Special Section on Phosphoproteomic Analysis of G Protein-Coupled Pathways - Axelrod Symposium—Minireview

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ABSTRACT

By limiting unrestricted activation of intracellular effectors, compartmentalized signaling of cyclic nucleotides confers specificity to extracellular stimuli and is critical for the development and health of cells and organisms. Dissecting the molecular mechanisms that allow local control of cyclic nucleotide signaling is essential for our understanding of physiology and pathophysiology, but mapping the dynamics and regulation of compartmentalized signaling is a challenge. In this minireview we summarize advanced imaging and proteomics techniques that have been successfully used to probe compartmentalized cAMP signaling in eukaryotic cells. Subcellularly targeted fluorescence resonance energy transfer sensors can precisely locate and measure compartmentalized cAMP, and this allows us to estimate the range of effector activation. Because cAMP effector proteins often cluster together with their targets and cAMP regulatory proteins to form discrete cAMP signalosomes, proteomics and phosphoproteomics analysis have more recently been used to identify additional players in the cAMP-signaling cascade. We propose that the synergistic use of the techniques discussed could prove fruitful in generating a detailed map of cAMP signalosomes and reveal new details of compartmentalized signaling. Compiling a dynamic map of cAMP nanodomains in defined cell types would establish a blueprint for better understanding the alteration of signaling compartments associated with disease and would provide a molecular basis for targeted therapeutic strategies.

SIGNIFICANCE STATEMENT

cAMP signaling is compartmentalized. Some functionally important cellular signaling compartments operate on a nanometer scale, and their integrity is essential to maintain cellular function and appropriate responses to extracellular stimuli. Compartmentalized signaling provides an opportunity for precision medicine interventions. Our detailed understanding of the composition, function, and regulation of cAMP-signaling nanodomains in health and disease is essential and will benefit from harnessing the right combination of advanced biochemical and imaging techniques.

Introduction

Cyclic nucleotides are intracellular second messengers that function to transmit a signaling event initiated in the extracellular environment throughout the intracellular space to reach defined intracellular compartments. The cyclic nucleotide messaging system is universally used, from prokaryotic cells to highly specialized eukaryotic cells, such as cardiomyocytes in the human heart (Michel et al., 1990; Berman et al., 2005). Cyclic nucleotides are molecules that are small and hydrophilic, which are biochemical properties that are compatible with their free diffusion through the cell

(Hunter, 2000). However, it is now clear that depending on the stimulus, the same cyclic nucleotide activates a distinct set of downstream signaling events. Such stimulus-specific effects are achieved via compartmentalization of the cyclic nucleotide signals within the cell (Buxton and Brunton, 1983; Rich et al., 2001; Zaccolo and Pozzan, 2002).

To illustrate how these signaling compartments may be established, it is worth taking a closer look at the cAMP-signaling pathway. We currently assume that the free concentration of this cyclic nucleotide within a cell depends on the rate of its synthesis, the rate of breakdown, and any potential intracellular buffering systems, such as cAMP-binding proteins or molecularly crowded cellular substructures that may slow cAMP diffusion (Nikolaev and Lohse, 2006; Agarwal et al., 2016; Richards et al., 2016; Bers et al., 2019). The synthesis rate

ABBREVIATIONS: AC, adenylyl cyclase; AKAP, A-kinase–anchoring protein; FRET, fluorescence resonance energy transfer; GPCR, G-protein–coupled receptor; IBMX, 3-isobutyl-1-methylxanthine; PDE, phosphodiesterase; PGE2, prostaglandin E2; PKA, protein kinase A; PKAC, PKA catalytic subunit; PKA-R, PKA regulatory subunit; SERCA, sarcoplasmic/endoplasmic reticulum calcium ATPase; SKIP, sphingosine kinase type 1–interacting protein.

of cAMP is governed through the activity of adenylyl cyclases (ACs). AC activity is modulated by activated G-proteins, which are released when a G-protein–coupled receptor (GPCR) binds its specific ligand. The breakdown rate of cAMP depends on the local activity of phosphodiesterases (PDEs) that hydrolyze cAMP to AMP. Regulation of local PDE activity is complex and heavily depends on the nature of the PDE isoforms present; the mammalian PDE superfamily is composed of 21 genes and multiple transcriptional splice variants or alternative transcription initiation sites, resulting in a very large number of isoforms. Different PDE isoforms often localize to distinct subcellular structures via protein-protein or protein-lipid interactions (Omori and Kotera, 2007). Depending on the extracellular ligand, a GPCR signaling event may activate distinct subsets of AC. The cAMP signal generated can engage diverse PDE isoforms, and these isoforms can be characteristically found in distinct cellular compartments (Johnstone et al., 2018). PDEs are the only enzymes that degrade cAMP, and thus the pattern of PDE isoforms involved in the degradation of cAMP in response to a specific signaling event becomes critical. Being able to limit the concentration of cAMP in their vicinity, the local activity of different PDE isoforms can lead to cAMP concentration gradients across a cell (Baillie, 2009; Houslay, 2010; Brescia and Zaccolo, 2016). On the other hand, inhibition of PDEs abolishes local differences in PDE activity and leads to a loss of cAMP compartmentalization (Jurevicius and Fischmeister, 1996; Zaccolo and Pozzan, 2002; Mongillo et al., 2004). PDEs therefore emerge as key regulators of compartmentalized signaling, as the unique array of PDEs that impinge on a defined subcellular location is what dictates the local level of cAMP and the activation of local effectors.

The theme of conferring specificity through localization continues downstream of cAMP synthesis and hydrolysis, with many cAMP target proteins enriched at distinct cellular sites. Local changes in cAMP directly affect the function of exchange proteins directly activated by cAMP, cyclic nucleotide–gated channels, and Popeye domain–containing proteins, all of which can localize to specific subcellular locations (Brady et al., 2004; Harnett et al., 2015; Pereira et al., 2015; Brand, 2018). Perhaps the best-studied cAMP effector is protein kinase A (PKA), which is anchored to specific cellular structures via A-kinase–anchoring proteins (AKAPs) (Kritzer et al., 2012; Scott et al., 2013; Taylor et al., 2013). Anchoring to a specific site increases the local rate of PKA substrate phosphorylation and can thus lead to distinct cellular patterns of phosphorylation. By providing binding sites for both the cAMP effector kinase PKA and protein phosphatases that can reverse PKA phosphorylation, AKAPs provide a structural basis for tight regulation of cAMP-dependent phosphorylation events (Feschenko et al., 2002; Dodge-Kafka et al., 2010; Nygren and Scott, 2015; Sanderson et al., 2018).

Maintaining compartmentalization of cyclic nucleotide signaling is critical for an accurate physiologic response to external stimuli. Accordingly, any perturbation of compartmentalized signaling may lead to developmental defects and disease (Zaccolo, 2011). For instance, specific functions can be attributed to distinct PDE isoforms, and dysregulation of PDE activity has been associated with infertility, cardiac arrhythmia, cardiac hypertrophy, heart failure, stroke, depression, dementia, and inflammation (Gretarsdottir et al., 2003; Halene and Siegel, 2007; Shen et al., 2010; Aye et al., 2012;

Ghigo et al., 2012; Zoccarato et al., 2015). Genetic alteration in AKAPs has been associated with increased risks of infertility, perinatal death, microcephaly, learning deficits, cardiac development defects, Long Q-T syndrome, cardiac arrhythmias, cardiac hypertrophy, breast cancer, prostate hyperplasia, schizophrenia, major depressive disorder, autism, and autoimmune disease [reviewed in Tröger et al. (2012)].

With the importance of spatial and temporal compartmentalization of cAMP signaling in health and disease well-established, current research focuses on mapping cAMP-signaling pathways in space and time across different cellular systems. Family-selective PDE inhibitors are Food and Drug Administration–approved for the treatment of congestive heart failure, thrombocythemia, chronic obstructive pulmonary disease, psoriasis, psoriatic arthritis, atopic dermatitis, intermittent claudication, erectile dysfunction, and pulmonary arterial hypertension (Baillie et al., 2019). However, some of these therapeutic agents are associated with significant side effects that, at least in part, are causally linked to their inability to discriminate between isoforms within the same PDE family. Thus, directly targeting individual cAMP-signaling domains via modulation of individual PDE isoform activity, as opposed to nonselective elevation of cAMP levels with available pharmacological inhibitors, has been proposed as a viable approach to mitigate side effects and safety concerns (Baillie et al., 2019). To evolve medicines targeting the cAMP-signaling pathway from a one-size-fits-all model to precise regulatory agents for specific cellular functions, we need a detailed understanding of the organization and regulation of cAMP nanodomains.

This minireview focuses on the contribution of high-resolution imaging techniques and proteomics to understanding localized cAMP-signaling domains. Advances in biochemical and imaging methodologies have refined our understanding of the spatial dimensions of cAMP-signaling compartments, providing evidence that the size of some of these domains is in the nanometer range (Surdo et al., 2017). Within these compartments, cAMP effectors and regulatory proteins cluster into functional units sometimes defined as signalosomes. We are only beginning to understand the components of signalosomes in different cell types and cellular compartments (Maurice et al., 2014). We predict that combining advanced imaging methods, such as live-cell fluorescence microscopy using targeted reporters (Barbagallo et al., 2016; Surdo et al., 2017) and super-resolution imaging (Ni et al., 2018), with proteomics approaches will be instrumental in identifying additional signaling components and novel signalosomes.

Defining cAMP Nanodomains Using Real-Time Imaging

Our understanding of cAMP compartmentalization in living cells has advanced with the development of genetically encoded fluorescent probes that allow monitoring of cAMP levels in real time upon cellular stimulation (Zaccolo et al., 2000). These sensors consist of a cAMP-binding element and two fluorescent proteins whose spectral properties are permissive for fluorescence resonance energy transfer (FRET), provided that they are in close proximity. In FRET-based cAMP sensors, binding of cAMP to the cAMP-binding element induces a conformational change that impacts the distance

and relative orientation of the two fluorophores. Depending on the design of the sensor, cAMP binding leads to either dissociation of the fluorophores and associated decrease in FRET or coming together of the fluorophores, resulting in an increase in FRET. Changes in FRET can be quantified with a fluorescence microscope. The high temporal and spatial resolution of this technique enables measurement of differential changes in cAMP concentration across adjacent cellular sites and allows it to be established that cAMP signaling in living cells is compartmentalized within restricted subcellular domains (Zaccolo and Pozzan, 2002). FRET-based sensors that report PKA-dependent phosphorylation (Lin et al., 2019) are based on a similar principle and are useful complementary tools.

Genetically encoded cAMP and PKA-activity sensors can be engineered to localize at defined cellular sites by introducing a unique targeting sequence (DiPilato et al., 2004; Di Benedetto et al., 2008, 2013; Herget et al., 2008; Sprenger et al., 2015; Barbagallo et al., 2016; Surdo et al., 2017). Targeting the same cAMP-FRET reporter to the plasma membrane and the mitochondria revealed differential dynamics of cAMP signaling in these two compartments. Local cAMP concentrations increase much faster in response to adrenaline at the membrane than in the cytoplasm (DiPilato et al., 2004). Using the dimerization/docking domain of PKA regulatory subunits (PKA-Rs) type I and type II as a targeting domain for the same FRET sensor in cardiomyocytes demonstrated differential regulation of cAMP levels in PKA-RI and PKA-RII compartments, which was dependent on the extracellular stimulus. This mechanistic insight into PKA-RI and PKA-RII substrate selectivity suggested that isoform-specific activators and inhibitors of PKA could be used to target subsets of PKA effectors in cardiac myocytes (Di Benedetto et al., 2008). The important role of PDEs in establishing cAMP compartments in response to specific signaling events has driven the development of cAMP sensors targeted to compartments known to be under the regulation of specific PDE isoforms (Herget et al., 2008). Fusion of the FRET sensor to the PDE itself as a targeting modality is associated with strong limitations for the analysis of any cAMP-signaling events downstream of the selected PDE. Overexpression of an active PDE may in itself disrupt cAMP-signaling events in the compartment studied, and overexpression of a catalytically inactive form may displace endogenous PDE enzyme activity. Therefore, it is pertinent to find alternative means to target FRET sensors to PDE-regulated compartments. An in-depth molecular understanding of the composition of each PDE compartment may facilitate the design of such targeted sensors. For example, targeting a FRET sensor to the cardiac sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA) 2a by means of a short targeting peptide derived from the sequence of its known interactor phospholamban allowed the development of a biocompatible targeted sensor that was introduced as a transgene to generate a viable mouse model (Sprenger et al., 2015). This mouse model was used to compare cAMP signaling in the vicinity of SERCA in healthy and hypertrophic murine hearts and revealed the detrimental effect of hypertrophy specifically on PDE4 activity in this compartment.

Targeting the cAMP-FRET reporter to specific subcellular sites has revealed that the size of individual cAMP domains may be significantly smaller than previously thought. In a recent study, the molecular understanding of signaling complexes at the cardiomyocyte plasmalemma, sarcoplasmic reticulum, and myofilaments was exploited to target the cAMP sensor cAMP Universal Tag for imaging experiments to the L-Type calcium channels at the plasmalemma, the SERCA2a/phospholamban complex at the sarcoplasmic reticulum, and the troponin complex at the myofilament using AKAP79, AKAP18δ, and troponin I as targeting domains, respectively. These proteins are well-established components of multiprotein assemblies at the three subcellular locations. To allow direct comparison of FRET signals at the three sites, the cAMP Universal Tag for imaging experiments reporter was engineered to increase the distance between the targeting domain and the FRET fluorophore pair, a design that minimizes steric hindrance from the targeting domain to the FRET module (Chao et al., 2019). By targeting the FRET sensors to these distinct macromolecular complexes, it emerged that in cardiomyocytes physiologically relevant cAMP signals operate within the nanometer range (Surdo et al., 2017). The operational diameter of a cAMP domain may therefore lie below the resolution limit of conventional optical microscopy. This is an important observation because it suggests that at least some of the subcellular cAMP-signaling domains may not be detectable by optical microscopy unless a cAMP sensor is strategically targeted within that nanodomain.

cAMP effector proteins often cluster together with their targets and cAMP regulatory proteins to form discrete cAMP-signaling hubs termed "signalosomes" (Maurice et al., 2014; Laudette et al., 2018). This is of considerable importance for cAMP compartmentalization, because it leads to local concentration and amplification of cAMP effects. With our understanding of the molecular composition of many signalosomes constantly evolving, we might soon be able to model comprehensive maps of all cAMP-signaling domains within a given cell type. Such a map would allow us to integrate signaling events from different GPCRs into a unified model. This will require further identification of proteins suitable for targeting FRET sensors to each nanodomain and quantification of cAMP signaling within them. In compartments that are very well-characterized on a molecular level, identification of signalosome components can be based on prior knowledge of the compartment. However, to identify additional components of known signalosomes and to uncover novel cAMP signalosomes, unbiased approaches may be advantageous. Proteomics have long been used to discover proteins associated with a given disease state or physiologic function (O'Reilly et al., 2018). We propose that proteomics is equally well-suited as a means to define the subcellular map of cAMP nanodomains.

Using Chemical Proteomics and Interactomes to Define cAMP-Signaling Domains

Proteomics Can Be Used to Identify New Components of cAMP Signalosomes. Different experimental approaches have been used to study cAMP signaling using targeted proteomics (Table 1). To analyze proteins that interact with cAMP itself, a chemical proteomics approach can be used. This involves chemical immobilization of the second messenger molecule onto agarose beads via flexible linkers and use of these cAMP beads for optimized affinity pull-downs. In rat-heart ventricular tissue lysates, mass spectrometry analysis

TABLE 1
Different approaches to targeted proteomics are used to study cAMP signaling

Targeting Approach	Description	References
Affinity resin with chemically immobilized cAMP	cAMP is chemically immobilized onto agarose beads and used for affinity purification	Scholten et al., 2006; Kovanich et al., 2010; Aye et al., 2012; Burgers et al., 2012
Affinity resin with chemically immobilized IBMX	The nonselective PDE inhibitor IBMX is chemically immobilized onto agarose beads and used for affinity purification	Corradini et al., 2015
Protein tag–mediated immunoprecipitation	A protein of interest is tagged with a peptide or fluorescent protein and expressed in the target cells or tissues. The recombinant protein is purified from whole-cell lysates using antibodies against the peptide/protein tag	Means et al., 2011; Schmoker et al., 2018
Enzyme-mediated proximity proteomics	A protein of interest is tagged with an engineered promiscuous biotin ligase and expressed in the target cells or tissues. All biotinylated proteins are purified from whole-cell lysates using streptavidin beads	Smith et al., 2018

confirmed that many interactors were genuine cAMP-binding proteins, including PKA, PDEs, and AKAPs (Scholten et al., 2006). Using a cAMP affinity–based chemical proteomics strategy in human heart and platelets uncovered novel AKAPs, including sphingosine kinase type 1–interacting protein (SKIP) and small membrane AKAP (Scholten et al., 2006; Kovanich et al., 2010; Burgers et al., 2012).

Chemical proteomics has also been performed using chemical inhibitors as a bait immobilized on agarose beads. A global PDE-capturing resin was synthesized based on the nonselective PDE inhibitor 3-isobutyl-1-methylxanthine (IBMX) (Corradini et al., 2015). By incubating this resin with HeLa cell lysates, it was possible to isolate several PDEs. To refine the interactome results and identify PDE family–specific interactors, selective PDE inhibitors, such as the PDE3 inhibitor cilostamide, were used to competitively elute PDE3 and its interacting proteins off the IBMX beads. Besides known interactors, such as the family of 14-3-3 proteins identified in pilot experiments with HeLa cell lysates, PDE3 was found to associate with a serine/threonine-protein phosphatase 2A complex composed of a regulatory, scaffold, and catalytic subunit, which highlights this as a method of interest to identify new mediators of PDE signaling in complex cell lysates (Corradini et al., 2015).

To further characterize the composition of known cAMP signalosomes, interactomes of specific proteins can be obtained by combining immunoprecipitation of the protein of interest with mass spectrometry. For instance, polypeptide protein tag–mediated immunoprecipitation of SKIP followed by mass spectrometry showed that the SKIP interactome comprises several members of a large multiprotein complex at the mitochondria that is important for inner membrane architecture and called the mitochondrial contact site and cristae-organizing system complex. This led to the discovery that SKIP is indeed enriched at the inner mitochondrial membrane, where it associates with coiled-coil-helix-coiled-coil-helix domain containing 3 (ChChd3), a prominent PKA substrate (Means et al., 2011). In human embryonic kidney 293 cells, immunoprecipitation of yellow fluorescent protein–labeled PKA catalytic subunit (PKAC) α was used to characterize the crosstalk between cAMP and Src family kinase signaling (Schmoker et al., 2018). Results from these experiments demonstrate that the activity of the Src family

kinase Fyn influences the docking of PKA to specific cellular scaffolds and suggest that Fyn may affect the downstream substrates targeted by PKA.

A slightly different approach to further interrogate known cAMP signalosomes in cellular model systems is enzyme-mediated proximity proteomics (Roux et al., 2012; Branon et al., 2018). With this approach, cells are transfected with a known signalosome protein that is tagged with an engineered promiscuous biotin ligase, leading to efficient biotinylation of proteins within a radius of ~5–10 nm from the tagged signalosome constituent. All biotinylated proteins can subsequently be separated out using streptavidin beads and submitted to proteomics analysis. This technique was used to characterize the distinct binding partners of compartment-selective variants of AKAP18 (Smith et al., 2018). The authors first showed that AKAP18γ is retained in the cytoplasm in a PKA-dependent manner and that single nucleotide polymorphisms in the PKA-anchoring domain of this AKAP lead to its nuclear translocation in HeLa cells. They then used enzyme-mediated proximity proteomics in human embryonic kidney 293T cells to show that nuclear AKAP18 isoforms strongly associate with the RNA-splicing machinery, whereas cytoplasmic forms can be found to interact with proteins involved in translational control and cell cycle progression.

When using proteomics to identify novel cAMP signalosome components, it is worth bearing in mind that these complexes may be cell type–specific and that the components of a functional signalosome may vary between organisms, tissues, and cell types. Thus, targeted proteomic studies in physiologically relevant cells and tissues are likely to be essential to complement the present findings. To further validate targeted proteomics experiments, gene-editing technologies, including CRISPR/CRISPR-associated protein 9 approaches (Doudna and Charpentier, 2014), could help assess the functional relevance of novel interactions in specific cell types.

Proteomics Reveals Disruption of cAMP-Signaling Domains in Heart Failure. Using chemical proteomics directly applied to human left ventricular free wall tissue from patients with dilated cardiomyopathy and control heart tissue revealed that, in the failing human heart, PKA association with AKAPs is severely altered (Aye et al., 2012). cAMP pull-downs were performed using either tissue lysates from normal hearts or lysates from dilated hearts and

subjected to quantitative proteomics analysis. The abundance of primary and secondary cAMP-interacting proteins was quantified. Evaluating ion volume and spectral counts for the primary cAMP binders showed that the amount of PKA-Rs was decreased in human dilated cardiomyopathy, whereas the amount of PDE2A was increased. The abundance of specific isoforms of PKA-R, which are primary binding partners of cAMP, was then compared with the abundance of their corresponding PKA-R isoform–specific AKAPs, which constitute secondary cAMP-binding partners. This analysis showed that the association profile of PKA-R with several AKAPs was severely altered in the failing hearts. These altered PKA-AKAP–binding profiles further substantiate a model in which the organization of cAMP-signaling modules is critical to maintain cell function and health. Molecular-level information on the reorganization of cAMP signaling in the failing heart may provide a resource to inform the development of specific molecular therapies that serve to locally rescue dysfunctional signaling. For instance, restoring local cAMP pools by downregulating PDE2 activity in a mouse model of cardiac pressure overload can counteract hypertrophic growth

that, in the long term, would lead to cardiac dilation and heart failure (Zoccarato et al., 2015).

Using Phosphoproteomics to Define cAMP-Signaling Domains

Mapping out the protein components of cAMP signalosomes within a cell will further support the development of new targeted cAMP-FRET sensors in which fusion of a signalosome-specific protein to the reporter directs its targeting to the specific signalosome and thus allows precise measurement of cAMP changes within that discrete cellular nanodomain (Fig. 1). Moreover, proteomics can help elucidate functional aspects of cAMP signaling by quantifying the net effects of downstream activity of PKA and PDEs. Subcellularly targeted FRET reporters can precisely define compartmentalized regulation of cAMP, and this allows us to estimate the range of PKA activation as a response. To confirm the downstream effects and map out the proteins targeted by PKA activation, identification of cAMP-dependent phosphorylation sites is
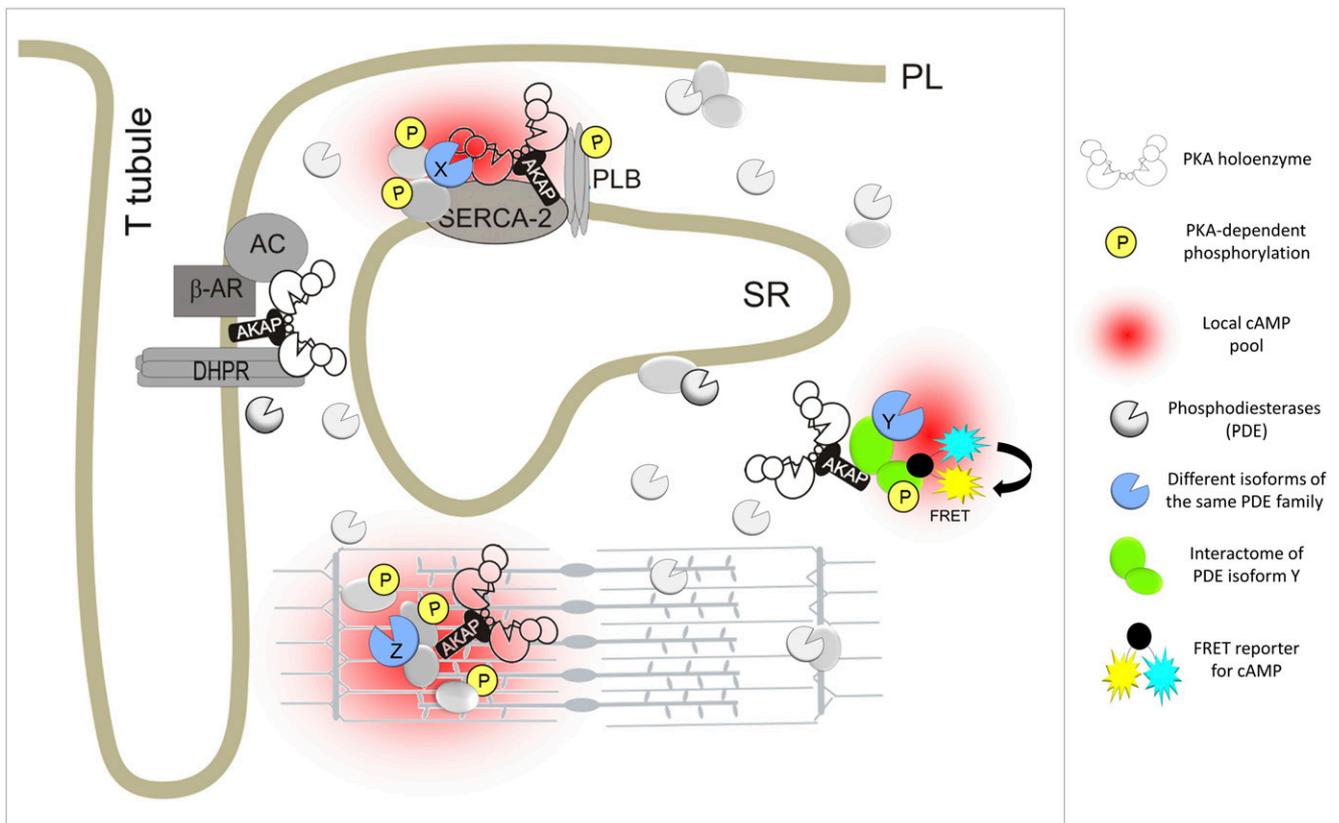
Fig. 1. Combining proteomics and imaging approaches to define a map of intracellular cAMP nanodomains. Cyclic AMP signaling in cardiomyocytes is compartmentalized in spatially confined signalosomes. Local changes in cAMP levels are dictated by the local activity of different PDEs that localize at specific sites mainly via protein-protein or protein-lipid interactions. The schematic shows three subcellular domains where cAMP levels are regulated by three different isoforms (X, Y, and Z) of a specific family of PDEs (in blue). The local increase in cAMP (represented as a red shaded area) generated by pharmacological inhibition of the members of this PDE family results in local activation of PKA and phosphorylation of local target proteins. The study of the phosphoproteome that results from inhibition of this specific PDE family of enzymes allows the identification of the sites of action of the PDE isoforms belonging to this family and can reveal the location of previously unidentified cAMP signalosomes. Because current PDE inhibitors are family-selective but cannot discriminate between different isoforms within the same PDE family, phosphoproteomics studies cannot define which isoform is responsible for the regulation of which local cAMP domain. One approach to overcome this limitation is to study the PDE isoform–specific interactomes. For example, pull-down of PDE isoform Y will allow identification of the proteins (shown in green in the schematic) that selectively interact with PDE isoform Y from those that interact with isoforms X and Z. By combining the analysis of PDE family–specific phosphoproteomes and of PDE isoform–specific interactomes, the composition of site-specific cAMP signalosomes can be defined with high resolution. Specific interactors of individual PDE isoforms can then be used to target cAMP and PKA-activity FRET reporters to the newly identified signalosomes for validation studies. β-AR = beta adrenergic receptor; DHRP = Dihydropyridine receptor; PLB = phospholamban; SR = sarcoplasmic reticulum

key. Phosphoproteomics can help link this cAMP-mediated PKA regulation to downstream effector molecules and biologic functions (Beltejar et al., 2017). Mass spectrometry can identify thousands of phosphosites with high precision (Altelaar et al., 2013), and quantitative mass spectrometry has been shown to be sensitive to dynamic changes in protein phosphorylation after GPCR activation (Williams et al., 2016). To maximize the coverage of phosphoproteins in a complex sample, phosphorylated proteins and peptides need to be separated from their nonphosphorylated counterparts using an enrichment strategy and combined with a quantification approach (Table 2).

Just like the interactome-based proteomics approaches described above, phosphoproteomics can identify novel substrates of cAMP signaling (Chu et al., 2004; Imamura et al., 2017; Isobe et al., 2017). Phosphoproteins identified by proteomics may be completely uncharacterized, but more often they will have been characterized in different contexts. In the latter case, protein data base information is associated with each identification, including information on function, subcellular localization, and known interactors (UniProt Consortium, 2019). By associating novel phosphosites with a specific stimulus, such as GPCR signaling, PKA activity, PDE activity, or phosphatase activity, we can derive hypotheses on the localization of the signaling domains regulated by these signaling events.

Quantitative phosphoproteomics was used to identify 670 site-specific phosphorylation changes in mouse hearts under β-adrenergic stimulation. This included previously unknown phosphorylation sites involved in myocardial contractility, suggesting that phosphoproteomics can identify potential targets for the treatment of heart disease and hypertension (Lundby et al., 2013). Furthermore, phosphoproteomic approaches can document the temporal regulation of cellular phosphosites controlled by PKA and any downstream kinases in response to an extracellular stimulus. In one study, Jurkat T cells stimulated by prostaglandin E2 (PGE2) over six different time points were harvested and subjected to phosphoproteomics analysis to reveal downstream PGE2-signaling dynamics in T cells (de Graaf et al., 2014). After PGE2 stimulation, several pathways became only transiently activated, whereas substrates in other pathways only showed significantly elevated phosphorylation after chronic stimulation with PGE2.

To comprehensively identify PKA substrates in kidney epithelial cells in a proteomics experiment, functional PKA protein was completely eliminated from these cells using CRISPR/CRISPR-associated protein 9 genome editing of the catalytic regions of PKACα and PKACβ in mouse mpkCCD cells (Isobe et al., 2017). Using stable isotope labeling with amino acids in cell culture–based quantitative phosphoproteomics, 229 PKA target sites were identified in the PKA functional knockout cells. Interestingly, PKA deletion was not only accompanied by a decrease in phosphorylation of direct PKA substrates but also by an increase in phosphorylation of a distinct set of proteins, highlighting the complex interplay of kinases and phosphatases that operate downstream on cAMP signaling. Thus, it will be interesting to similarly interrogate in future experiments how the abundance of phosphatases may shape the PKA phosphoproteome.

TABLE 2
Enrichment and quantification strategies used to study cAMP-dependent phosphoproteins using phosphoproteomics

Technique	Description	References
Phosphopeptide enrichment		
[32]P-labeling and 2D gel electrophoresis	Cells are incubated with radioactive phosphate prior to protein isolation. Cellular proteins are separated using 2D gel electrophoresis, and labeled bands corresponding to phosphoproteins are isolated for mass spectrometry	Chu et al., 2004
HAMMOC resin	Phosphoproteins are enriched using resins with hydroxy acid–modified metal oxides	Imamura et al., 2017
MOAC matrix	Phosphoproteins are enriched using a matrix of metal oxides or hydroxides (e.g., titanium dioxide)	Lundby et al., 2013; Williams et al., 2016; Isobe et al., 2017
IMAC resin	Phosphoproteins are enriched using IDA or NTA resins with associated metal ions. Because the metal ions are positively charged, they interact with and retain negatively charged phosphate groups on phosphoproteins	de Graaf et al., 2014; Golkowski et al., 2016; Williams et al., 2016; Beltejar et al., 2017; Isobe et al., 2017
Protein quantification		
Label-free quantification	All samples are prepared for mass spectrometry and individually analyzed by LC-MS/MS. Quantification is based on the comparison of peak intensity of the same peptide or the spectral count of the same protein	Lundby et al., 2013; de Graaf et al., 2014; Beltejar et al., 2017
SILAC labeling	Cells are incubated with isotopically labeled amino acids prior to protein isolation. Samples are combined for LC-MS/MS analysis, allowing for relative quantitation of protein content between up to three samples based on their differential masses	Golkowski et al., 2016; Williams et al., 2016; Imamura et al., 2017
TMT labeling	Proteins from up to 16 samples are digested for mass spectrometry and then labeled with isobaric chemical tags allowing for relative quantitation of protein content between samples	Imamura et al., 2017

HAMMOC, hydroxy acid–modified metal oxide chromatography; IDA, iminodiacetic acid; IMAC, immobilized metal-ion affinity chromatography; MOAC, metal oxide affinity chromatography; LC-MS/MS, liquid chromatography tandem mass spectrometry; NTA, nitrilotriacetic acid; SILAC, stable isotope labeling with amino acids in cell culture; TMT, tandem mass tag.

Phosphoproteomics can be used to understand the impact of a change in the cAMP signal in different compartments. Selective, inhibitor-dependent phosphoproteome analysis can help dissect the roles of different PDEs in the regulation of cyclic nucleotide signaling (Beltejar et al., 2017). Because PDEs remain the only known route of cAMP degradation, their local activity contributes to intracellular cAMP gradients. Family-selective PDE inhibitors locally increase cAMP levels in those compartments where members belonging to that PDE family are localized. The resulting local increase in cAMP activates PKA in the vicinity. Thus, analysis of the phosphopeptides generated on family-selective PDE inhibition can provide novel information on the location and function of cAMP-signaling domains under the control of members of that particular PDE family. Beltejar and colleagues (2017) used highly selective inhibitors for PDE1, PDE3, PDE4, PDE7, and PDE8 families, alone and in combination, to perturb cAMP nanodomains in Jurkat T cells costimulated with low PGE2 (1 nM). Proceeding with the combination of PDE inhibitors that caused the greatest increases in global cAMP, which was coinhibition of PDE3 and PDE4 for Jurkat cells, the authors found that the PDE-regulated phosphoproteomes under the control of PDE3 and PDE4 were remarkably distinct from the phosphoproteome recorded upon global PDE inhibition. These differences in PDE-regulated phosphoproteomes are predicted to lead to the regulation of different biologic processes in the T cells, depending on the number and nature of active PDEs. Thus, the approach of using selective, inhibitor-dependent phosphoproteome analysis can be used as a method to dissect the roles of different PDEs in the regulation of cAMP signaling.

Similarly, a phosphoproteomic study in Leydig cells showed that coinhibition of PDE4 and PDE8 in this cell type can lead to synergistic effects on cellular signaling pathways (Golkowski et al., 2016). Strikingly, although the PDE8-regulated phosphoproteome only comprised 54 phosphorylation sites, and PDE4 inhibition caused only minor effects, the concomitant deactivation of both PDE families led to the detection of 749 regulated sites, which was nearly 14 times as many as with PDE8 inhibition alone.

Combining Interactomics, Phosphoptoteomics, and Real-Time FRET Imaging

Identifying the phosphorylation sites that are regulated downstream of a cellular signaling event provides us with an understanding of the molecular targets of that signaling event. Our knowledge of the regulatory effects of phosphorylation at these sites, based on previous experiments or further investigation, can then inform hypotheses on downstream cellular functions affected. However, to truly understand the spatial boundaries within which these downstream signaling events take place, there is a need to combine the functional information inherent in the phosphoproteome with network-level information on the direct and indirect interaction partners of the proteins involved in the signaling event. Interactomes can provide such detailed understanding of protein interaction networks and may be used to capture in detail the composition of cAMP signalosomes. We have previously shown that a deep molecular understanding of the components of cAMP signalosomes translated into practical

molecular tools, such as targeted FRET sensors, allows us to dissect cAMP signaling within a cell with unprecedented precision (Surdo et al., 2017). A line of investigation that we are currently pursuing combines PDE family–selective phosphoproteomics and PDE isoform–specific interactome analysis with FRET imaging technology (Fig. 1).

When applying a PDE family–selective pharmacological inhibitor, a rise in cAMP is expected to occur selectively in those subcellular domains that are under the control of the different isoforms within that PDE family. Such local pools of high cAMP lead to local activation of PKA and to phosphorylation of local targets. Analysis of the changes in a cell's phosphoproteome upon inhibition of a PDE family can provide information on the localization and function of cAMP nanodomains. However, a caveat to this approach is that it does not discriminate between individual isoforms within a PDE family, as isoform-selective inhibitors are not currently available. The analysis of PDE isoform–specific interactomes can overcome this limitation because it provides specific information on the subcellular localization of individual enzymes and on the location of cAMP pools under their specific control. Combining the analysis of PDE family–specific phosphoproteomes and PDE isoform–specific interactomes has the additional advantage that crossreferencing candidate signalosome proteins from the two data sets provides a means to validate the results and to identify the proteins that are most likely to be true protein markers of previously unidentified cAMP nanodomains. These markers can then be used as targeting domains to direct the cAMP or PKA-activity FRET reporters to that specific site for further validation.

With this combined approach, we aim at building a physical and functional map of cAMP-signaling events in a cell that will allow new insights into the formation and temporal dynamics of cAMP nanodomains in response to activation of specific GPCRs. In the future, this knowledge could be applied to inform the design of targeted therapies for conditions that involve dysregulation of the cAMP pathway. In some of these conditions, it may be required to target several cellular compartments in specific combinations for the therapy to be most effective. Other conditions may require a very targeted approach at a one-compartment level to increase specificity of the treatment that minimizes off-target effects and maximizes patient safety.

Conclusions

GPCRs are the largest and most diverse group of membrane receptors in eukaryotes, and they are expressed in virtually all tissues in the body. Their crucial role in physiology and pathophysiology makes them one of the most targeted molecules in drug development (Bjarnadóttir et al., 2006). More than half of the approved GPCR-targeting drugs perturb the cAMP-signaling pathway (Sriram and Insel, 2018).

We only begin to appreciate how these diverse extracellular signals integrate to establish unique patterns of compartmentalized cAMP-signaling domains within a given cell type. Although the medicines we use to modulate the cAMP pathway are helping patients, they are also associated with unwanted adverse effects. To increase the specific efficacy of our current armamentarium of drugs targeting this signaling pathway, we need a detailed understanding of the complexity of the system. To achieve this, we must strive to map out all

cellular cAMP signalosomes on a nanometer scale. The unique combination of phosphoproteomics, interactome analysis, and FRET imaging may support the discovery of new pharmacological targets by increasing the likelihood of identifying biologically meaningful interactions. Functional phosphoproteomic analysis downstream of specific GPCRs or PDEs may help define novel signalosomes by identifying new signaling targets. Interactomes of new or known signalosome components may support identification of suitable targeting domains for FRET sensors that can then be used to characterize cAMP signaling at that site in space and time. With a detailed model of the spatiotemporal distribution of cyclic nucleotides in healthy versus diseased cells, we may be able to design specific targeted interventions, such as signalosome disruptor peptides or small molecules, to correct pockets of aberrant cyclic nucleotide signaling for precision medicine.

Authorship Contributions

Wrote and contributed to the writing of the manuscript: Schleicher, Zaccolo.

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