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Published in:
Trends in Pharmacological Sciences

DOI:
[10.1016/j.tips.2017.11.006](https://doi.org/10.1016/j.tips.2017.11.006)

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2018

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Musheshe, N., Schmidt, M., & Zaccolo, M. (2018). cAMP: From Long-Range Second Messenger to Nanodomain Signalling. *Trends in Pharmacological Sciences*, 39(2), 209-222.
<https://doi.org/10.1016/j.tips.2017.11.006>

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Review

cAMP: From Long-Range Second Messenger to Nanodomain Signalling

Nshunge Musheshe,^{1,2} Martina Schmidt,^{1,3} and Manuela Zaccolo^{2,*}

How cAMP generates hormone-specific effects has been debated for many decades. Fluorescence resonance energy transfer (FRET)-based sensors for cAMP allow real-time imaging of the second messenger in intact cells with high spatiotemporal resolution. This technology has made it possible to directly demonstrate that cAMP signals are compartmentalised. The details of such signal compartmentalisation are still being uncovered, and recent findings reveal a previously unsuspected submicroscopic heterogeneity of intracellular cAMP. A model is emerging where specificity depends on compartmentalisation and where the physiologically relevant signals are those that occur within confined nanodomains, rather than bulk changes in cytosolic cAMP. These findings subvert the classical notion of cAMP signalling and provide a new framework for the development of targeted therapeutic approaches.

cAMP Signalling

cAMP is one of a small number of intracellular second messengers that relay the information carried by hormones, neurotransmitters, and other extracellular cues to the intracellular environment. Inside the cell, cAMP triggers a chain of biochemical events that results in the appropriate cellular reaction to the specific extracellular stimulus. The basic molecular components of the cAMP pathway are well established. The signal is generated on ligand binding to a G_s-protein-coupled receptor (G_sPCR) at the plasma membrane. This leads to activation of adenylyl cyclases (AC), also located at the plasma membrane, which synthesise cAMP from ATP. The signal is turned off by receptor desensitisation and by the action of phosphodiesterases (PDEs), a large superfamily of metallohydrolases that degrade the cyclic nucleotides [1] (Figure 1). Given the broad spectrum of cellular functions regulated by cAMP, this pathway has attracted significant interest for potential therapeutic applications. Examples of current therapeutics that target cAMP signalling include the PDE3 inhibitors amrinone, milrinone, and enoximone (for the treatment of acute heart failure) and cilostazol (for the treatment of intermittent claudication); and the PDE4 inhibitors apremilast (for the treatment of psoriatic arthritis), roflumilast [for chronic obstructive pulmonary disease (COPD)] and crisaborole (for atopic dermatitis). In addition, several β -adrenergic receptor blockers are in use for the treatment of arrhythmias, congestive heart failure, and glaucoma, and for the prophylaxis of migraine. Molecules that interfere with cAMP levels are being investigated for their potential therapeutic applications in a variety of other pathological conditions [2] and are at the centre of major drug discovery programs. cAMP was identified in 1957 by Earl Sutherland [3]. He was studying the hormonal regulation of glycogenolysis and found that cAMP is the molecule responsible for the activation of glycogen phosphorylase in response to adrenaline. This observation essentially led him to conceive the idea of a second messenger and of intracellular signal transduction, for which he was awarded the Nobel Prize in 1971. Over the following decades, it became clear that cAMP is not only responsible for the ancestral fight-or-flight response to catecholamines, but also mediates the action of a multitude of other hormones and

Highlights

Refinements of fluorescence-based imaging methods for real-time detection of cAMP signals in intact cells are providing novel insight into the subcellular organisation of this complex and multifunctional signalling pathway

Compartmentalisation of cAMP signals appears to be more extreme than previously thought. Evidence is emerging that physiologically relevant cAMP/protein kinase A (PKA) signals are those constrained within subcellular compartments with submicroscopic dimension.

A complex pattern comprising multiple cAMP signals with distinct amplitude and kinetics and with a nonometre range of action can be generated by the activation of an individual G-protein-coupled receptor (GPCR).

The nanoheterogeneity of cAMP/PKA signals is required for fine-tuning of cellular function and is altered in animal models of disease.

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Figure 1. Molecular Components of the cAMP Signalling Pathway. The schematic illustrates the traditional view of cAMP as a long range-acting second messenger, whereby cAMP is generated at the plasma membrane (PM) and diffuses inside the cell to activate its effector protein kinase A (PKA), which in turn phosphorylates intracellular targets. The red-shaded area represents the homogeneous diffusion of cAMP in the cytosol. Abbreviations: AC, adenylyl cyclase; ER, endoplasmic reticulum; GPCR, G_s-protein-coupled receptor; PDE, phosphodiesterase.

neurotransmitters and is involved in most cellular functions. The broad spectrum of cAMP functions was difficult to reconcile with its ability to generate distinct, hormone-specific cellular effects. To explain this property of cAMP, researchers hypothesised that physically segregated pools of cAMP are required to activate separate arms of this pathway, which are confined to distinct subcellular compartments [4]. However, direct demonstration of the spatial confinement of cAMP was difficult to achieve and the enigma remained for several decades. In more recent years, the development of FRET-based reporters and imaging of cAMP with high spatial and temporal resolution in intact, living cells has represented a turning point in the field. Application of these new technologies has revolutionised our understanding of cAMP signalling and is now starting to provide novel insight into cell physiology that may be harnessed to develop better therapies.

The Problem of cAMP-Dependent Hormonal Specificity

In the model proposed by Sutherland, cAMP is synthesised at the plasma membrane and diffuses inside the cell to activate intracellular effectors that act at distant intracellular sites to induce a specific function, for example, the activation of an enzyme or of a transcription factor (Figure 1). This model of cAMP as a long-range second messenger stemmed from the original observation by Sutherland and his colleagues that the response to hormones could be separated into a membrane-associated step and a cytosolic step. In their experiments, they used fractions from cell homogenates and found that application of the hormone directly to the

supernatant (cytosolic) fraction had no effect. However, when the hormone was applied to the particulate (membrane) fraction, an active factor, cAMP, was generated and it was the addition of this factor to the supernatant that resulted in the increased activity of the enzyme phosphorylase [3]. The model of cAMP as a long range-acting messenger was also supported by the observation, years later, that the diffusion constant of cAMP measured in cells can be as high as that measured in water [5], arguing for the ability of this second messenger to equilibrate rapidly throughout the cell (Figure 1). Based on this model, cAMP has often been considered to serve a long-distance, integrative role as opposed to Ca^{2+} , another second messenger that is well known to predominantly have a short range of action [6]. The idea of cAMP as a long-range second messenger has remained the prevalent view for several decades and it is still the model currently proposed by most textbooks.

However, there is an obvious problem with this model (Figure 2). The same cell can express several G_s PCRs that respond to different hormones and mediate different cellular functions but that all act via the generation of cAMP. In addition, the most-extensively studied effector of cAMP, protein kinase A (PKA), a tetrameric enzyme where cAMP binding to the two regulatory subunits (R) releases their inhibitory effect on two catalytic subunits (C), is a highly promiscuous enzyme that can phosphorylate a multitude of different targets within the same cell [7]. These include multiple metabolic enzymes, transcription factors, receptors, channels, transporters, and signalling and structural proteins. In addition, in several cell types, cAMP directly binds and modulates the activity of two isoforms of the protein EPAC [8], several cyclic nucleotide-gated ion channels (CNGC) [9], and the more-recently identified Popeye domain-containing (POPDC) proteins [10]. Each of these effectors is responsible for a separate additional set of cAMP-dependent functions, further adding to the complexity of the system (Figure 2). The difficulty in reconciling hormonal specificity with the action of a freely diffusible, long range-acting second messenger and of a catalytic subunit 'swimming about, happily phosphorylating a variety of cellular constituents whether they need it or not' [11] was recognised early on. However, it is clear that the cell is capable of producing hormone-specific effects in response to cAMP, as made apparent in classical experiments where an increase in contractility was observed in the heart when isoproterenol, but not prostaglandin, was applied, despite the fact that both hormones induced the synthesis of a similar level of intracellular cAMP and PKA activity [12].

FRET-Based Imaging Probes and Direct Visualisation of cAMP Compartmentalisation

How does the cell resolve this conundrum? The notion inferred from early studies that cAMP spreads homogeneously from the site of synthesis at the plasma membrane into the cell was overturned by the introduction of fluorescent indicators for cAMP based on FRET [13] (Box 1). These probes are characterised by proximity-dependent changes in the fluorescence signals of a donor and an acceptor fluorophore (typically two spectral variants of GFP) that are fused to a cAMP-binding domain (CBD). cAMP binding results in a conformational change of the sensing domain that modifies the distance between the two fluorophores and, as a consequence, their fluorescent emission. The resulting change in FRET efficiency can easily be monitored using an optical microscope that collects the emitted fluorescent light. The unique benefit offered by these sensors is that they are genetically encoded and can be expressed in living cells. Therefore, the changes in cAMP level are reported in real time, as they happen in the complex intracellular chemical environment and within the intact microarchitecture of the cell. The high spatial and temporal resolution of these sensors overcame a major limitation of previously available methods to assess cAMP. Conventional biochemical approaches, which are typically *in vitro* competitive-binding assays, measure total rather than free cAMP, have limited temporal resolution, and provide no information on the subcellular location where the biochemical events

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Figure 2. The cAMP Signalling Pathway Is Highly Complex. The schematic illustrates the intricacy of the cAMP signalling pathway with its multiple effectors (in red). The promiscuity of protein kinase A (PKA) is illustrated by black arrows (activation) and blunted lines (inhibition). The extensive crosstalk between the cAMP signalling pathway and other signalling pathways is shown by blue lines. For simplicity, the multiple targets of PKA (in grey) are not named. Although all the elements included in the schematic represent experimentally validated components of the pathway, only a minor fraction of all the known PKA targets and of the possible crosstalk interactions is represented. Abbreviations: AC, adenylyl cyclase; EPAC, exchange factor directly activated by cAMP; ER, endoplasmic reticulum; G_sPCR, G_s-protein-coupled receptor; PDE, phosphodiesterase; PM, plasma membrane; POPDC, Popeye domain-containing protein.

under investigation occur. A further advantage of the FRET-based reporters is that they can be expressed in living organisms as transgenes [14,15], with the potential to provide a readout of cAMP signalling in the free moving animal.

Studies using FRET-based imaging clearly demonstrated that cAMP does not diffuse homogeneously within the cell (i.e., it is 'compartmentalised') and that the spatial regulation of the second messenger and of its effectors and regulators is what warrants the specificity of the hormonal response. FRET-based reporters for cAMP have now been available for almost two decades and multiple versions have been tailored over time to help address specific questions [13]. The unprecedented spatiotemporal resolution of this approach has provided a wealth of information in support of the local regulation of cAMP signalling, converting the concept of

Box 1. FRET-Based Sensors for Detection of Local cAMP

FRET-based reporters exploit the ability of a donor fluorescent molecule to transfer by resonance part of its excited state energy to a nearby fluorescent acceptor, the absorption spectrum of which overlaps at least in part with the emission spectrum of the donor. The efficiency of this process (E) depends on the inverse sixth distance between donor and acceptor, as described by Equation 1:

$$E = 1 / \left[1 + (R/R_0)^6 \right] \quad [1]$$

where R_0 is the distance at which half of the energy is transferred. Doubling of the distance between R_0 to $2R_0$ decreases the efficiency of transfer from $E = 50\%$ to $E = 1.5\%$. Therefore, FRET provides a sensitive measure of intermolecular distances and of conformational changes.

FRET-based reporters for cAMP typically are based on a cAMP-binding domain (CBD) sandwiched between the cyan (CFP)- and the yellow (YFP)-emitting variants of GFP as the donor and acceptor, respectively (Figure 1A). Binding of cAMP to the CBD changes its conformation and the relative position of donor and acceptor. The resulting change in the distance between the fluorophores affects the efficiency of energy transfer. Typically, the ratio between the fluorescence intensity of donor and the acceptor is used as a read-out of cAMP concentration [75].

These sensors can be fused to short polypeptides or to protein domains to target them to specific subcellular sites. Figure 1B illustrates a selection of these targeted sensors where specific localisation was achieved by fusion to a short peptide or protein domain for nuclear localisation [32], targeting to membrane lipid rafts and nonraft domains [31,35], or localisation to the mitochondria [41]. Fusion to full-length proteins that are part of localised macromolecular complexes has been another successful strategy, for example, for targeting the sensor to multiprotein complexes at the plasma membrane [43], sarcoplasmic reticulum [30,43] or the subcortical cytoskeleton [16].

Figure 1. cAMP Nanodomains. The targeting domain is shown in red. Abbreviations: CBN, cyclic nucleotide-binding domain; ER, endoplasmic reticulum; PM, plasma membrane.

cAMP compartmentalisation from something ‘researchers advocate when they can’t make sense of their results’ into a widely accepted model [2].

Direct visualisation of cAMP in the intact cell unequivocally demonstrated that this second messenger does not homogeneously distribute in the cell (Figure 3, Key Figure). Tight spatial regulation of its concentration results in cAMP levels being different in different subcellular compartments. Evidence shows clearly that a major determinant of cAMP compartmentalisation is its degradation by PDEs. The PDE superfamily includes 11 families (PDE1–11), with several families comprising multiple genes and several genes expressing multiple splice variants, resulting in >100 PDE isoforms. Each isoform displays a unique combination of enzyme kinetics, regulatory mechanisms, and subcellular localisation properties. Seven PDE families (PDE1, 2, 3, 4, 7, 8, and 11) hydrolyse cAMP into inactive 5'-AMP, thus terminating the cAMP signal. The different localisation and distinct modes of regulation of the multiple PDE isoforms result in different rates of cAMP degradation at different sites (Figure 3). Thus, PDEs regulate the localisation, duration, and amplitude of cAMP signals within subcellular domains, control its diffusion to neighbouring compartments, and prevent unnecessary PKA activation [16,17]. The role of individual PDEs in shaping local cAMP levels has been reviewed elsewhere [18,19]. A second important feature of compartmentalised signalling is the subcellular localisation of the cAMP effectors. For example, PKA is largely bound, via its R subunits, to A kinase-anchoring proteins (AKAPs), a group of structurally diverse proteins that localise to different subcellular sites [20,21] and anchor PKA to macromolecular complexes that often include, or

Key Figure

cAMP Signalling Is Organised in Nanocompartments

Figure 3. The schematic shows, as an example, a cardiac myocyte (top) and a zoomed-in detail that includes a T tubule, which is an invagination of the plasma membrane (PL), part of the sarcoplasmic reticulum (SR), and a section of the myofilaments (MF). Protein kinase A (PKA) is anchored to A-kinase-anchoring proteins (AKAPs). Yellow circles indicate PKA-dependent phosphorylation. Activation of the β -adrenergic receptor (β -AR) by the agonist isoproterenol (ISO) generates multiple, spatially distinct cAMP pools. The intensity of the red-shaded areas indicates the concentration of cAMP. Phosphodiesterases, shown in green, contribute to shaping the pattern of cAMP signals. The space where cAMP is above the threshold for PKA activation is limited to submicroscopic domains.

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are in close proximity to, PKA phosphorylation targets [22]. AKAPs can also bind PDEs and phosphatases, providing local elements for signal termination. The spatial arrangement of regulators, effectors, and targets results in a patterned increase in cAMP and unique stimulus-specific local signals [16,22–24] (Figure 3). Only at some locations does the concentration of the second messenger exceed the activation threshold of the local effector protein, thereby setting off the appropriate cellular response. The amplitude and duration of the extracellular stimulus may also contribute to the resulting cAMP pattern, because larger amounts of the second messenger or persistent elevation may saturate the local PDEs, resulting in cAMP spillover to neighbouring compartments [25]. FRET-based reporters provide a means to directly image these spatially confined subcellular domains of cAMP and to establish the critical role of PDEs in shaping them. FRET imaging has also enabled researchers to gauge the size of the local cAMP domains, and early estimates indicated that they could be as small as a few μm [26–28].

Targeted cAMP Reporters

One approach that has been exploited successfully to dissect cAMP compartmentalisation involves targeting FRET-based cAMP sensors to specific subcellular sites [16,23,29,30] (Box 1). For example, targeting of the indicators to the plasma membrane showed that the cAMP signal close to the membrane tends to be higher than the cAMP signal in the bulk cytosol [29,31]. This may not be that surprising, because the plasma membrane is one of the sites where cAMP is generated. However, targeting of the sensor to the centrosome [28], nucleus [29,32], sarcoplasmic reticulum (SR) [30] showed that sites that are located deep inside the cell might also sense higher second messenger levels compared with the bulk cytosol. These findings indicate that the compartmentalisation of cAMP does not simply comprise a uniform gradient where the signal progressively dissipates as it moves away from the site of synthesis. Further studies supported the notion of a more-complex patterning of cAMP domains. By using short peptides that can be differentially lipidated, it is possible to target proteins alternatively to lipid rafts or to nonlipid raft regions [33]. Using these peptides to target the FRET-based sensors to raft and nonraft domains revealed that the signal at the plasma membrane is heterogeneous and that its modulation is different at these two subplasma membrane compartments [34,35]. Given that membrane receptors are known to be differentially distributed between raft and nonraft regions [36] and that there are differences in the membrane distribution of ACs isoforms [37], it is not entirely unexpected that synthesis of cAMP at the plasma membrane occurs at defined spots. However, what the experiments using raft and nonraft-targeted FRET reporters showed is that, once generated by a receptor–AC combination located in the raft compartment, cAMP cannot reach a sensor that is localised outside the rafts, indicating tight control of the lateral propagation of the cAMP signal. These findings also imply that the dimension of cAMP domains can be submicroscopic, because the estimated size of lipid rafts is 10–200 nm.

Targeted FRET-based reporters have also been instrumental in demonstrating compartmentalised cAMP signalling at mitochondria. Recent evidence using sensors targeted to the mitochondrial matrix (MM) or to the outer mitochondrial membrane (OMM) indicated that not only are mitochondria a site where cAMP is regulated independently from cAMP in the bulk cytosol, but that MM and OMM also constitute two distinct cAMP domains. In the MM, cAMP is thought to be generated by a resident soluble AC [38], an isoform of the enzyme that is insensitive to hormonal stimulation and is activated by HCO_3^- [39]. The inner MM (IMM) is impermeable to cAMP and provides a physical barrier that isolates the MM from the influx of cAMP generated at the plasma membrane [40,41]. The IMM also blocks any efflux of matrix-generated cAMP into the cytosol, although there is evidence of a PDE localised to the matrix that can terminate the signal in this compartment [38]. A completely distinct cAMP domain appears to be localised at the OMM. This site relies on the hormonal-dependent synthesis of cAMP and on its degradation by PDEs bound

to the mitochondrial membranes. These two distinct submitochondrial cAMP domains appear to control completely different functions, with the arm of the pathway located to the MM affecting oxygen consumption and ATP production, and the OMM arm regulating mitochondrial morphology, mitochondrial membrane potential, and apoptosis [38,40,42].

From Microdomains to Nanodomains of cAMP

One limitation of the studies using targeted reporters to assess differences in cAMP signals at different subcellular sites is that fusion of different targeting domains often modifies the properties of the sensor in a targeting sequence-specific manner [30,43]. This means that different targeting domains affect FRET efficiency to a different degree and, without accurate calibration of the reporters, it is difficult to draw unequivocal conclusions on the differences in the level of cAMP in the compartments under investigation [44]. In a recent study, this limitation was overcome by engineering a FRET sensor that was less susceptible to hindrance from the targeting sequence [43]. The sensor, named CUTie (for cAMP universal tag for imaging experiments), was targeted to the plasmalemma (PL), SR, and myofilaments (MF) in cardiac myocytes, three sites that are nodal points in the regulation of cardiac excitation–contraction coupling. Specific targeting in each of these sites was achieved by fusion of CUTie to AKAP79, AKAP18δ, and troponin I (TPNI), respectively. AKAP79 is known to organise a multiprotein complex at the PL that includes, in addition to PKA and the phosphatase calcineurin, the β-AR, AC5/6, and the L-type Ca²⁺ channel (CaV1.2). This complex regulates cAMP synthesis and the influx of Ca²⁺ that triggers cardiac myocyte contraction [45]. AKAP18δ localises at the SR, interacts with the SERCA/PLB complex and regulates Ca²⁺ reuptake in the SR during cardiac myocyte relaxation [22]. TPNI is part of the troponin complex at MFs and its phosphorylation by PKA also promotes relaxation. A careful ‘in cell’ calibration of the three targeted sensors confirmed that they all reacted with the same FRET changes to a given concentration of cAMP, allowing reliable comparison of the signals detected at the three sites. Imaging of cardiac myocytes expressing these sensors revealed an unexpected heterogeneity in the catecholamine-dependent cAMP response. While cAMP increased simultaneously and to a similar extent at the PL and SR, the cAMP signal was delayed and attenuated at the MFs [43] (Figure 3). This result was unforeseen: these three sites are all targeted by PKA phosphorylation to promote excitation–contraction coupling and, because they are part of the same ‘functional domain’, one would expect them to sense the same cAMP signal. The study showed that this is a simplistic view and demonstrated that such heterogeneity of cAMP serves an important functional role because it is required to achieve maximal stimulated contractility: when PDE inhibitors were applied and the compartmentalisation abolished, the contractile response was significantly reduced, despite similar amounts of cAMP being generated and the same amount of Ca²⁺ being mobilised [43]. Therefore compartmentalisation of cAMP provides greater contractile benefit for the same Ca²⁺ enhancement. When cardiac myocytes from failing hearts were analysed using targeted reporters and FRET imaging, the compartmentalisation of cAMP appeared to be altered [43]. These findings not only confirmed the functional significance of cAMP compartmentalisation, but also indicated that its disruption may be involved in the pathogenesis of heart failure, a role that has been suggested for several other disease states, such as obstructive lung disorders and antimicrobial resistance [46] (Boxes 2 and 3). They also provide a new facet to the complex cAMP signalling system: not only different hormones, via activation of different receptors, generate distinct pools of cAMP, but multiple cAMP signals, with distinct amplitude and kinetics, can also be generated by activation of the same receptor, a feature that warrants consideration when screening for compounds that target GPCRs.

Given the architecture and geometry of cardiac myocytes, an upper limit to the distance between PL, SR, and MF can be fixed to approximately 300 nm, indicating that the size of the cAMP

Box 2. Profound Changes in Airway cAMP Compartmentalisation in COPD

Obstructive lung disorders (asthma and COPD) are among the leading causes of morbidity and mortality worldwide. Their prevalence is expected to increase due to lifestyle factors, exposure to noxious pollutants, and cigarette smoke. Oxidative stress induced by inflammatory cells or inhaled particles is particularly important in COPD. Inflammatory cells recruited to the diseased airways initiate reactive oxygen species production, which in turn activates inflammatory transcription (such as NF- κ B) and drives abnormal lung repair, mucus hypersecretion, airway hyper-responsiveness, airflow limitation, and lung ageing.

Oxidative stress is a feature of COPD exacerbations triggered by respiratory (viral) infections, air pollution, or allergens, a process strongly linked to dysfunctions in the energy-generating mitochondria. No curative treatment for COPD is currently available. β -AR agonists are widely used in the treatment of airflow limitation, and anti-inflammatory treatment in COPD relies on (among others) PDE inhibitors. Recent studies demonstrated that, next to β -AR and PDE, Epac and members of the AKAP superfamily contribute to both the development and progression of obstructive lung disorders. Experimental models of COPD exhibited a profound alteration in the expression profile of PDEs, Epac, and a subset of AKAPs. In airway smooth muscle, expression of Epac1 [76,77] and both AKAP5/12 (both known to regulate β_2 -AR recycling) [71] were reduced and, in parallel, expression of PDE3/PDE4 [78] was increased. The increased expression of PDE3/4 leads to reduced cAMP. The consequent reduced activation of PKA and Epac (the expression of which is also reduced) results in increased phosphorylation of MLC and airway constriction. At the same time, reduced Epac activity leads to increased phosphorylation of Akt and ERK1/2, and increased activity of NF- κ B, resulting in cell proliferation and inflammation (excessive production of the neutrophil marker interleukin-8). In airway epithelial cells, expression of AKAP9 and the adherens junction marker E-cadherin was found to be decreased [79], leading to a loss in barrier function. These findings point to a role that is played by disrupted compartmentalisation of cAMP in COPD (Figure I).

Figure I. Compartmentalisation of Airway cAMP in Experimental Models of Chronic Obstructive Pulmonary Disease (COPD). Schematic illustration of airway epithelial and smooth muscle cell functioning in experimental models of COPD. The red-shaded area illustrates the profound alterations in cAMP compartmentalisation in disease conditions induced by cigarette smoke, leading to an increase in contractility, proliferation, and inflammation in airway smooth muscle cells and disruption of the barrier function in airway epithelial cells. Abbreviations: AC, adenylyl cyclase; AKAP, A-kinase anchoring protein; Akt, p70S6K; Epac, exchange protein directly activated by cAMP; ERK1/2, signalling kinases; G_sPCR, G_s-protein-coupled receptor; MLC, myosin light chain; MLCK, MLC kinase; MLCP, MLC phosphatase; NF- κ B, nuclear factor kappaB; PDE, phosphodiesterase.

Box 3. Profound Changes in Airway cAMP Compartmentalisation in Antimicrobial Resistance

Antimicrobial resistance (AMR) is an increasingly pervasive problem worldwide, and represents one of the greatest challenges to global public health today. The WHO released a new report on AMR, saying that susceptibility to common bacterial infections has reached alarming levels in many parts of the world. *Aspergillus fumigatus* is an opportunistic fungus that causes approximately 90% of the systemic infections due to *Aspergillus* spp. The primary site of infection is the lung. The process of *A. fumigatus* internalisation into pulmonary epithelial cells is a key step in the cause of aspergillosis.

Pulmonary epithelial cells act not only as a mechanical barrier, but also as a first line of defense of the host innate immune system. However, until recently, the mechanisms leading to the internalisation of *A. fumigatus* within pulmonary epithelial cells were largely unknown. Recent studies demonstrated that β -1,3-glucan and gliotoxin, both produced by *A. fumigatus*, increase the internalisation of the fungus into pulmonary epithelial A549 cells by inducing host cellular phospholipase D (PLD) activation. PLD of *A. fumigatus* itself is a virulence factor and improves internalisation. The phosphorylation status of the actin regulator cofilin in the host cell determines the internalisation of *A. fumigatus* (X. Han, PhD Thesis, University of Groningen, 2017). Intriguingly, cofilin and PLD are interconnected by the cAMP effector Epac. Phosphorylated cofilin activates PLD, whose activity can be further elevated by Epac. DHN-melanin, another main component of *A. fumigatus*, reduces host cell cAMP and elevates the expression of Epac, but not of PKA. As a consequence, cortical actin dynamics increase, leading to the weakening of cell-cell contacts and disruption of the antimicrobial barrier function of the pulmonary epithelium. Such recent findings illustrate that pathogenic mechanisms of *A. fumigatus* invasion and responses in the host are closely paralleled by alterations in airway cAMP compartmentalisation, and open novel pathways for the development of drugs for AMR (Figure 1).

Figure 1. Compartmentalisation of Airway cAMP in Antimicrobial Resistance. Schematic illustration of pulmonary epithelial cells and the invasion of *Aspergillus fumigatus*. The red-shaded area illustrates the profound alterations in cAMP compartmentalisation in disease conditions induced by *A. fumigatus* invasion, leading to a reduction in cAMP and an increase in actin dynamics, and resulting in a loss of barrier function. Abbreviations: 14-3-3, stabilises phospho-cofilin; AC, adenylyl cyclase; cofilin, actin regulator; Epac, exchange protein directly activated by cAMP; G_sPCR, G_s-protein-coupled receptor; PLD, phospholipase D.

domains imaged in this study was in the nanometre range [43]. However, the fact that the level of cAMP detected at these three sites was different from the level in the bulk cytosol [43] suggests that the actual size of the cAMP nanodomains are even smaller and limited to the space immediately surrounding each individual macromolecular complex targeted by the sensor.

Nanodomains of PKA Activity

One general conclusion that can be drawn based on the current data is that it is the local, rather than the global cytosolic cAMP that undergoes the fine, stimulus-specific regulation. Therefore, it appears that the functional outcome does not rely on 'bulk cAMP' changes and that the physiologically relevant cAMP signals are those that occur within individual nanodomains. This notion is supported by studies investigating PKA activity. Contrary to generally held dogma (but

see also [47]), recent evidence indicates that the C subunit of PKA may not necessarily 'swim about' in the cytosol because it also appears to display a short range of action. Electron microscopy structural analysis of PKA/AKAP complexes revealed an intrinsic flexibility of the PKA holoenzyme that would allow phosphorylation of PKA targets embedded in the PKA/AKAP complex without a requirement for the C subunit to be released from the R subunit [48]. Based on these studies, association of PKA with AKAPs would limit the action of C subunits within a range of 15–25 nm centred around the AKAP [49]. In another study, although the authors challenged the notion that C subunits remain tethered to R subunits on cAMP elevation, further evidence was provided in support of a limited ability of C subunits to diffuse in the cytosol. In a large variety of cells, R subunits were found to be one order of magnitude in excess of their C counterparts, a feature that would promote high rates of R–C association [50]. The idea that, on cAMP binding, the C subunit is released but rapidly recaptured by a nearby R subunit is also supported by separate investigations [51] where a new class of fluorescent indicators was used to provide super-resolution visualisation of PKA activity. The data indicated that, at the plasma membrane, PKA activity is not uniform across the lipid bilayer but is localised in clusters of approximately 250 nm in diameter. These clusters were shown to colocalise with AKAP79 and were dissipated in the presence of the synthetic peptide STAD-2, which specifically disrupts the interaction between AKAPs and the PKA regulatory subunit RII [52]. Given that the PKA activity clusters were larger than the colocalised AKAP clusters and exceeded the intrinsic flexibility of the PKA holoenzyme, these findings support a release-and-recapture mechanism. One would expect the location of these PKA 'active zones' to coincide with cAMP nanodomains, although this has not yet been directly assessed. Therefore, multiple data appear to show that cAMP signalling operates in the nanometre range. This does not mean that cAMP cannot diffuse in the cell and serve integrative functions by coordinating multiple inputs away from the site of synthesis. However, the final functional outcome appears to be dictated by the level of cAMP that is achieved in the restricted environment surrounding a spatially constrained effector, rather than by the overall change in cellular cAMP.

Unresolved Issues

Although the model of cAMP compartmentalisation is now widely accepted, many questions remain (see Outstanding Questions). One point that has been particularly debated in the literature has to do with the fact that numbers do not appear to add up. Given the relatively slow rates at which cAMP is generated by AC (~ 20 cAMP molecule s^{-1}) [53] and the fast diffusion of cAMP in the cytosol ($10\text{--}450 \mu m^2 s^{-1}$, depending on the approach used for the measurements and on the cell type [54–56]), it is difficult to envisage how concentration gradients of cAMP can be maintained, particularly in cells with simple architecture. In addition, given the apparent slow rate at which PDEs degrade the fast-diffusing cAMP (between 0.09 and 450 cAMP molecule s^{-1} , depending on the isoform) [57] and the reported high sensitivity of PKA to cAMP (EC_{50} for activation in the 100–300 nM range [58]), one would predict that unnecessary activation of PKA at selected sites is hard to avoid. Multiple experimental and computational studies have explored these issues and the results consistently confirm that PDE activity is an essential factor in determining cAMP compartmentalisation (reviewed in [59]). However, simulations fail to predict meaningful localised cAMP gradients unless rates of synthesis and degradation of cAMP are set at least 100 times higher than the values measured experimentally [59], the PKA activation threshold is increased at least ten times [60], or the diffusion of cAMP is significantly reduced [61,62]. Several factors may act in concert to slow down the diffusion of cAMP within specific subcellular domains. These may include a high buffering capacity [54], a high local protein density and molecular crowding, as well as physical obstacles [56], all of which may have particular relevance in anatomically restricted spaces [63]. Some of the discrepancies between models and experimental observations may also result

from the fact that enzyme rate constants and binding affinities that are used in the mathematical models have been determined *in vitro*, but the actual values in the intact cell may be significantly different. For example, a recent study used a variety of FRET-based reporters for cAMP concentrations and PKA activity expressed in intact living cells to demonstrate that the activation threshold of PKA is approximately 20 times higher when measured in the cell compared with values determined *in vitro* using purified enzyme [57]. This means that a somewhat sluggish PDE activity may still be adequate to keep the level of cAMP below the activation threshold of PKA. In addition, it is possible that rate constants and binding affinities values that are available for PDEs (so far only determined *in vitro*) may also be inaccurate. Biochemical methods to determine enzyme kinetics parameters are usually based on the Michaelis–Menten equation, which is valid when the substrate concentration [S] greatly exceeds that of the enzyme [E], a condition that is fulfilled in *in vitro* measurements. However, in the cell, total [E] is often close to [S] and [E]/[S] may be even greater than one in restricted subcellular domains. For example, the overall intracellular concentration of PKA has been estimated to be approximately 0.2 μM in skeletal muscle [64], close to the concentration of cAMP [57,64,65]. Within spatially defined domains, the [PKA]/[cAMP] or the [PDE]/[cAMP] value could be significantly higher as a consequence of clustering of the enzyme within local signalosomes and the reduced accessibility of cAMP to some sites. For high [E]/[S] values, the Michaelis–Menten equation becomes increasingly inadequate to describe the reaction equilibrium constants [66]. Therefore, it will be important to develop mathematical models that integrate explicit architectures of nanodomains with realistic geometries and distribution of signalling components (e.g., cAMP, PDEs, PKA, and AKAPs) and reaction rates determined *in vivo*. These models will be of paramount importance in helping dissect the bewildering complexity of the cAMP signalling system.

Concluding Remarks

The arrangement of the cAMP signalling pathway in a network of multiple coexisting domains, only a fraction of which are involved in the response to any given stimulus, provides opportunities to intervene therapeutically with increased precision by selectively targeting function at individual sites. Indeed, there is accumulating evidence indicating that disrupted compartmentalisation of cAMP participates in the pathogenesis of disease [43,67–72]. With a full understanding of the organisation, regulation, and function of individual cAMP domains, it may be possible to develop precision medicine strategies to target individual cAMP pools, rather than global intracellular cAMP levels, with greater therapeutic efficacy and specificity. This could be achieved, for example, via selective local manipulation of PDEs activity at specific subcellular sites. Family-selective pharmacological inhibitors of PDEs currently available do not discriminate between the multiple isoforms within a family and, therefore, may not provide sufficient selectivity. Isoform-selective inhibitors are difficult to develop due to high structural similarity between the isoforms. An alternative approach to targeting individual PDE isoforms is to displace them from their subcellular anchor site, a manoeuvre that has been proved to result in local elevation of cAMP and activation of specific PKA-dependent functions [73,74]. To move beyond the proof-of-concept stage and assess whether this approach holds any translational potential, a detailed cell type-specific map of the cAMP subcellular domains and a mechanistic understanding of their regulation and functional significance are necessary (see Outstanding Questions). Given the complexity of the system, this may appear a formidable task and will undoubtedly require several years of intense effort. Further refinement of the cAMP probes and of real-time imaging methodologies will continue to have an important role in enabling further progress. cAMP reporters for super-resolution applications will be especially useful for defining the topography of cAMP domains with nanometre resolution. Development of robust sensors that can be imaged reliably in free-moving animals to assess signalling in intact organisms

Outstanding Questions

What are the exact topography, regulation, and function of cAMP/PKA nanodomains? What is the number and location of these compartments within a given cell? What are amplitude and kinetics of the cAMP/PKA signal within each domain?

Are the amplitude and location of these nanodomains fixed in a given cell or, more likely, are they affected by the current circumstances the cell is experiencing?

How does a specific GsPCR determine what part of the downstream signalling network is activated in order to achieve the required pattern of cAMP nanosignals?

What are the functional roles and coordination of signalling between different nanodomains?

What is the identity of individual PDE isoforms that impinge on each of these domains? How much do phosphatases contribute to the compartmentalisation of PKA signals?

How is cAMP/PKA compartmentalisation organised in human cells and how is it disrupted in human disease?

would also represent an important step forward. Defining the details of how cAMP nano-domains are organised in healthy and diseased human cells is another fundamental prerequisite for translational applications. Depending on the specific cell type and pathology, human samples are not always easily accessible. However, the growing number of models of disease that use human-derived pluripotent stem cells now provide the opportunity to undertake this work systematically. There are certainly exciting times ahead, given that the significance of cAMP compartmentalisation in health and disease is only starting to emerge.

Acknowledgements

This work was supported by the British Heart Foundation (PG/10/75/28537 and RG/12/3/29423), the BHF Centre of Research Excellence, Oxford (RE/08/004), the Graduate School of Science (GSSE), and Engineering and the Groningen Research Institute of Pharmacy (GRIP).

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