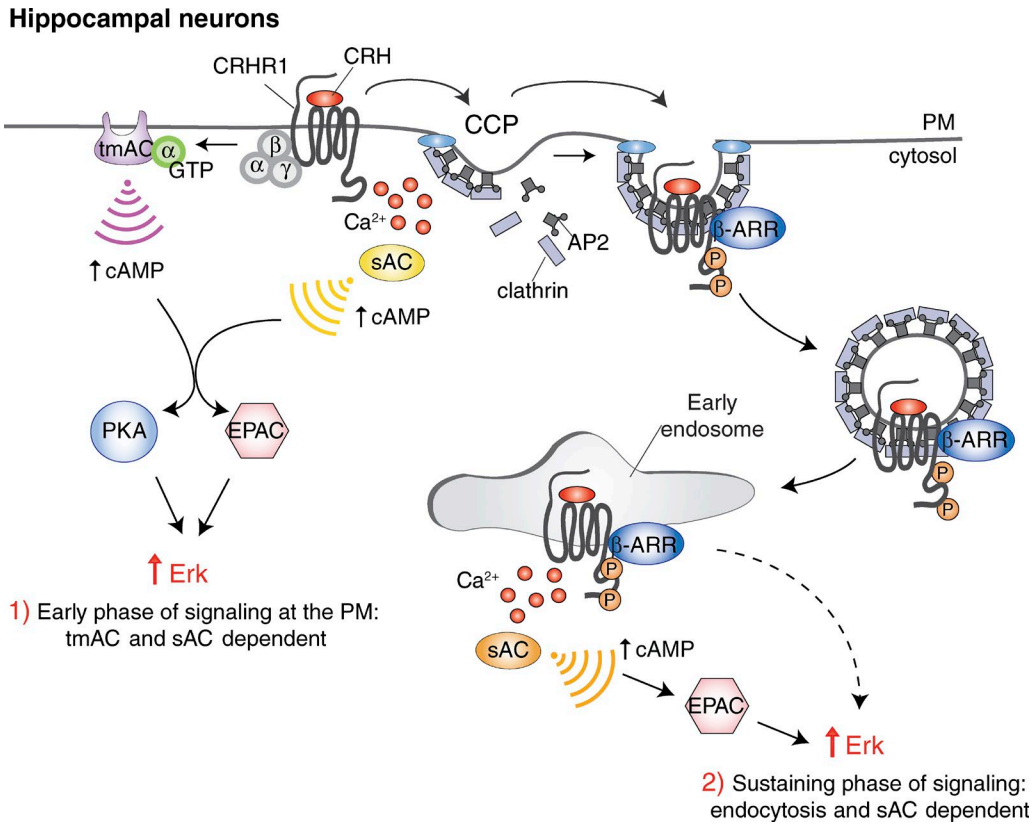

Figure 1. **Spatial and temporal control of GPCR signaling by ACs.** In hippocampal neurons, CRHR1, upon binding to agonist CRH, activates the trimeric G protein complex, composed of α, β, and γ subunits. Stimulatory Gα subunit is released and activates tmAC and cAMP production (Irannejad and von Zastrow, 2014). In parallel, activated GPCR can also induce local Ca^{2+} release that activates sAC, followed by an increase in cAMP levels (Inda et al., 2016). (1) These two sources of cAMP at the PM contribute to the early phase of ERK activation via the effectors protein kinase A (PKA) and EPACs (Inda et al., 2016). Activated GPCRs once released from the G proteins are phosphorylated and recruit β-arrestin (β-ARR) to be internalized via clathrin-coated pits (CCPs; Irannejad and von Zastrow, 2014). (2) At the endosomal station, GPCRs activate sAC to trigger a second wave of cAMP production and to sustain Erk signaling in an EPAC-dependent fashion. sAC activation at the PM and at endosomes requires Ca^{2+} and bicarbonate (not depicted for simplicity; Inda et al., 2016). The signaling cascade leading to Ca^{2+} and bicarbonate release (particularly at the endosomal station) and the exact mechanism of action of sAC are not completely characterized. The different forms of ACs, and the corresponding cAMP gradients generated by them, are depicted in different colors to highlight their different roles. Whether this specificity reflects different isoforms and/or regulation remains to be clarified.

sponse originating from the PM and a late response that is endocytosis dependent (Bonfiglio et al., 2013). In their new work, Inda et al. (2016) reinforce these findings, showing that distinct cAMP sources are differentially involved in the two phases of Erk signaling. Whereas tmAC and sAC both contribute to the acute Erk signaling response, sAC is specifically involved in the sustained "endocytic" phase of Erk signaling in hippocampal neuronal cells (Fig. 1). Using a Förster resonance energy transfer–based biosensor to measure cAMP production, Inda et al. (2016) demonstrate that cellular cAMP production by CRHR1 largely depends on endocytosis, as the inhibition of receptor internalization strongly affected cAMP production. Importantly, sAC, but not tmAC, is essential for endocytosis-dependent cAMP production at the endosomes. Thus, the idea that spatial signaling constraints are provided through the anchoring of signaling molecules to endomembranes is challenged by the work of Inda et al. (2016), which shows that a cytoplasmic molecule (sAC) can also provide spatial signaling resolution.

Importantly, Inda et al. (2016) identified the cAMP effectors upstream of Erk activation: protein kinase A and exchange proteins activated by cAMP (EPACs). These effectors both act downstream of tmACs and sAC in the first phase of Erk signaling at the PM. However, only EPAC appears to respond to cAMP produced at the endosome and it is the only cAMP effector required for the endocytosis-dependent phase of Erk signaling, again indicating a mechanism for spatial constraint (Fig. 1).

One major issue is how spatial restriction is achieved in the case of a soluble enzyme. Indeed, whereas tmACs are localized to membranes, and it is thus easy to conceptualize how spatial information is decoded (e.g., via recruitment to specific subcellular locations), sAC is localized throughout the cytosol, leaving open the issue of how specificity and the spatial restriction of signaling is achieved. Several scenarios provide a plausible explanation for how spatial restriction could be achieved in the case of sAC. For instance, sAC or its effectors could be anchored by specialized scaffolding proteins to specific endomembrane regions, thereby facilitating the spatial resolution of signaling. Such a mechanism was shown in the case of the A-kinase anchoring protein ezrin, which recruits sAC at the PM (Watson et al., 2015). However, endosomal-specific scaffolds have not yet been identified. Furthermore, sAC, in contrast to tmACs, can be directly activated by calcium (Jaiswal and Conti, 2003), which is released locally by activated CRHR1. This again indicates how the differential regulation of signaling might occur as a result of subcellular location. Finally, the local activation of CRHR1 could generate gradients of cAMP

from the PM or from the endosomes that can then be spatially decoded. Indeed, once produced, cAMP can be confined by regulated diffusion or turnover. Whether these mechanisms are all acting in the cell, and how they are integrated, remains an open issue that will require further investigation in the future.

Another important question to arise from the work by Inda et al. (2016) is whether the spatial control of cAMP signaling is common to all cells or whether it is specific to neurons given the long distance between soma and distal axons, which could provide greater opportunity to decode cAMP gradients generated by sAC. In this regard, Inda et al. (2016) showed that cell context is relevant because sAC is not required for CRHR1 signaling in NIH3T3 fibroblasts, despite these cells having a comparable response in terms of calcium release and cAMP production to hippocampal neurons. Additionally, their findings show that there is also receptor specificity, as sAC is not required for isoproterenol-elicited cAMP rise via the β-adrenergic receptor, suggesting that not all GPCRs signal through sAC.

The discovery by Inda et al. (2016) of the existence of yet another source of cAMP raises several other issues: How do the two different forms of AC interplay at the PM and/or at the endosomes? How are they regulated by different GPCRs? Does signaling generated by distinct GPCRs depend on different forms of ACs? Given that many GPCRs trigger second messenger responses, which in turn control a downstream transcriptional program (Irannejad and von Zastrow, 2014), we predict that the work of Inda et al. (2016) will have widespread physiological implications on how signaling from distinct GPCRs is diversified and regulated in space and time. It remains to be clarified whether the subtle effects observed in vitro on Erk activation will turn out to be relevant to determine the final biological output of a given GPCR, and whether they could be better unmasked in a more physiological in vivo context.

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