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Title Page

Minireview

Axelrod Symposium 2019: Phosphoproteomic Analysis of G Protein-Coupled Pathways

Defining a cellular map of cAMP nanodomains

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Running Title Page

Running title: **Defining a cellular map of cAMP nanodomains**

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Abbreviations

AC, adenylyl cyclase

AKAP, A-kinase anchoring protein

AMP, adenosine monophosphate

cAMP, 3',5'-cyclic adenosine monophosphate

CNG channel, cyclic nucleotide-gated channel

CRISPR, clustered regularly interspaced short palindromic repeats

CUTie, cAMP Universal Tag for imaging experiments

Epac, exchange protein activated by cAMP

FDA, Food and Drug Administration

FRET, fluorescence resonance energy transfer

GPCR, G-protein-coupled receptor

HAMMOC, hydroxy acid-modified metal oxide chromatography

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IBMX, 3-isobutyl-1-methylxanthine

IDA, iminodiacetic acid

IMAC, immobilized metal-ion affinity chromatography

LC, liquid chromatography

MICOS, mitochondrial contact site and cristae-organizing system complex

MOAC, metal oxide affinity chromatography

MS/MS, tandem mass spectrometry

NTA, nitrilotriacetic acid

PDE, phosphodiesterase

PGE2, prostaglandin E2

PKA, protein kinase A

PKA-R, PKA regulatory subunit

PP2A, serine/threonine-protein phosphatase 2A

SERCA, sarcoplasmic/endoplasmic reticulum calcium ATPase

SILAC, Stable Isotope Labelling with Amino Acids in Cell Culture

SKIP, sphingosine kinase type1-interating protein

TMT, tandem mass tag

YFP, yellow fluorescent protein

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Abstract

By limiting unrestricted activation of intracellular effectors, compartmentalised signalling 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 signalling is essential for our understanding of physiology and pathophysiology but mapping the dynamics and regulation of compartmentalised signalling is a challenge. In this Minireview we summarise advanced imaging and proteomics techniques that have been successfully used to probe compartmentalised 3',5'-cyclic adenosine monophosphate (cAMP) signalling in eukaryotic cells. Subcellularly targeted fluorescence resonance energy transfer (FRET) sensors can precisely locate and measure compartmentalised cAMP, and this allows us to estimate the range of effector activation. As 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 signalling 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 compartmentalised signalling. Compiling a dynamic map of cAMP nanodomains in defined cell types would establish a blueprint for better understanding the alteration of signalling compartments associated with disease and would provide a molecular basis for targeted therapeutic strategies.

Significance Statement

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

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Introduction

Cyclic nucleotides are intracellular second messengers that function to transmit a signalling 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 specialised eukaryotic cells, such as cardiomyocytes in the human heart (Michel *et al.*, 1990; Berman *et al.*, 2005). Cyclic nucleotides are small and hydrophilic molecules, 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 signalling events. Such stimulus-specific effects are achieved via compartmentalisation of the cyclic nucleotide signals within the cell (Buxton and Brunton, 1983; Rich *et al.*, 2001; Zaccolo and Pozzan, 2002).

To illustrate how these signalling compartments may be established it is worth taking a closer look at the 3',5'-cyclic adenosine monophosphate (cAMP) signalling 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 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 hydrolyse 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 result in a very large number of isoforms. Different PDE isoforms often localise to distinct subcellular structures via protein-protein or protein-lipid interactions (Omori and Kotera, 2007). Depending on the extracellular ligand, a GPCR signalling event may activate distinct subsets of AC. The cAMP signal generated can engage

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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 signalling 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 compartmentalisation (Jurevicius and Fischmeister, 1996; Zaccolo and Pozzan, 2002; Mongillo *et al.*, 2004). PDEs therefore emerge as key regulators of compartmentalised signalling 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 localisation 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 (Epac), cyclic nucleotide-gated (CNG) channels, and Popeye domain containing (POPDC) proteins, all of which can localise 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 compartmentalisation of cyclic nucleotide signalling is critical for an accurate physiological response to external stimuli. Accordingly, any perturbation of compartmentalised

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signalling 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 have 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 compartmentalisation of cAMP signalling in health and disease well established, current research focusses on mapping cAMP signalling pathways in space and time across different cellular systems. Family-selective PDE inhibitors are FDA-approved for the treatment of congestive heart failure, thrombocythaemia, 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 which, at least in part, are causally linked to their inability to discriminate between isoforms within the same PDE family. Thus, directly targeting individual cAMP signalling domains via modulation of individual PDE isoform activity, as opposed to non-selective 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 signalling pathway from a one-size-fits-all model to precise regulatory agents for specific cellular functions we need a detailed understanding of the organisation and regulation of cAMP nanodomains.

This Minireview focusses on the contribution of high-resolution imaging techniques and proteomics to understanding localised cAMP signalling domains. Advances in biochemical and imaging

methodologies have refined our understanding of the spatial dimensions of cAMP signalling 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 signalling components and novel signalosomes.

Defining cAMP nanodomains using real-time imaging

Our understanding of cAMP compartmentalisation 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 allowed to establish that cAMP signalling in living cells is compartmentalised 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 localise at defined cellular sites by introducing a unique targeting sequence (DiPilato *et al.*, 2004; Di Benedetto *et al.*, 2008; Herget *et al.*, 2008; Di Benedetto *et al.*, 2013; 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 signalling 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 dimerisation/docking domain of PKA regulatory (PKA-R) subunits 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 signalling 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 signalling events downstream of the selected PDE. Overexpression of an active PDE may in itself disrupt cAMP signalling 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 2a (SERCA2a) 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 signalling 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 signalling complexes at the cardiomyocyte plasmalemma, sarcoplasmic reticulum and myofilaments was exploited to target the cAMP sensor CUTie (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 as targeting domains AKAP79, AKAP18δ and troponin I, 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 CUTie reporter was engineered to increase the distance between the targeting domain and the FRET fluorophore pair, a design that minimises 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 as it suggests that at least some of the subcellular cAMP signalling 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 signalling hubs termed signalosomes (Maurice *et al.*, 2014; Laudette *et al.*, 2018). This is of considerable importance for cAMP compartmentalisation, 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 signalling domains within a given cell type. Such a map would allow

us to integrate signalling events from different GPCRs into a unified model. This will require further identification of proteins suitable for targeting FRET sensors to each nanodomain and quantify cAMP signalling within them. In compartments that are very well characterised 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 physiological 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 signalling domains

Proteomics can be used to identify new components of cAMP signalosomes

Different experimental approaches have been used to study cAMP signalling using targeted proteomics (**Table 1**). To analyse proteins that interact with cAMP itself, a chemical proteomics approach can be used. This involves chemical immobilisation of the second messenger molecule onto agarose beads via flexible linkers and use of these cAMP beads for optimised affinity pull-downs. In rat heart ventricular tissue lysates, mass spectrometry analysis 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 type1-interating protein (SKIP) and small membrane (sm)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 immobilised on agarose beads. A global PDE-capturing resin was synthesised based on the non-selective 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 PP2A complex composed of a regulatory, scaffold and catalytic subunit, which highlights this as a method of interest to identify new mediators of PDE signalling in complex cell lysates (Corradini *et al.*, 2015).

To further characterise 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, called the mitochondrial contact site and cristae-organizing system (MICOS) complex. This led to the discovery that SKIP is indeed enriched at the inner mitochondrial membrane where it associates with Chchd3, a prominent PKA substrate (Means *et al.*, 2011). In HEK293 cells, immunoprecipitation of YFP-labelled PKA catalytic subunit (PKACα) was used to characterise the cross-talk between cAMP and Src family kinase signalling (Schmoker *et al.*, 2018). Results from these experiments demonstrated 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). Here, 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 characterise 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 HEK293T cells to show that nuclear AKAP 18 isoforms strongly associate with the RNA splicing machinery, while 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 clustered regularly interspaced short palindromic repeats/Cas9 (CRISPR/Cas9) approaches (Doudna and Charpentier, 2014), could help assess the functional relevance of novel interactions in specific cell types.

Proteomics reveals disruption of cAMP signalling 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-R subunits was decreased in human dilated cardiomyopathy, while the amount of PDE2A was increased. The abundance of specific isoforms of PKA-R, which are primary binding partners of cAMP, was then compared to 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 organisation of cAMP signalling modules is critical to maintain cell function and health. Molecular-level information on the re-organisation of cAMP signalling in the failing heart may provide a resource to inform the development of specific molecular therapies that serve to locally rescue dysfunctional signalling. 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 signalling domains

Mapping out the protein components of cAMP signalosomes within a cell will further support the development of new targeted cAMP FRET sensors where 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 (**Figure 1**). Moreover, proteomics can help elucidate functional aspects of cAMP signalling by quantifying the net effects of downstream activity of PKA and PDEs. Subcellularly targeted FRET reporters can precisely define compartmentalised 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 key. Phosphoproteomics can help link this cAMP-mediated PKA regulation to downstream effector molecules and biological 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 following GPCR activation (Williams *et al.*, 2016). To maximise the coverage of phosphoproteins in a complex sample, phosphorylated proteins and peptides need to be separated from their non-phosphorylated 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 signalling (Chu *et al.*, 2004; Imamura *et al.*, 2017; Isobe *et al.*, 2017). Phosphoproteins identified by proteomics may be completely uncharacterised, but more often they will have been characterised in different contexts. In the latter case, protein database information is associated with each identification, including information on function, subcellular localisation and known interactors (UniProt Consortium, 2019). By associating novel phosphosites with a specific stimulus, such as GPCR signalling, PKA activity, PDE activity, or phosphatase activity, we can derive hypotheses on the localisation of the signalling domains regulated by these signalling 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 signalling dynamics in T cells (de Graaf *et al.*, 2014). Following 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-Cas9 genome editing of the catalytic regions of the PKA catalytic subunits PKA-Cα and PKA-Cβ in mouse mpkCCD cells (Isobe *et al.*, 2017). Using SILAC-based quantitative phosphoproteomics, 229 PKA target sites were identified in the PKA functional knock-out 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 signalling. Thus, it will be interesting to similarly interrogate in future experiments how the abundance of phosphatases may shape the PKA phosphoproteome.

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 signalling (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 localised. 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 signalling domains under the control of members of that particular PDE family. Beltejar and colleagues used highly selective inhibitors for PDE1, PDE3, PDE4, PDE7 and PDE8 families, alone and in combination, to perturb cAMP nanodomains in Jurkat T cells co-stimulated with low PGE2 (1 nM). Proceeding with the combination of PDE inhibitors that caused the greatest increases in global cAMP, which was co-inhibition 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 biological 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 signalling.

Similarly, a phosphoproteomic study in Leydig cells showed that co-inhibition of PDE4 and PDE8 in this cell type can lead to synergistic effects on cellular signalling pathways (Golkowski *et al.*, 2016). Strikingly, while 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, nearly fourteen 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 signalling event provides us with an understanding of the molecular targets of that signalling 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 signalling 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 signalling 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 signalling 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 localisation and function of cAMP nanodomains. However, a caveat

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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 as it provides specific information on the subcellular localisation 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 cross-referencing candidate signalosome proteins from the two datasets 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 signalling 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 minimises off-target effects and maximises 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 signalling pathway (Sriram and Insel, 2018).

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We only begin to appreciate how these diverse extracellular signals integrate to establish unique patterns of compartmentalised cAMP signalling domains within a given cell type. While 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 signalling 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 nanometre 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 signalling targets. Interactomes of new or known signalosome components may support identification of suitable targeting domains for FRET sensors that can then be used to characterise cAMP signalling at that site in space and time. With a detailed model of the spatio-temporal 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 signalling for precision medicine.

Authorship Contributions

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

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Legends for Figures

Figure 1

Combining proteomics and imaging approaches to define a map of intracellular cAMP nanodomains. Cyclic AMP signalling in cardiomyocytes is compartmentalised in spatially confined signalosomes. Local changes in cAMP levels are dictated by the local activity of different phosphodiesterases (PDE) that localise 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. As 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 isoforms 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.

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Tables

TABLE 1. Different approaches to targeted proteomics are used to study cAMP signalling.

Targeting approach	Description	References
Affinity resin with chemically immobilised cAMP	cAMP is chemically immobilised 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 immobilised IBMX	The non-selective PDE inhibitor IBMX is chemically immobilised 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

cAMP, 3′,5′-cyclic adenosine monophosphate; IBMX, 3-isobutyl-1-methylxanthine; PDE, phosphodiesterase

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

Technique	Description	References
Phosphopeptide enrichment		
^{32}P-labelling and 2D gel electrophoresis	Cells are incubated with radioactive phosphate prior to protein isolation. Cellular proteins are separated using 2D gel electrophoresis and labelled 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)	Isobe et al., 2017; Lundby et al., 2013; Williams et al., 2016
IMAC resin	Phosphoproteins are enriched using IDA or NTA resins with associated metal ions. As the metal ions are positively charged, they interact with and retain negatively charged phosphate groups on phosphoproteins	Beltejar et al., 2017; de Graaf et al., 2014; Golkowski et al., 2016; Isobe et al., 2017; Williams et al., 2016
Protein quantification		
Label-free quantification	All samples are prepared for mass spectrometry and individually analysed 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	Beltejar et al., 2017; de Graaf et al., 2014; Lundby et al., 2013
SILAC labelling	Cells are incubated with isotopically labelled 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; Imamura et al., 2017; Williams et al., 2016

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TMT labelling	Proteins from up to 16 samples are digested for mass spectrometry and then labelled 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; LC, liquid chromatography; MOAC, metal oxide affinity chromatography; MS/MS, tandem mass spectrometry; NTA, nitrilotriacetic acid; SILAC, Stable Isotope Labelling with Amino Acids in Cell Culture; TMT, tandem mass tag

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Figures

Figure 1