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G Protein–Coupled Receptor Signaling: New Insights Define Cellular Nanodomains

Martin J. Lohse,[1,2] Andreas Bock,[2] and Manuela Zaccolo[3]

[1]ISAR Bioscience Institute, Planegg/Munich, Germany; email: Martin.Lohse@Isarbioscience.de

[2]Rudolf Boehm Institute of Pharmacology and Toxicology, Leipzig University, Leipzig, Germany

[3]Department of Physiology, Anatomy and Genetics and National Institute for Health and Care Research Oxford Biomedical Research Centre, University of Oxford, Oxford, United Kingdom; email: Manuela.zaccolo@dpag.ox.ac.uk

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Abstract

G protein–coupled receptors are the largest and pharmacologically most important receptor family and are involved in the regulation of most cell functions. Most of them reside exclusively at the cell surface, from where they signal via heterotrimeric G proteins to control the production of second messengers such as cAMP and IP_3 as well as the activity of several ion channels. However, they may also internalize upon agonist stimulation or constitutively reside in various intracellular locations. Recent evidence indicates that their function differs depending on their precise cellular localization. This is because the signals they produce, notably cAMP and Ca^{2+}, are mostly bound to cell proteins that significantly reduce their mobility, allowing the generation of steep concentration gradients. As a result, signals generated by the receptors remain confined to nanometer-sized domains. We propose that such nanometer-sized domains represent the basic signaling units in a cell and a new type of target for drug development.

INTRODUCTION

Signaling by G protein–coupled receptors (GPCRs) is usually thought to affect the entire cell in a uniform manner based on two generally held views: First, most GPCRs appear to be uniformly distributed at the cell surface, and second, their second messengers—notably 3′,5′-cyclic adenosine monophosphate (cAMP), inositol 1,4,5-triphosphate (IP_3), and Ca^{2+}—are water soluble and therefore expected to be freely diffusible. However, a significant body of evidence indicates that often both of these assumptions are not correct: Receptors may be concentrated in distinct locations on the cell surface but also in cell organelles, and concentrations of cAMP and Ca^{2+} may show marked subcellular gradients. Signaling via spatially confined calcium signals has been long appreciated (1–4). Abundant Ca^{2+}-binding proteins in cells provide high buffering capacity and significantly slow diffusion of Ca^{2+} away from Ca^{2+} channels that mediate its influx into the cell or release from internal stores (5).

In contrast, evidence for cAMP buffering has been lacking, and cAMP has generally been considered to act in a long-distance manner, typically from cell surface receptors across the entire cell (6). More recent evidence, however, indicates a conspicuous intracellular buffering capacity for cAMP, making it largely immobile in unstimulated cells (7–9). Buffered diffusion provides the conditions for the generation of highly confined cAMP signals upon GPCR activation. The highly compartmentalized nature of cAMP signaling, together with GPCR signaling to multiple effectors and from multiple cellular sites, has completely transformed our appreciation of how these receptors operate (7, 10–15). Evidently, they activate complex signaling patterns, whereby many local signaling domains contribute to a cell's responses to external stimuli.

CELLULAR LOCALIZATION OF GPCRs

Cell Surface Localization

Upon synthesis, GPCRs are inserted cotranslationally into the membrane of the endoplasmic reticulum (16), aided, for some GPCRs, by signal or pseudosignal peptides (16–18). There, the receptors undergo an N- to C-terminal folding process (19, 20) and various posttranslational modifications, including *N*- and *O*-linked glycosylation (mostly in the N terminus) and palmitoylation (mostly at the C terminus) (21). Among other effects (21), both modifications improve targeting to the cell surface, where most GPCRs normally reside (21–24). Receptor targeting to the cell surface may be further aided by interactions with receptor accessory proteins such as receptor activity–modifying proteins (RAMPs) (25–28) or melanocortin 2 receptor accessory proteins (MRAPs) (29), which also affect ligand specificity and downstream signaling. Only a few GPCRs, for example, the chemokine CXCR7 receptor (30), remain intracellularly and move to the surface only under certain circumstances.

While receptor distribution at the cell surface often appears uniform, it can in fact be quite uneven. For example, receptors can be concentrated at presynaptic sites (31–33) to inhibit neurotransmitter release (34), and β_2-adrenergic receptors on cardiac myocytes appear to reside exclusively in the T-tubules (35). The mechanisms of such selective localization are still incompletely understood. Interactions between the receptors and elements of the cytoskeleton that restrict mobility have been demonstrated for some GPCRs (36–41). Other mechanisms that have general effects on membrane protein distribution may also promote specific receptor localization, including membrane curvature, geometry, and lipid composition (42–45). In addition, GPCRs may be localized in a dynamic fashion into specific membrane domains such as caveolae, lipid rafts, and clathrin-coated pits (46, 47).

GPCRs can move at the cell surface with diffusion coefficients in the order of $D \approx 0.10\ \mu m^2/s$, that is, free Brownian diffusion (48–53). However, in many cases, fractions with different diffusion patterns are detected, with some receptors being essentially immobile and others moving by directed diffusion (54–57). When such receptor movements are followed over time, membrane regions of higher and lower receptor density become apparent (54). The same diffusive behavior and patterning occur for heterotrimeric G proteins. These inhomogeneities in receptor distribution define small cell membrane domains where receptors and G proteins preferentially meet and interact and where GPCR-triggered signaling can occur (54). The stability of these domains over time is unknown.

Mobile receptors may still show specific localization (and function). Thus, presynaptic μ-opioid receptors (μORs) move along the surface of an axon, but they become phosphorylated and internalized at presynaptic locations and subsequently recycle locally and reinsert into the presynaptic terminal to control transmitter release (58).

GPCRs in Endosomes

In addition to their cell surface localization, many GPCRs are found in intracellular organelles. The function of intracellular GPCRs is best studied for endosomes, but GPCRs have also been reported in the endoplasmic reticulum and Golgi apparatus, as well as in mitochondria and the nuclear membrane (**Figure 1**). However, it remains to be established how physiologically relevant signaling may occur in the latter locations.

Many (but not all) cell surface GPCRs become internalized upon prolonged stimulation with their agonists (13) in a process that begins with receptor phosphorylation by G protein–coupled receptor kinases (GRKs) (59), followed by phosphorylation-dependent binding of β-arrestins (60) (**Figure 1**). While this process was initially thought to mediate receptor desensitization by terminating signaling to G proteins (60), this process was found to allow additional signaling pathways to emerge. Upon binding to receptors, β-arrestins adopt an active conformation that enhances binding to clathrin heavy chain, β2-adaptin, and phosphatidylinositol 4,5-bisphosphate (PIP2) (13, 61–64) and also to multiple kinases in a manner that may depend on the activating receptor (61, 65, 66). These multiple interactions allow β-arrestins to mediate two major events: receptor endocytosis and signaling via protein kinases. As a result, endosomes containing β-arrestin-bound GPCRs become signaling hubs that trigger a second wave of signaling by kinases following the first G protein–mediated wave at the cell surface (67). A third wave of signaling, also emanating from endosomes and possibly other organelles, involves new encounters with G proteins located there and the production of second messengers from intracellular sites (67).

There are numerous variations in the patterns of GPCR endocytosis. Lateral receptor diffusion in the plasma membrane may be regulated by agonists, resulting in different degrees of their accumulation in clathrin-coated pits and subsequent internalization; such a differentiation has been shown for the μOR, for which the peptide agonist [D-Ala^2,*N*-MePhe^4,Gly-ol]-enkephalin (DAMGO), but not morphine, promotes receptor accumulation in clathrin-coated pits and subsequent internalization (68). Some receptors appear to become internalized in a clathrin-independent manner. This may occur with the help of other adapter proteins, for example, endophilin, which promotes rapid clathrin-independent internalization of β_1-adrenergic receptors (β_1-ARs) (69). Organelles distinct from endosomes may become involved in GPCR internalization; for example, thyroid-stimulating hormone (TSH) and β_1-ARs have been found colocalized with Golgi markers (70–72) (**Figure 1**). Subcellular locations of GPCRs can be modulated by different agonists: Peptide agonists for the μOR stimulate receptor accumulation in early endosomes, whereas synthetic opioid drugs additionally promote receptor sorting into the Golgi apparatus (73).

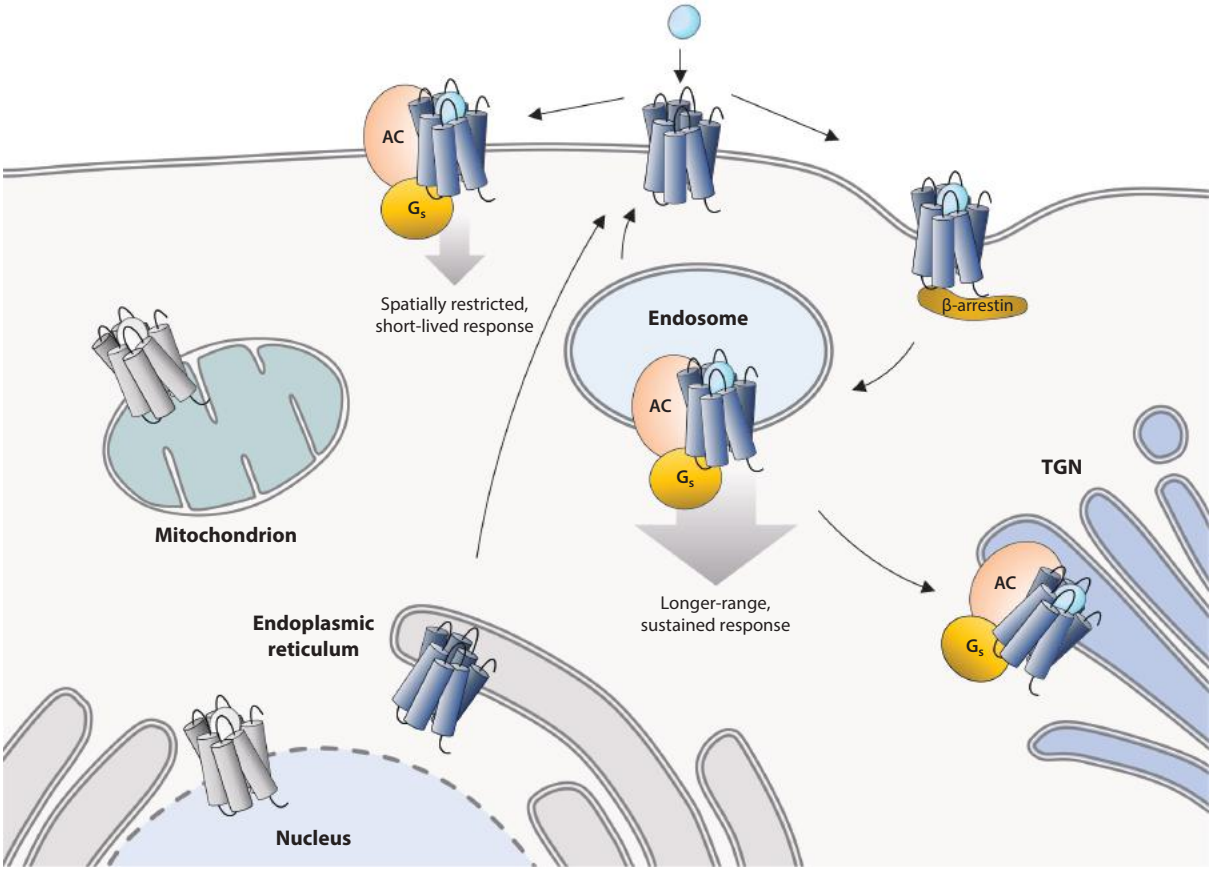

Figure 1

Subcellular localization of GPCRs and consequences of spatiotemporal signaling. GPCRs reside primarily in the plasma membrane but have also been found in several subcellular compartments. GPCR activation by extracellular ligands (*blue circles*) causes G protein activation (*yellow circles*) at the plasma membrane, resulting in a spatially restricted, short-lived second messenger response. Prolonged agonist stimulation promotes β-arrestin recruitment and subsequent endocytosis into endosomes, the TGN, and Golgi apparatus. From such intracellular compartments, GPCRs can reinitiate G protein signaling that may result in a more sustained and longer-range response. Abbreviations: AC, adenylyl cyclase; GPCR, G protein–coupled receptor; TGN, *trans*-Golgi network.

Following endocytosis, GPCRs may be sorted in complex ways and become either degraded or resensitized and get recycled back to the cell surface (13, 74–76). Again, this sorting process and the proteins involved appear to be highly receptor and cell specific (13).

GPCRs in Other Cell Organelles

Much less is known about the localization of GPCRs in other organelles. Mitochondria and the nuclear membrane have been reported to contain GPCRs, and functional responses have been observed in some instances (77) (**Figure 1**).

Mitochondria are reactive to both Ca^{2+} and cAMP and are affected by changes in their cytosolic concentrations (78). Mitochondria take up Ca^{2+} from the cytosol but also actively release Ca^{2+}, thereby contributing to cytosolic Ca^{2+} gradients (79). In contrast, mitochondria seem to respond to two distinct cAMP systems: While the outer and inner mitochondrial membranes directly sense

cytosolic cAMP generated upon activation of cell surface GPCRs, the inner membrane is impermeable to cAMP. However, cAMP is generated within the matrix by a soluble adenylyl cyclase (AC) that is stimulated by bicarbonate and Ca^{2+} (80). The outer mitochondrial membrane is sensitive to cytosolic cAMP, but it appears to represent a cAMP domain that is independent from the neighboring cytosol because of unique regulation by mitochondria-resident cAMP-degrading phosphodiesterases (PDEs) (81, 82) plus attenuated dephosphorylation of protein kinase A (PKA) targets by phosphatases (80, 83).

Several GPCRs have been detected in mitochondria and have been linked to defined downstream responses, including CB1 cannabinoid receptors (84, 85), MT1 melatonin receptors (86), multiple purinergic (87) and serotonin (88) receptors, and the entire angiotensin receptor system (89) (**Figure 1**). However, their exact signaling pathways and links to downstream responses are still largely elusive. It remains to be demonstrated whether the signaling networks elicited by mitochondrial GPCRs are the same as those present at the cell surface.

Likewise, there are several reports of GPCRs in nuclear membranes, including adrenergic, adenosine, angiotensin, bradykinin, and chemokine receptors (reviewed in 77). Activation of these receptors has been reported to cause a number of effects in the nucleus, notably changes in Ca^{2+} (90) and in gene transcription (91). Activation of gene transcription in osteoblasts requires interaction with β-arrestins (91), and it was suggested that these data might indicate direct transcriptional modulation by nuclear GPCRs. The details of nuclear GPCR signaling systems, the localization of their components, and their functional interactions need to be worked out conclusively.

SIGNALING BY INTERNALIZED GPCRs

Endocytosis or internalization of GPCRs was initially regarded as a mechanism of receptor desensitization (92, 93). However, more detailed kinetic analyses showed that, compared to the actions of PKA, GRKs, and β-arrestins, endocytosis was too slow to contribute significantly to desensitization (93–95). Two alternative roles were then assigned to endocytosis: sorting toward lysosomes, leading to receptor degradation and long-term downregulation (92), or resensitization via dephosphorylation and subsequent recycling to the cell surface (75, 76).

The findings that β-arrestins—particularly in their active state—can interact with and activate multiple protein kinases have identified their role as scaffolding proteins and expanded their function from mere inhibitors of G protein–mediated signaling to promoters of endocytosis and signaling proteins in their own right (61, 62, 96, 97). Kinases that they may locally activate include the Src protein kinase (98) and Src family members (99), Jun kinases (100), Akt (101), extracellular signal–regulated kinase 1/2 (ERK1/2) (102), and phosphatidylinositol-3 (PI3) kinase (103). After recruitment to β-arrestins, these kinases trigger local phosphorylation-mediated signals. An alternative pathway for GPCR-mediated activation of intracellular kinases has recently been proposed: Studies conducted in genetically modified human embryonic kidney (HEK) cells lacking either G protein α subunits or β-arrestins suggested that GPCR-mediated kinase signaling from endosomes requires the presence of active G proteins (104–107).

By recruiting other enzymatically active proteins, β-arrestins can even further mediate local signaling processes. A well-documented example is the recruitment of cAMP-PDEs, which can change local cAMP levels. Although some early publications that reported an interaction between PDE4 members and β-arrestins were retracted, there is evidence indicating that this may be a mechanism for enhanced cAMP degradation in the vicinity of an active GPCR (108). Local, receptor-associated cAMP degradation would create a domain of low cAMP, where cAMP targets are protected from activation. The opposite effect, an increase in local cAMP by recruitment of a PDE4, has been recently reported for the nucleus: Endosomal GPCRs bind β-arrestin-2, which

in turn recruits PDE4D5 to the endosome and depletes PDE4D5 from the nucleus, thereby increasing nuclear cAMP and cAMP-dependent transcription of immediate early genes, which is required for learning and memory (109).

In addition to these noncanonical signaling pathways triggered from endosomes, it has subsequently become clear that endosomal GPCRs also reinitiate a further wave of G protein–mediated signaling (67). Internal G protein–mediated signaling was first observed in yeast, where a noncanonical G protein mediates activation of a PI3 kinase by the Ste2p pheromone receptor (110). The phosphatidylinositol 3-phosphate generated by this process is retained in the adjacent membrane, and thus its signaling remains local.

In 2009, three studies reported sustained G protein–mediated responses from internal sites: by sphingosine-1-phosphate receptors (S1PRs) via G_i in a Golgi-associated compartment (111), and by two peptide hormone receptors, the G_s-dependent parathyroid hormone 1 receptor (PTH1R) in endosomes (112) and the G_s-coupled TSH receptor in Golgi-associated membranes (70). Thus, internalized GPCRs can meet G proteins on internal membranes—endosomes or Golgi-associated vesicles—and assemble to form a fully competent signaling complex (14, 113) that initiates an additional wave of signaling via second messenger production at intracellular sites (67) (**Figure 1**). In thyroid cells, the TSH receptors traffic retrogradely to the *trans*-Golgi network (TGN) where they activate G_s proteins residing in the retromer-coated compartment that brings them to the TGN (71). These complex cell structures are apparently not active in HEK293 cells, in which no such sustained cAMP responses were elicited (114).

Other receptors that were subsequently found to signal from internal sites include the G_s-coupled dopamine D_1 receptor (115) and β_2-ARs (116), as well as the luteinizing hormone (LH) receptor in ovarian follicles (117). An interesting twist to these findings is that in some instances the ligands are not cointernalized together with their receptors but are provided separately. Notably, it has been shown for the G_s-coupled dopamine D_1 receptor that the ligand dopamine enters cells via an organic cation transporter and reaches the intracellularly located receptors via the cytosol (118). Such mechanisms may form the basis for signaling from newly synthesized receptors located in the Golgi apparatus while they are still on their biosynthetic pathway (119). **Table 1** provides an overview of GPCRs that have been shown to signal from internal sites.

The most interesting aspect of endosomal and Golgi signaling by GPCRs is the observation that the functional consequences differ from those triggered by GPCRs at the cell surface (14, 120) (**Figure 1**). First, the receptor location affects the kinetics of the second messenger signal, with the signal often being more sustained when triggered by intracellular receptors, for both G_s- (70, 112) and G_i-mediated (73) responses. Such sustained signaling may depend on the formation of a so-called megaplex that comprises the receptor bound to both G protein and β-arrestin (121), which may be further stabilized by phosphoinositide binding (122). Second, GPCR activation at internal sites may have distinct physiological consequences, notably in endocrine cells where only internal signals trigger adequate responses, such as increased production of the rate-limiting hydroxylase for vitamin D synthesis in response to parathyroid hormone (PTH) (123) or cytoskeletal rearrangements required for thyroid hormone secretion in response to TSH (70). Optogenetic activation of cAMP production revealed more robust changes in the phosphoproteome when cAMP was generated at endosomes rather than at the cell surface; this was particularly apparent for proteins that were dephosphorylated in response to cAMP by selective activation of protein phosphatase 2A (124).

Intracellular G protein–mediated GPCR signaling may not be limited to cAMP. Neurokinin 1 receptors (NK_1Rs) appear to activate cytosolic protein kinase C (PKC) and nuclear ERK by a mechanism that requires NK_1R internalization (125). Using subcellularly targeted ERK activity biosensors, researchers showed that β_2-ARs activate ERK signaling exclusively from endosomes,

Table 1 GPCRs that signal from subcellular locations

Receptor[a]	Endogenous ligand(s)	G protein preference[a]	Signaling initiated from internal sites	Subcellular localization of active receptors	Functional implication	Reference(s)
5-HT_{2A}	5-Hydroxytryptamine (serotonin)	$G_{q/11}$, $G_{i/o}$	ND	Golgi	Neuroplasticity	224
ADGRG2, GPR64	Peptides derived from the Stachel sequence: TSFGVLLDLSRT-SLPP	G_s	cAMP, CREB	Early endosomes	ND	225
AT_1R	Angiotensin II	$G_{q/11}$, $G_{i/o}$	p63RhoGEF	Early endosomes	ND	226
β_1-AR	Adrenaline, noradrenaline	G_s, $G_{i/o}$	cAMP, PKA, PI4P hydrolysis	Golgi	Cardiac hypertrophy	72, 119
β_2-AR	Adrenaline, noradrenaline	G_s, $G_{i/o}$	cAMP, ERK, CREB	Early endosomes	Gene transcription	107, 116, 120
B_2R	Bradykinin and related peptides	$G_{q/11}$, $G_{i/o}$, G_s	p63RhoGEF	Early endosomes	ND	226
CLR	CGRP (with RAMP1), adrenomedullin (with RAMP2 or RAMP3)	G_s, $G_{q/11}$	PKC, ERK	Early endosomes	Nociception	192
CTR	Calcitonin, CGRP, adrenomedullin	G_s, $G_{q/11}$	cAMP	ND	ND	227
CXCR4	CXCL12	$G_{i/o}$	Akt	Early endosomes	Suppression of anoikis/apoptosis	228
D_1R	Dopamine	G_s	cAMP, PKA	Golgi	ND	115, 118
DOR	β-Endorphin, [Leu]enkephalin, [Met]enkephalin	$G_{i/o}$	cAMP, PKC, ERK	Golgi, endosomes	Pain relief, no gene transcription, distinct protein phosphorylation	73, 193, 229
GLP-1R	Glucagon-like peptide 1	G_s	cAMP	Endosomes	Glucose-stimulated insulin secretion	230
KOR	Dynorphin A	$G_{i/o}$	cAMP	Different endosomes	ND	231
LHR	LH, HCG	G_s	cAMP	Endosomes	Meiosis resumption in oocytes/ ovulation	117
M_3R	ACh	$G_{q/11}$	p63RhoGEF	Early endosomes	ND	226
μOR	β-Endorphin, [Leu]enkephalin, [Met]enkephalin	$G_{i/o}$	cAMP	Golgi, endosomes	ND	73, 229
NK_1R	Substance P, neurokinin A, neurokinin B	$G_{q/11}$, G_s	PKC, ERK	Endosomes	Nociception	125
OTR	Oxytocin, vasopressin	$G_{q/11}$, $G_{i/o}$	p63RhoGEF	Early endosomes	ND	226

(*Continued*)

Table 1 (*Continued*)

Receptor[a]	Endogenous ligand(s)	G protein preference[a]	Signaling initiated from internal sites	Subcellular localization of active receptors	Functional implication	Reference(s)
PAC_1R	PACAP-27, PACAP-38, VIP	G_s, $G_{q/11}$	PKC, ERK	Endosomes	Cardiac neuron excitability, chronic pain	232–234
PAR2	Trypsin, tryptase, TF/VIIa, Xa, elastase, cathepsin S	$G_{q/11}$	PKC, ERK	Endosomes	Chronic pain	194
PTH1R	PTH, PTHrP	G_s, $G_{q/11}$	cAMP	Endosomes	Calcemic effects, increase of serum Ca^{2+} and vitamin D	112, 123
S1PR	Sphingosine-1-phosphate	$G_{i/o}$	cAMP	Endosomes	Chemokinetic migration	111
TPR	Thromboxane A_2, various prostaglandins	$G_{q/11}$	p63RhoGEF	Early endosomes	ND	226
TSHR	TSH	G_s, $G_{q/11}$	cAMP, PKA, CREB	Early endosomes, Golgi	Thyroid function	70, 71
V_2R	Vasopressin, oxytocin	G_s	cAMP, PKA	Early endosomes	Antidiuretic and antinatriuretic effects	124, 216

[a]Receptor nomenclature and G protein preference are given according to Reference 235.

Abbreviations: $5\text{-}HT_{2A}$, 5-hydroxytryptamine 2A receptor; β-AR, β-adrenoceptor; μOR, μ-opioid receptor; ACh; acetylcholine; ADGRG2, adhesion G protein–coupled receptor G2; AT_1R, angiotensin 1 receptor; B_2R, bradykinin B2 receptor; cAMP, cyclic adenosine monophosphate; CGRP, calcitonin gene–related peptide; CLR, calcitonin receptor–like receptor; CREB, cAMP response element-binding protein; CTR, calcitonin receptor; CXCR4, chemokine CXCR4 receptor; D_1R, dopamine D_1 receptor; DOR, δ-opioid receptor; ERK, extracellular signal–related kinase; GLP-1R, glucagon-like peptide 1 receptor; GPCR, G protein–coupled receptor; HCG, human chorionic gonadotropin; KOR, κ-opioid receptor; LH, luteinizing hormone; LHR, LH receptor; M_3R, muscarinic M_3 acetylcholine receptor; ND, not determined; NK_1R, neurokinin 1 receptor; OTR, oxytocin receptor; p63RhoGEF, $G_{q/11}$-activated guanine nucleotide exchange factor with specificity for RhoA; PAC_1R, pituitary adenylate cyclase–activating peptide receptor; PAR2, proteinase-activated receptor 2; PI4P, phosphatidylinositol-4-phosphate; PKA, protein kinase A; PKC, protein kinase C; PTH, parathyroid hormone; PTH1R, PTH 1 receptor; PTHrP, PTH-related peptide; RAMP, receptor activity–modifying protein; S1PR, sphingosine-1-phosphate receptor; TPR, thromboxane A_2 receptor; TSH, thyroid-stimulating hormone; TSHR, TSH receptor; V_2R, vasopressin 2 receptor; VIP, vasoactive intestinal peptide.

and endosomal ERK activation appears to propagate into the nucleus to modulate gene transcription and cell proliferation (107). These studies suggest that localized signaling may be a universal feature of GPCR signaling pathways.

SIGNALING BY cAMP NANODOMAINS

GPCRs can perform different functions depending on their location, implying that the second messengers they produce must preserve the receptor's local action and operate in a spatially restricted manner. While compartmentalized Ca^{2+} signaling has been appreciated for a long time (1–4), a large body of evidence now also supports cAMP compartmentalization.

cAMP had long been known to play a pleiotropic role in regulating various cellular functions, and the canonical linear cascade model connecting ligand binding to GPCRs, cAMP generation, PKA activation, and substrate phosphorylation was recognized to be inadequate to explain cAMP's ability to relay distinct signals from different extracellular stimuli. However, the mechanisms by

which cAMP mediates hormone-specific cellular effects were unclear until the last 20 years, when evidence began accumulating for an alternative model. This model involves compartmentalization of cAMP signaling pathways within cells, where spatial confinement is critical for hormonal specificity. Activation of G_s-coupled receptors generates distinct cAMP pools that activate specific subsets of PKA that are immobilized by A-kinase anchoring proteins (AKAPs) in proximity to their specific targets (126). AKAPs nucleate signaling hubs, or signalosomes, by interacting with multiple signaling molecules, including GPCRs (127, 128), adenylyl cyclases (ACs) (129), phosphatases (130), and cAMP-hydrolyzing PDEs (131), resulting in unique combinations of signaling components that regulate specific cellular functions. Evidence for this model comes predominantly from investigations on β-AR signaling in cardiac myocytes, but this subcellular organization is present across various cell types (132–136).

Real-time monitoring of intracellular signaling in intact cells with high spatial and temporal resolution using fluorescence resonance energy transfer (FRET) tools (11, 137–140) enabled the direct demonstration that, upon GPCR activation, cAMP levels are not uniform within a cell but differ at different sites, directly revealing compartmentalized cAMP signaling. While compartmentalized cAMP signaling had been proposed to explain different outcomes of hormones acting via cAMP (141–145), the first direct evidence of nonuniform cAMP distribution in cells was shown through FRET imaging experiments in cardiac myocytes (11). A reporter based on FRET between the PKA regulatory and catalytic subunits fused to cyan (CFP) and yellow fluorescent proteins (YFPs), respectively, revealed that β-AR stimulation elicits changes in cAMP concentration specifically in subcellular compartments where PKA is anchored to AKAPs. A sensor variant in which the regulatory subunit component lacks the domain necessary for AKAP anchoring (11) failed to pick up any significant cAMP signal in response to β-AR stimulation, confirming the extremely localized nature of the cAMP increase (**Figure 2**).

Signalosomes organize PKA in close proximity to phosphorylation targets, while compartmentalized cAMP allows for the activation of PKA effectors within specific signalosomes. For instance, in cardiac myocytes, activation of β-ARs leads to increased PKA-dependent phosphorylation of phospholamban and troponin I, while activation of the prostaglandin EP receptor does not—despite a similar overall amount of cAMP being generated by the activation of the two receptors (143, 144). Imaging experiments using targeted FRET reporters demonstrated that the phosphorylation of distinct PKA targets upon application of catecholamines and prostaglandin depends on the ability of the two stimuli to generate a rise in cAMP at different subcellular sites where distinct AKAP-PKA signalosomes are located (146). Targeted reporters also showed that not only do different GPCRs generate spatially distinct cAMP signals but the cAMP response elicited by the activation of a specific receptor is also heterogeneous. For example, activation of cardiac β-ARs results in a larger cAMP response at the plasmalemma and sarcoplasmic reticulum compared to the cAMP signal generated at the myofilaments. Such heterogeneity is functionally relevant, as forcing a homogeneous cAMP increase through pharmacological inhibition of PDEs results in an attenuated inotropic response (147).

The observation that the amplitude and kinetics of the cAMP response to β-AR activation differ at the plasmalemma, sarcoplasmic reticulum, and myofilaments provided a first indication that adjacent cAMP domains are delimited by very narrow boundaries, as these structures are in very close proximity to each other (10). Based on early imaging experiments in cardiac myocytes, the size of these cAMP subcellular compartments was estimated to be in the micrometer range, since the cAMP pools detected appeared to align along the sarcomere Z lines, which are spaced about 2 μm apart (11) (**Figure 2**). However, recent studies have shown that cAMP subcellular domains can be much smaller. Nanometer-sized cAMP domains were found in HEK293 cells, a cell type with a much simpler subcellular structure than cardiac myocytes. Nanodomains of up to 60 nm

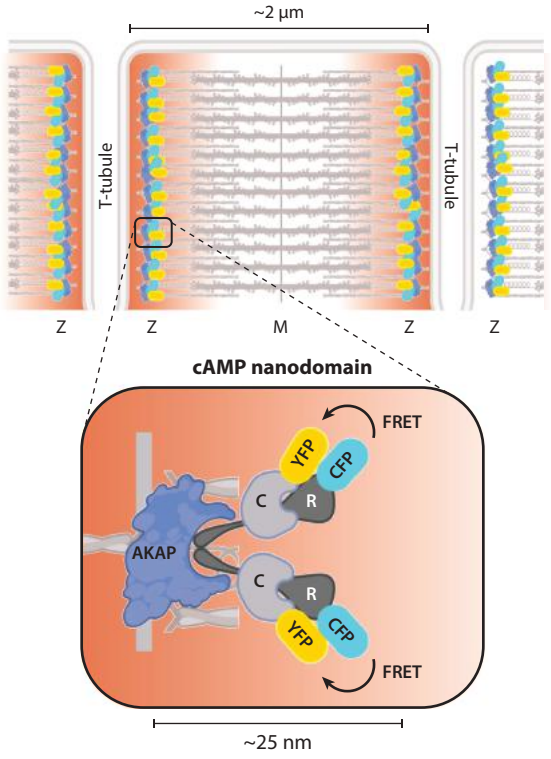

Figure 2

Visualization of cAMP nanodomains using targeted sensors. Imaging of cAMP subcellular compartments in cardiac myocytes with a PKA holoenzyme FRET sensor. The sensor is anchored to AKAPs and shows a striated pattern in cardiac myocytes due to localization at sarcomere Z lines. Activation of β-adrenergic receptors leads to a larger increase in cAMP concentration in the regions where the sensor is localized, estimated initially in the ~1-µm range corresponding to the distance between Z lines. The actual size of the cAMP pools is much smaller and below optical resolution (~20–25 nm). Abbreviations: AKAP, A-kinase anchoring protein; C, catalytic subunit of PKA; cAMP, cyclic adenosine monophosphate; FRET, fluorescence resonance energy transfer; PKA, protein kinase A; R, regulatory subunit of PKA.

in diameter were identified using low agonist concentrations in HEK293 cells expressing FRET cAMP sensors that incorporate spacers of known length between a distinct GPCR and the sensor (so-called nanorulers) (7) (**Figure 3**). These nanodomains were found to operate independently of each other and to be protected from cAMP influx from outside the domain. These domains, named receptor-associated independent cyclic adenosine monophosphate nanodomains (RAINs), undergo a local cAMP increase and receptor-associated PKA activation, which requires AKAP-tethering of PKA, suggesting that RAINs constitute individual signalosomes. By using analogous constructs to separate the cAMP sensors from PDEs, even smaller nanodomains, with radii as small as <10 nm (8), of lower cAMP surrounding the PDE were defined. Strikingly, low cAMP domains in the nano-space surrounding a PDE were still detectable in cytosolic preparations (8), which ruled out the requirement for a physical barrier to achieve cAMP compartmentalization.

The size of cAMP pools measured with nanorulers matches that of a single AKAP:PKA complex, estimated to be 20–25 nm (148) (**Figure 2**). AKAP:PKA clusters imaged at the plasma membrane of Chinese hamster ovary (CHO) cells using super-resolution microscopy were reported to have a mean diameter of approximately 100 nm (149), which is also compatible with the

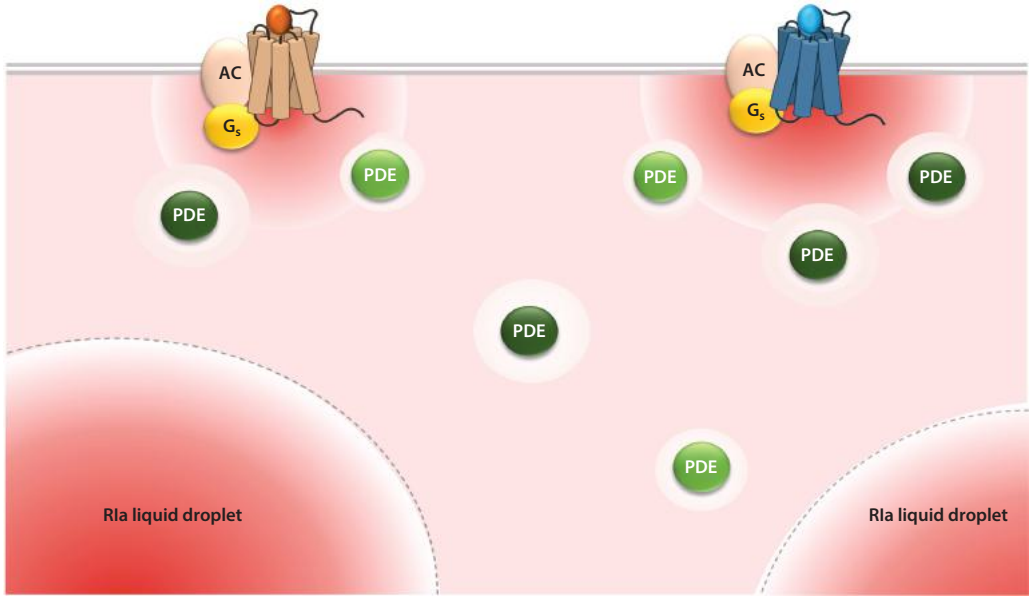

Figure 3

Spatiotemporal GPCR signaling at the nanometer scale. GPCR stimulation with low agonist concentrations creates RAINs, which extend up to 30–60 nm away from receptors. PDEs play a role in shaping the size of RAINs, and RAINs have limited exchange with other subcellular compartments. cAMP can additionally be compartmentalized in the cell cytosol through the formation of micrometer-sized biomolecular condensates. These liquid droplets contain PKA-RIα, the catalytic subunit of PKA, and an excess of cAMP, and their formation, size, and buffering capacity are dependent on GPCR activation. Red haloes indicate cAMP pools. Abbreviations: AC, adenylyl cyclase; cAMP, cyclic adenosine monophosphate; GPCR, G protein–coupled receptor; PDE, phosphodiesterase; PKA-Riα, protein kinase A regulatory subunit Iα; RAIN, receptor-associated independent cAMP nanodomain.

cAMP nanodomain size. Thus, the cAMP nanodomains appear to extend by as much as required to achieve activation of individual signalosomes. To ensure that phosphorylation is restricted to nearby targets, the assumption is that, on cAMP binding, either the catalytic PKA subunit dissociates and rapidly reassociates to regulatory subunits within the domain (9) or substrate phosphorylation can occur without complete dissociation of PKA (148).

The nanoscale dimension of cAMP subcellular domains means that they are below optical resolution, making them invisible to conventional microscopy techniques. Only by targeting a sensor strategically within the domain can these domains be optically detected (**Figure 2**). In fact, early attempts at imaging cAMP using freely diffusible reporters failed to confirm highly localized signals (150), initially raising some skepticism about the concept of cAMP compartmentalization (151, 152).

Spatial confinement through inclusion in local signalosomes as a mechanism to secure specificity of response is not exclusive to PKA and has also been shown for other cAMP effectors, including the exchange factor directly activated by cAMP (EPAC) proteins (153–156) and the Popeye domain–containing (POPDC) proteins (157, 158). Given that these effectors modulate their own distinct cellular functions, they are also expected to be activated locally by confined pools of cAMP. Notably, EPAC embedded in an mAKAPβ/PLCε scaffold at the nuclear envelope/Golgi interface in cardiac myocytes was shown to be selectively activated by cAMP generated by β-ARs that were resident at the Golgi apparatus. Activation of these internal receptors generated a signal that was divergent from that generated by cell surface β-ARs and stimulated EPAC/PLCε-dependent phosphatidylinositol-4-phosphate (PI4P) hydrolysis and cardiac hypertrophy (72).

MECHANISMS OF cAMP LOCALIZATION

Several distinct mechanisms have been proposed to contribute to the compartmentalization of signaling, including limitations to diffusion of second messengers and/or their target proteins, rapid degradation of second messengers, and binding reactions that give rise to buffering (i.e., restrictions to mobility because of binding to specific sites).

It has long been established that small compartments exist for Ca^{2+} that control, for example, vesicle release at presynaptic sites or excitation-contraction coupling in the heart (1, 2, 159). Both experiments and mathematical models have established that the decisive mechanism to allow the establishment of intracellular Ca^{2+} gradients is the presence of buffers, that is, sites in the cell that bind Ca^{2+} and thereby limit its diffusion (3, 160). For example, presynaptic vesicle release can be controlled by a single Ca^{2+} channel, giving rise to a very local domain of high Ca^{2+} that extends over only tens of nanometers (nanodomain). The domains from several channels may overlap and give rise to a larger microdomain (4, 159). While it has not been proven whether the same mechanisms apply to Ca^{2+} signals elicited by GPCRs, it is reasonable to assume that they do.

The concept of cAMP compartmentalization has been more difficult to establish, as it is at odds with observations that cAMP can freely diffuse in the cytosol. Measurements of its mobility in cells have consistently given diffusion coefficients of more than 100 $\mu m^2/s$ up to almost 800 $\mu m^2/s$, which corresponds to free diffusion in aqueous media (138, 150, 161, 162). Mathematical modelling revealed a mismatch by four orders of magnitude between the fast cAMP diffusion rates and the rate of cAMP degradation by PDEs and suggested that cAMP concentrations should rapidly equilibrate across a cell, precluding the formation of any significant cAMP gradients (163). However, more recent research has indicated that a combination of various mechanisms, as discussed below, may lead to sufficient cAMP buffering and allow for the formation of cAMP compartments within cells.

Phosphodiesterases

Extensive evidence supports a critical role of cAMP-degrading PDEs in establishing confined cAMP pools (164–167), including the consistent observation that PDE inhibition results in the dissipation of cAMP gradients imaged using fluorescent reporters (11, 145, 147, 168). PDEs are a superfamily of enzymes consisting of 11 families (PDE1–11) with over 100 isoforms. These isoforms differ in terms of their affinity for cyclic nucleotides, catalytic activity, tissue distribution, and subcellular localization. When cAMP synthesis is triggered by a GPCR, a PDE can create a region of low cAMP concentration around itself and inhibit the activation of PKA within that area (8). The range of PDE's action varies depending on the enzymatic properties of the isoform involved: PDE4A1, a low-turnover but high-affinity enzyme (169), can create a low cAMP domain with a radius of 10 nm. PDE2A3, which has a faster turnover but lower affinity for cAMP (169), can deplete cAMP in a radius of more than 30 nm (8).

The PDE system's complexity allows for sophisticated regulation of local cAMP concentration. Local control may be achieved by one or multiple PDEs that may act synergistically (170, 171). Each PDE can be uniquely regulated by Ca^{2+}, cyclic guanosine monophosphate, and various post-translational modifications. These modifications include PKA-dependent phosphorylation, which enables short-term feedback regulatory loops (165). For some PDE isoforms, cAMP-dependent enhancement of gene transcription has also been demonstrated, providing longer-term feedback regulation (172, 173). The resident PDE's identity, activation/inhibition status, and conditions that promote its recruitment or release from a specific location determine the cAMP level within that domain and the cellular response to the extracellular stimulus. Ultimately, the localization of different PDE isoforms in a cell determines the subcellular topography of the cAMP nanodomains

(174), which is expected to be dynamic and to undergo remodeling in pathological conditions (14, 175–177).

Although it is clear that PDEs are a key determinant of cAMP compartmentalization, how exactly they achieve this despite the mismatch between their catalytic rate and the speed of cAMP diffusion has remained unresolved for several decades.

cAMP Buffering

While buffering has long been established as the key mechanism of localized Ca^{2+} signaling, it had not been considered significant for cAMP, because of multiple measurements of its rapid diffusion in cells (12). This view changed when it became apparent that all measurements of cAMP diffusion had been carried out at very high levels of cAMP, which raised the possibility that cellular binding sites had been saturated.

More recent experiments using a fluorescent analog of cAMP showed that at low, physiological concentrations, cAMP is essentially completely bound and, thus, immobile (8). While the nature of the binding sites remains to be elucidated, their abundance was determined to be well above 10 μM, indicating that they suffice to completely bind cAMP at basal and even moderately elevated concentrations. Robust elevation of cAMP, however, such as strong receptor stimulation or inhibition of PDEs combined with the AC stimulator forskolin, overcame the binding capacity and converted cAMP from an immobile to a fully mobile molecule, with a diffusion coefficient consistent with free diffusion (8). Because of this significant binding to proteins, the concentration of free cAMP in cells is very low and, as mentioned above, this allows PDEs to create substantial concentration gradients over very short distances (**Figure 3**).

Liquid-Liquid Phase Separation

Liquid-liquid phase separation (LLPS) has been recently suggested as an additional mechanism that may lead to low levels of free cytosolic cAMP (178). LLPS occurs when, above a certain concentration threshold, a molecule in solution demixes into two phases—a concentrated, condensed phase and a dilute phase (179). A recent study (178) shows that the PKA regulatory subunit RIα can phase separate, dynamically sequestering cAMP within liquid condensates. The formation of phase-separated RIα bodies is thought to occur via multivalent interactions involving the RIα dimerization/docking domain and linker region, and activation of cAMP signaling appears to drive the process by rapidly inducing RIα condensation. Disruption of RIα phase separation resulted in the flooding of nanodomains with low cAMP in the immediate surrounding of a PDE, as detected by using targeted sensors (178, 180).

In summary, both cAMP buffering via binding proteins and PKA-RIα condensation appear to contribute to nanodomain signaling at physiological cAMP concentrations (**Figure 3**). At the same time, flooding of these nanodomains by high cAMP concentrations provides a means to achieve far-reaching cAMP effects, such as diffusion to the nucleus—effects that are, for example, observed upon long-term stimulation of cardiac β_1-ARs (181, 182).

PHYSIOLOGICAL RELEVANCE OF LOCALIZED GPCR SIGNALING

The fact that GPCR signals are not uniformly distributed throughout a cell but rather activate a limited subset of downstream effectors in a compartmentalized manner allows for precise information transmission to the correct intracellular machinery and the appropriate cellular effect. The compartmentalized nature of GPCR signaling has at least two other important functional implications. First, the protein-protein and protein-lipid interactions, the membrane dynamics,

and the buffering systems that underpin compartmentalization can be disrupted in pathological conditions, and understanding the location where pathways and processes are disturbed should be a primary concern when one studies mechanisms of disease. Second, the organization of GPCR signaling in subcellular compartments provides a unique opportunity for targeted therapeutic interventions, as drugs that affect an individual domain are expected to have better efficacy and reduced side effects.

Various mechanisms have been proposed to link local, sustained cAMP signals elicited at internal sites to specific physiological responses. cAMP buffering limits the cAMP nanodomains at RAINs to a few tens of nanometers (7); there are no comparable measurements for endosomal nanodomains, but it is plausible they have a similar size. Thus, for signaling to occur at locations that are further away from the site of cAMP generation, the strength of the stimulus needs to be strong enough to saturate cAMP binding sites and thereby flood the nanodomains so that cAMP becomes diffusible. It should be noted, however, that only a relatively small amount of diffusible cAMP would be required to elicit a response: Four molecules of free cAMP would be sufficient to activate a nanodomain formed by one PKA holoenzyme anchored to one AKAP and to achieve phosphorylation of a nearby substrate (183).

As an alternative mechanism to nanodomain flooding, other components must become diffusible for signaling to occur. For example, the catalytic subunit of PKA may be a diffusible element that enters the nucleus of HEK293 cells (184), while in neurons a PDE4 was reported to diffuse out of the nucleus to cause nuclear cAMP enrichment (109). A highly mobile AKAP7γ was suggested to contribute to the fast spreading of β-AR signals via diffusion along the surface of the sarcoplasmic reticulum (185). Interestingly, in these examples it is not cAMP that diffuses but proteins that are involved in cAMP signaling.

Distinct functional downstream effects of cell surface versus internal cAMP signals have been reported in different systems. We discuss three examples: endocrine function, pain signaling, and cardiac function.

Endosomal Receptors in Endocrine Functions

In various endocrine systems, signals for peptide and proteohormone agonists, such as PTH, TSH, and LH, have been shown to be biochemically and functionally different when generated from intracellular sites (following internalization of receptors together with their ligands) compared to signals at the cell surface. In all three cases, intracellularly generated cAMP responses are sustained and are required for functional hormone responses, such as PTH-induced increases in vitamin D synthesis (via increased production of the rate-limiting hydroxylase) and regulation of blood Ca^{2+} levels (15, 123), TSH-induced thyroid hormone secretion (70, 71), and LH-induced oocyte maturation in ovarian follicles (117).

In this context, two questions remain to be answered: What are the mechanisms that make endosomal cAMP signals sustained, and how is sustained cAMP signaling linked to the functional endocrine response? As for the distinct mechanisms of endosomal signaling, we have alluded above to the possible contribution of a complex consisting of the endosomal GPCRs together with G protein and β-arrestin (121). In the case of the PTHR, Gβγ subunits liberated by activation of G_q (which occurs via the same PTHRs) may contribute to the formation of PTHR–β-arrestin–Gβγ complexes (123); Gβγ exchange between different receptors had been proposed as a mechanism of cross talk between activated receptors (186). The specific nature of the activating complex may depend not only on the specific receptor involved but also on factors such as phospholipids that may stabilize the complex (122). In the PTHR system, specific agonists as well as specific receptor mutants have been identified that either are or are not capable of

eliciting internal signaling and endocrine responses (187). This knowledge may help to develop more specific drugs that can activate or restore endocrine functions.

Endosomal Receptors in Pain Suppression

Several examples of GPCR signaling from internal sites have been described to be involved in the mediation and transmission of inflammatory and pain signals (188, 189). The receptors mediating these responses appear to be located on endosomes and to bind both β-arrestins and G proteins (189). Such dual binding is more prominent for GPCRs that can stably bind β-arrestins via their C terminus and form more stable complexes with β-arrestins. Such GPCRs were formerly termed class B receptors, defined as GPCRs that cointernalize with β-arrestins (190).

The pattern of internal receptor signaling reported for these processes is remarkably uniform: In each case, inhibition of receptor internalization resulted in abrogation of the pathological response. Inhibition of inflammatory effects and/or pain perception with internalization blockers has been observed in spinal cord neurons for NK_1Rs, which mediate the effects of substance P (125, 191), and for calcitonin receptor–like receptors (CLRs) stimulated by calcitonin gene–related peptide (192). Likewise, the antinociceptive effects of δ-opioid agonists have been attributed to signaling by endosomal receptors (193). These effects were observed in spinal cord slices and even in living mice when inhibitors were injected intrathecally.

Similar findings were obtained for inflammatory processes in irritable bowel syndrome. The protease-activated receptor 2 (PAR2) in primary sensory neurons or nociceptors is activated by proteolytic cleavage and then internalized into endosomes. From there, the receptor activates ERK, which elicits persistent hyperexcitability from nociceptors (194). Inhibitors of clathrin- and dynamin-dependent endocytosis and of the ERK cascade prevented this hyperexcitability. PARs can be cleaved by different proteases, which results in different types of receptor activation (195, 196). Only the effects of proteases that cause PAR2 internalization were affected by endocytosis inhibitors (194).

These observations underline the potential importance of endosomal signaling in inflammatory processes and pain perception. These data, which await confirmation by other teams, intriguingly suggest that it might be possible to develop new types of drugs that selectively target endosomal versus cell surface signaling or that inhibit internalization of the relevant receptors.

Localized cAMP Signaling in Cardiac Function

Several examples of how compartmentalized GPCR signaling is relevant to pathophysiology can be drawn from the cardiovascular field. β-ARs have been studied extensively for their role in heart failure, in which increased sympathetic drive is a characteristic feature. Cardiomyocytes primarily express β_1-ARs and fewer β_2-ARs, both of which work through G_s and cAMP but have different functional impacts (197). In healthy hearts, β_1-ARs enhance cardiac frequency, contractility, and relaxation, while β_2-ARs have modest effects on frequency and no effects on relaxation (198, 199). However, in heart failure, chronic β_1-AR stimulation leads to cardiomyocyte hypertrophy and apoptosis, while β_2-AR signaling may even be cardioprotective (200, 201).

The way β_1-ARs and β_2-ARs compartmentalize cAMP may contribute to their varying effects. β_2-ARs are highly localized in cardiomyocytes, mainly in caveolae and T-tubules (35, 168, 202), resulting in a more confined cAMP signal compared to that of β_1-ARs, which generate a robust cAMP signal that spreads throughout cardiomyocytes (150, 168). The signal generated by β_2-ARs is regulated by multiple PDEs, while the signal of β_1-ARs is predominantly controlled by PDE4 isoforms (150). Notably, both the more confined distribution of β_2-ARs at the plasma membrane and their more confined cAMP signals are compromised in heart failure (168)—an effect that adds

to the many disturbances of the β-AR system in heart failure, which include downregulation of β_1-ARs at the protein and messenger RNA level and altered coupling of β_2-ARs (203–208).

β_2-ARs also differ from β_1-ARs in that they can activate β-arrestin-mediated pathways and couple to G_i in addition to G_s (209). These receptors can also signal from internal membranes, resulting in specific functional outcomes (210). β_1-ARs are present at the nuclear membrane where they may regulate gene transcription (211). Recent research suggests that β_2-ARs activate G_s from early endosomes, promoting prosurvival cues, while β_1-ARs activate G_s from Golgi membranes, leading to cAMP-dependent transcription of prohypertrophic genes (72, 119). These findings align with earlier studies showing that activation of the endocytosis machinery is essential for the cardiac remodeling induced by chronic β_1-AR activation (212) and the harmful effects triggered by mechanical stress (213).

The vasopressin receptor is another GPCR that can signal from endosomal membranes. It is activated by arginine vasopressin (AVP) and regulates water reabsorption and vasoconstriction, and vasopressin 2 receptors are potential targets for the treatment of heart failure and hyponatremia (214). When activated and phosphorylated, they interact with β-arrestin in a very stable megaplex, which involves the receptor, β-arrestin, and G_s (215), to generate a long-lasting cAMP signal, resulting in PKA-dependent phosphorylation of aquaporin-2 and its insertion into the apical membrane (216). Oxytocin, another physiological V_2 receptor ligand, triggers signaling at the same receptor but does not cause internalization or measurable antidiuretic and antinatriuretic effects (216).

Another example is S1PR, which regulates heart rate, cardiac contractility, and vascular tone (217–219). FTY720, an analog of S1PR that is approved for the treatment of multiple sclerosis (220, 221), triggers long-lasting G_i and ERK signals from the *trans*-Golgi network (111). FTY720 has been shown to have cardioprotective effects (220, 221), making it a potential tool for targeting compartmentalized G protein signaling to treat cardiovascular disorders.

OUTLOOK

Localized signaling by GPCRs has become a field of intensive research. While it has been appreciated for decades that localized signaling occurs for Ca^{2+}, such mechanisms have now also emerged for other signaling pathways and notably for protein kinase A and cAMP signaling. The recognition that cell signaling occurs in minute dimensions of nanometer size has a number of theoretical as well as practical implications.

First, the functioning of nanometer-sized signaling domains has to be understood in terms of the statistics of individual molecules rather than bulk solutions. As an example, RAINs normally contain only a few molecules (sometimes less than one) of cAMP but several cAMP binding sites, including four that are required to activate a single PKA. Thus, individual cAMP molecules might be delivered to PKA rather than being taken up from solution. Likewise, degradation of cAMP by PDEs then occurs one by one.

Second, the interactions between the many individual functional nanodomains in a cell need to be worked out. There may be individual ways of communication between them, but they may also become fused by flooding of their binding sites so that larger domains and eventually a whole cell may receive a second messenger stimulus.

Third, targeting specific nanodomains for therapeutic purposes is an exciting prospect. There are first examples of such drug developments, for example, by specifically targeting endosomal receptors with conjugated ligands (125, 192) or by targeting endocytosis (222), even though the latter approach is quite nonspecific. Ligands that are selective for the PTHR exist naturally and have been developed further to specifically target cell surface versus endosomal signaling (187).

A μ-opioid agonist has been designed that binds to receptors only at low pH (223); while this ligand was developed for inflamed tissues with low pH, it may also show prolonged signaling from endosomes, which become progressively acidified after their formation. A ligand that binds well to receptors at low pH might thus maintain binding in endosomes, while other ligands would dissociate from the receptors as the pH drops.

Lastly, while subcellular signaling may be selectively targeted, additional efforts are needed to establish whether modulation of signaling at the nanoscale may be therapeutically exploited. Robust assays of nanodomain-specific functions will be needed to integrate this knowledge in drug development.

Recent research involving new tools has progressed toward defining the elementary functional units of cell signaling, especially by GPCRs and cAMP. Investigating the mechanisms of such individual signaling units as well as their interactions will lead us toward the fundamental principles of cell signaling and regulation and may also reveal strategies for the development of new types of GPCR-directed drugs.

SUMMARY POINTS

1. It has been appreciated for decades that G protein–coupled receptor (GPCR) signaling via Ca^{2+} is spatially limited via strong buffering, that is, cellular binding sites for Ca^{2+} trap Ca^{2+} ions, preventing them from diffusing unless these sites are saturated. Recent data show that similar mechanisms regulate GPCR signaling via cyclic adenosine monophosphate (cAMP). Furthermore, cAMP is buffered and remains largely immobile at basal (ranging from high nanomolar to low micromolar) concentrations.
2. cAMP buffering allows the generation of cAMP concentration gradients and the establishment of nanometer-sized domains of high or low cAMP concentrations within a cell.
3. GPCR-stimulated adenylyl cyclases generate receptor-associated independent cAMP nanodomains (RAINs) with a radius of up to 60 nm, where cAMP concentrations are elevated and stimulation of cAMP-dependent effectors may occur. These RAINs are one example of the long-postulated signalosomes and represent basic units of GPCR/cAMP signaling.
4. Similarly, phosphodiesterases (PDEs) generate regions of low cAMP with a radius that can be as small as 10 nm. cAMP effectors located in these nanodomains are shielded from the influence of cellular cAMP. The localization of the different PDE isoforms within a cell determines a diverse range of cAMP concentrations, creating a complex signaling nanoarchitecture.
5. When cAMP levels exceed the buffering capacity of a cell, individual RAINs fuse, and eventually cAMP becomes a diffusible messenger that can activate its targets across an entire cell, including the nucleus.
6. Individual RAINs may be arranged in a spatially and temporally synchronized manner such as the cAMP nanodomains patterned along the Z lines of cardiac myocytes.
7. cAMP buffering also allows local signaling at internal sites in a cell. Such internal signaling can occur from organelles, most notably endosomes and Golgi-associated membrane compartments. GPCRs may reach these internal compartments by internalization together with their bound agonists. In some instances, GPCRs may already be localized at

internal sites, and the agonists can reach them through diffusion or facilitated transport across the cell membrane.

8. cAMP signaling from internal sites may have downstream consequences that are distinct from cell surface signals. This has been shown for multiple endocrine receptors [e.g., parathyroid hormone (PTH), thyroid-stimulating hormone (TSH), and luteinizing hormone (LH)], for which physiological responses require receptor internalization. Signaling from internal sites also appears to be required for some forms of inflammatory and pain signals.
9. Localized GPCR signaling provides a unique opportunity for drug development. This can be achieved by modulating receptor internalization, by drugs that preferentially reach the intracellular space, or by drugs that preferentially modulate receptors at internal sites. Another approach involves manipulating cAMP concentrations by selectively targeting PDEs that are resident within individual signaling domains.

FUTURE ISSUES

1. What is the molecular composition of individual cyclic adenosine monophosphate (cAMP) nanodomains [e.g., receptor-associated independent cAMP nanodomains (RAINs) or other signalosomes as well as domains of low cAMP around phosphodiesterases (PDEs)], and how does the composition differ from one receptor type to another and from one PDE type to another?
2. How does cAMP act in nanodomains at the single-molecule level? Are individual cAMP molecules handed over from one protein (e.g., adenylyl cyclases) to the next [e.g., protein kinase A (PKA)], or does cAMP reach its sites of action by diffusion in solution?
3. How do individual G protein–coupled receptors (GPCRs) precisely couple with the intracellular molecular machinery to ensure that cAMP levels rise only in the appropriate compartment, particularly if signalosomes activated by cAMP are located far from the cell surface? How do internalized receptors identify the correct location to stop during their journey after internalization so that they reach relevant targets? How is PDE activity orchestrated such that specific stimuli generate an effective cAMP signal only in the appropriate nanodomain? And how does the intracellular texture impact and regulate GPCR signaling?
4. Can nanodomains of high or low cAMP be selectively targeted for therapeutic purposes? What assays and tools are needed to uncover the molecular architecture and dynamics of these signaling domains, and can this knowledge be translated into novel drug discovery strategies? How can one implement assays that preserve and address the complexity of the cellular architecture in the drug discovery pipeline?

DISCLOSURE STATEMENT

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