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Themed Section: Novel cAMP Signalling Paradigms

REVIEW

Novel cAMP signalling paradigms: therapeutic implications for airway disease

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Since its discovery over 50 years ago, cAMP has been the archetypal second messenger introducing students to the concept of cell signalling at the simplest level. As explored in this review, however, there are many more facets to cAMP signalling than the path from Gs-coupled receptor to adenylyl cyclase (AC) to cAMP to PKA to biological effect. After a brief description of this canonical cAMP signalling pathway, a snapshot is provided of the novel paradigms of cAMP signalling. As in the airway the cAMP pathway relays the major bronchorelaxant signal and as such is the target for frontline therapy for asthma and COPD, particular emphasis is given to airway disease and therapy. Areas discussed include biased agonism, continued signalling following internalization, modulation of cAMP by AC, control of cAMP degradation, cAMP and calcium crosstalk, Epac-mediated signalling and finally the implications of altered genotypes will be considered.

LINKED ARTICLES

This article is part of a themed section on Novel cAMP Signalling Paradigms. To view the other articles in this section visit http://dx.doi.org/10.1111/bph.2012.166.issue-2

Abbreviations

AC, adenylyl cyclase; ASM, airway smooth muscle; CFP, cyan fluorescent protein; COPD, chronic obstructive pulmonary disease; CREB, cAMP response element binding; Epac, exchange protein directly activated by cAMP; GEF, guanine nucleotide exchange factor; GRK, G-protein coupled receptor kinase; hASM, human airway smooth muscle; IP_3, inositol trisphosphate; RACK1, receptors for activated C kinase 1; SERCA, sarco/endoplasmic reticulum Ca^{2+}-ATPase; SOCC, store-operated calcium channel; TSH, thyroid-stimulating hormone; YFP, yellow fluorescent protein

cAMP is the classical second messenger discovered by Earl W Sutherland Jr. and Theodore W Rall in 1956 (Sutherland and Rall, 1958). To date, research into cAMP has led to five Nobel awards (Beavo and Brunton, 2002) and is the subject of almost 100 000 publications on PubMed. In the last decade, in particular, the generation and enhanced use of fluorescent probes allowing quantification/visualisation of cAMP signalling at a pharmacological, spatial and temporal level has added significantly to our understanding of this pathway (Lohse *et al.*, 2008; Hill *et al.*, 2010).

In the airways, cAMP is a critical regulator of airway tone being the major pro-relaxant effector in airway smooth muscle (ASM) bundles. As such, it is a key therapeutic target in airway disease being the transducer of the signal induced by clinically used β_2-adrenoceptor agonists (e.g. indacaterol, formoterol, salmeterol) which results in bronchodilation and symptomatic relief. Although outside the scope of this review, it should be mentioned that in addition to its pro-relaxant role in ASM, cAMP modulates a range of diverse cellular events pertinent to airway function including the production and secretion of inflammatory mediators and extracellular matrix, proliferation, migration and in epithelial cells mucus secretion, wound healing, anion transport and ciliary beating (Salathe, 2002; Giembycz and Newton, 2006).

For the purpose of this review, we will give a brief outline of classically accepted cAMP signalling prior to exploring the novel paradigms that have added to our understanding of this critical pathway. We will consider the ways in which cAMP formation can be induced and degraded and look at the spatiotemporal nature of these events. We will then highlight some cellular modulators that have previously unrecognized impacts on cAMP signalling. Finally, an assessment of

the therapeutic implications of these novel paradigms will be presented within the context of airway disease. Whilst this review will be centred around cAMP signalling in ASM cells, where a key discovery or a paradigm shift has been reported in other cell systems, this will be included and discussed.

Canonical cAMP signalling

As shown in Figure 1, the classically described cAMP signalling pathway is initiated when an extracellular agonist binds to its requisite Gs-protein coupled receptor and induces a conformational change resulting in the Gs-protein complex dissociating into a Gα subunit and a Gβγ dimer (see review Billington and Penn, 2003). Whilst the Gβγ dimer is able to initiate specific signalling cascades of its own, it is the Gsα subunit that drives the canonical signalling pathway via activation of adenylyl cyclase (AC). Specific AC isoforms then catalyse the conversion of intracellular ATP to cAMP and pyrophosphate. Although historically, the major target for cAMP was solely cAMP-dependent protein kinase or PKA, the last two decades have confirmed the equal importance of Epac (exchange protein directly activated by cAMP) in this role (see Epac-mediated signalling). PKA is a holoenzyme comprising two regulatory and two catalytic subunits. An increase in intracellular cyclic AMP results in two cAMP molecules binding to each PKA regulatory subunit allowing the release of PKA catalytic units enabling them to be functionally active. In human airway smooth muscle (hASM), targets phosphorylated by the active PKA catalytic subunits include cAMP response element binding (CREB) transcription factor, phospholipase C (PLC), the inositol trisphosphate (IP_3) receptor, myosin light chain kinase (MLCK) and the β_2-adrenoceptor itself (Billington and Penn, 2003) with the net functional result being ASM relaxation.

Whilst the classically described elements of the cAMP pathway are as valid today as 40 years ago, it has become clear that the schematic shown in Figure 1 is far from comprehensive in terms of summarizing the complexities of the cAMP signalling pathway. In the next few sections, we will consider

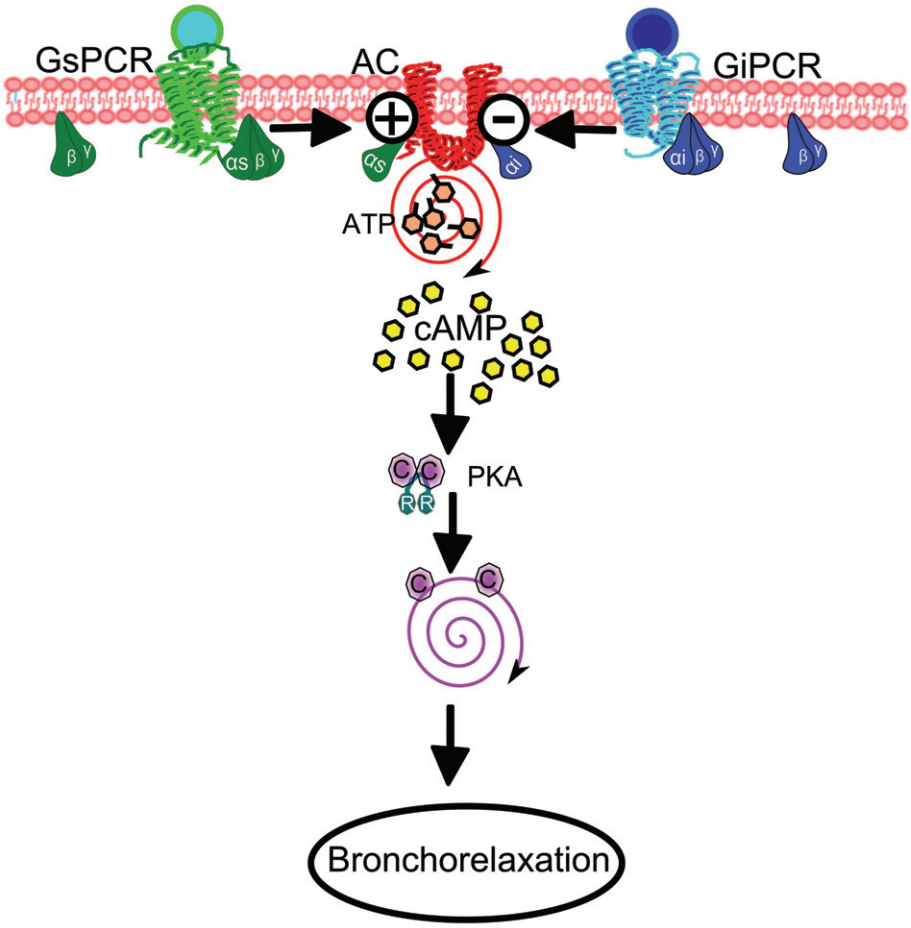

Figure 1

The canonical cAMP signalling pathway. Following the binding of agonist, a Gs-protein coupled receptor (GsPCR) such as the β_2-adrenoceptor, or a Gi-protein coupled receptor (GiPCR); for example, muscarinic M2 Acetylcholine receptor undergoes a conformational change promoting the dissociation of the Gα subunit from the Gβγ dimer. Whereas the Gαi-protein has an inhibitory effect on AC, the Gαs-protein stimulates AC inducing it to catalyse the formation of cAMP from ATP. Binding of cAMP to PKA results in the release of the PKA catalytic subunits allowing them to phosphorylate a wide range of cellular targets, with the net result being bronchorelaxation (see text for further details).

the novel paradigms emerging regarding specific aspects of cAMP signalling.

GPCR-mediated cAMP signalling: new considerations

Biased agonism

GPCRs constitute a significant target for pharmacological therapeutic intervention with ~30% of marketed small molecule drugs in clinical use directed at them (Hopkins and Groom, 2002). Whilst it has long been accepted that different agonists acting at the same receptor induce effects of different magnitude (e.g. formoterol is a full, whereas salmeterol is a partial, agonist), it is now becoming apparent that different agonists, or even stereoisomers of the same agonist (Woo *et al.*, 2009) acting at the same receptor can activate diverse downstream pathways to different extents. This phenomenon is known most commonly as biased agonism or ligand-directed signalling and has recently been reviewed both within the context of asthma (Walker *et al.*, 2011) and specifically with relation to the β_2-adrenoceptor (Evans *et al.*, 2010). One simple example of biased agonism is that observed in cardiomyocytes whereby the β_2-adrenoceptor preferentially induces Gs- versus Gi-mediated effects depending on the ligand involved; for example, whilst fenoterol appears selective for Gs-mediated contractile effects, salbutamol activates both Gs- and Gi-coupled pathways to a comparable degree (Xiao *et al.*, 2003). However, perhaps the best studied example of biased agonism and one that is relevant to airway cell signalling is the differential ability of β_2-adrenoceptor ligands to activate Gs-mediated cAMP production versus the β-arrestin-mediated MAPK pathway (Luttrell *et al.*, 1999). These findings are likely to be due to each ligand stabilizing a different activation state of the receptor (Walker *et al.*, 2011). The ability to take therapeutic advantage of biased agonism, that is, utilizing a ligand that signals solely via clinically beneficial pathways whilst avoiding clinically problematic pathways, is an obvious goal for future drug development. To facilitate this aim, Lefkowitz and colleagues recently proposed a novel methodology for quantifying biased signalling for a range of β_2-adrenoceptor ligands (Rajagopal *et al.*, 2011). Quantifying therapeutically positive versus negative properties is especially important for β_2-adrenoceptor ligands utilized in the treatment of asthma particularly due to (i) the possibility that chronic use of these agents could have adverse clinical effects (see review Sears, 2011) and (ii) suggestions that a β_2-adrenoceptor inverse agonist would be more appropriate for asthma therapy (Nguyen *et al.*, 2009; Penn, 2009; Page, 2011; Sears, 2011).

Recent studies from our own laboratory have explored the effects of biased agonism on the kinetics of signalling (Billington and Hall, 2011); that is, does the choice of ligand dictate how rapidly the β_2-adrenoceptor-AC-cAMP pathway is activated? Using a fluorescent Epac-based probe (CFP-Epac(dDEP,CD)-VENUS) (see Epac-mediated signalling section) as a readout for cAMP activation, we assessed the time taken for clinically relevant β_2-adrenoceptor agonists (salmeterol, salbutamol, formoterol and indacaterol) to (i) initiate a quantifiable activation of the probe and (ii) induce maximal activation of the probe. Surprisingly, despite having a significantly slower onset of action clinically (~30 min compared with <2 min for salbutamol) (Sears and Lotvall, 2005), salmeterol was no slower to initiate probe activation than the other agonists studied. In terms of ligand-mediated altered kinetics, we observed that once probe activation had been initiated, indacaterol and isoprenaline induced probe activation (via increased cAMP levels) significantly faster than salmeterol (Billington and Hall, 2011).

Continued signalling following internalization

Following agonist binding, many GPCRs, including the β_2-adrenoceptor, undergo desensitization [e.g. where receptor phosphorylation promotes the binding of GPCR kinases (GRKs) leading to the recruitment of β-arrestins and the cessation of downstream signalling] or internalization (where the GPCR is internalized via a clathrin-mediated mechanism). An exciting and previously unrecognized aspect of GPCR-mediated signalling is the ability of some GPCRs to continue to signal even after internalization has occurred. Utilizing fresh thyroid follicles from an Epac1-cAMP reporter transgenic mouse, Calebiro *et al.* (2010a) tracked intracellular cAMP changes in response to thyroid-stimulating hormone (TSH). Surprisingly, after 10 min TSH exposure, which is known to result in internalization of receptor into endosomes, rather than desensitization of the cAMP signal, a persistent cAMP signal was observed, which was unaffected by removal of agonist. Calebiro and colleagues reviewed this new model of GPCR activation last year, and further investigation will be needed to clarify whether this is a GPCR-specific event or common to all GPCRs (Calebiro *et al.*, 2010b). Whether or not similar signalling is specifically relevant to airway pharmacology is currently unknown.

ACs

ACs are the sole enzymes responsible for cAMP production. Although they are grouped in a family and subclassified in terms of their modulation by G-protein subunits and calcium, they constitute a very diverse set of enzymes in terms of signalling properties (with the exception of AC5 and 6, which share similar signalling properties). Of the nine membrane-bound AC subtypes identified, we and others have found the AC 5/6 subtypes to be key activators of cAMP in hASM cells with possible additional roles for 2 and 4 (Billington *et al.*, 1999; Xu *et al.*, 2001; Bogard *et al.*, 2011). It has been hypothesized that, as reported in cardiac myocytes (Post *et al.*, 1995), AC is the rate-limiting factor for the β_2-adrenoceptor–AC–cAMP pathway in hASM cells. In support of this theory, in separate studies, we and Bogard *et al.*, overexpressed AC6 in hASM cells and each found cAMP to be elevated versus empty vector controls (Billington *et al.*, 1999; Bogard *et al.*, 2011) and even versus hASM cells individually overexpressing the other key components of the pathway; β_2-adrenoceptor and Gsα (Billington *et al.*, 1999).

Wang *et al*. (2011) recently explored this *in vivo*, hypothesizing that transgenic mice overexpressing AC5 would exhibit augmented β_2-adrenoceptor-mediated airway relaxation. Intriguingly, the opposite was found with isoprenaline-induced airway relaxation and cAMP production being significantly reduced. This occurred in parallel with increased Gαi expression and ERK1/2 activation, suggesting that increased AC5 tips the Gs/Gi balance of β_2-adrenoceptor signalling firmly towards Gi. Wang *et al*. (2011) suggest that β_2-adrenoceptor signalling may operate within a set range and any alteration of signalling components leading to a deviation from this range would result in the induction of compensatory mechanisms to return to the 'homeostatic set point'. Interestingly, similar observations have been made in transgenic mice overexpressing the β_2-adrenoceptor, where airway responsiveness is actually increased with up-regulation of PLC signalling (McGraw *et al*., 2003).

It has become increasingly apparent that ACs are not important solely in terms of their enzymatic properties but also are critically important as scaffold proteins. Their role in driving microdomain-specific signalling has been systematically explored and reviewed by Willoughby and Cooper (2007) and is discussed further below ('Spatiotemporal control of cAMP').

Control of cAMP degradation

As cAMP is a rapid response second messenger, it is necessary for the cell to have a mechanism available to also dissipate the signal as rapidly as is required. This function is performed and tightly regulated by members of the PDE family whose importance has become increasingly apparent since their isolation in the 1970s (Uzunov and Weiss, 1972). To date, 11 distinct PDE families have been described each with multiple members (see review Francis *et al*., 2011). Isoforms in families 5, 6 and 9 are cGMP-specific, 4, 7 and 8 are cAMP-specific, whereas 1,2,3,10 and 11 can degrade both cAMP and cGMP.

Whilst the majority of PDE isoforms are expressed in airway cells, in particular, a clear functional role has been ascertained for members of the PDE4 family. The PDE4 family is responsible for the majority of cAMP degradation and has been subdivided into four groups (A–D), the genes of which encode over 20 distinct isoforms that can also be grouped by structure into (i) long, containing two regulatory domains namely upstream conserved regions (UCR)s 1 and 2; (ii) short, containing UCR2 but not UCR1; and (iii) supershort, containing a truncated UCR2 and no UCR1. The unique N-termini of each isoform have been observed to ensure specificity of function in terms of signalling and cellular localization (for comprehensive reviews, please refer to Houslay *et al*., 2005; 2007; 2010). Inhibitors of the PDE4 family such as roflumilast and cilomilast have been used in clinical trials or have been licenced for use in chronic obstructive pulmonary disease (COPD) (Spina, 2008) and have potential use in asthma.

In hASM cells, we have determined there to be a key role for PDE4D and in particular the PDE4D5 isoform in terms of cAMP-induced up-regulation at the level of gene expression, protein expression and protein activity (Le Jeune *et al*., 2002). Since cloning PDE4D5 nearly 15 years ago (Bolger *et al*., 1997), Houslay and colleagues have meticulously dissected the regulation and function of this PDE isoform, primarily in cardiomyocytes and HEK293 cell lines, and found it to play a key role in β_2-adrenoceptor signalling. In these systems, following β_2-adrenoceptor activation, PDE4D5 and β-arrestin are recruited, as a complex, to microdomains in close proximity to the β_2-adrenoceptor where PDE4D5 can exert tight local control on cAMP levels by specifically regulating PKA tethered to A-kinase Anchoring Protein (AKAP)79 (Lynch *et al*., 2005). Also in these systems, PDE4D5 has been shown to play a pivotal role in the switching of signalling between β_2-adrenoceptor-mediated cAMP production via Gs proteins and activation of the ERK pathway via Gi-mediated β-arrestin activity (Baillie *et al*., 2003; Bolger *et al*., 2003; 2006). Unlike any other PDE isoform, PDE4D5 is able to bind to the scaffold protein Receptors for Activated C Kinase (RACK)1, and this observation has led to the recent description of crosstalk between the cAMP and PKC signalling pathways (Bird *et al*., 2010). It is clear from these studies that the ability of PDE4D5 to tightly orchestrate such key cellular endpoints as relaxation and proliferation via cAMP/ERK would make it exceptionally important in airway function.

Specifically in the airways, the functional importance of PDE4D5 in hASM cells was further confirmed by utilizing siRNA to specifically knockdown the PDE4D5 isoform, resulting in significantly enhanced basal and isoprenaline-induced cAMP production (Billington *et al*., 2008). In addition to modulating cAMP in terms of total cytosolic content, knocking down PDE4D5 increased the speed with which a maximal cAMP response was observed (Billington *et al*., 2008). Since highlighting the functional importance of PDE4D5 in hASM cells, Hu *et al*. (2008) further confirmed this observing an upregulation of PDE4D5 to be associated with PGE_2-induced pro-asthmatic changes including β_2-adrenoceptor desensitization, impaired cAMP production and hyperresponsiveness to acetylcholine. In keeping with increased levels of PDE4D being associated with 'pro-asthmatic' changes, an increase in PDE4D expression was recently reported in hASM cells from asthmatic patients when compared with non-asthmatic individuals (Trian *et al*., 2011).

cAMP and calcium crosstalk

Despite the evidence for crosstalk between calcium and cAMP signals being over 40 years old (Rasmussen, 1970), these critical second messenger pathways are all too often viewed as largely separate entities. However, in addition to the direct control of AC isoforms by calcium, there is an increasing body of work surrounding the relationship between cAMP and store-operated calcium channel entry (SOCC).

SOCC entry probably constitutes the major long term functional regulator of calcium signalling in ASM and in recent years this pathway has been shown to be modified by cAMP signalling and vice versa. In porcine ASM, Ay *et al*. (2006) reported cAMP to negatively regulate SOCC-mediated calcium influx, and in 2009, Lefkimmiatis *et al*. (2009) reported that depletion of calcium in the endoplasmic reticulum (ER) induced recruitment of ACs and cyclic AMP accumulation. A key player in this process appears to be Stim1, a calcium sensor located in the ER membrane that has been shown to relocate to a region of the ER membrane in close

proximity to the plasma membrane when intracellular calcium stores are depleted (Zhang *et al.*, 2005) and whose functional importance in SOCC signalling we have reported in hASM cells (Peel *et al.*, 2006; 2008). As has been described in a number of cell systems (see review Smyth *et al.*, 2010), in hASM cells, we have observed calcium depletion to induce recombinantly expressed Stim1 fused to yellow fluorescent protein (YFP) and the calcium-release activated calcium modulator 1 (Orai1) fused to cyan fluorescent protein (CFP) to co-localize into punctae, which can be dispersed upon re-addition of calcium (Billington *et al.*, 2010). Intriguingly in HEK293 cells, Martin *et al.* (2009) observed Stim and Orai to also colocalize with AC8 in lipid rafts, providing further evidence of the interaction between SOCC and cAMP signalling.

Another key player in calcium modulation, transient receptor potential cation channel C6 (TRPC6), has recently been found to alter cAMP in HEK293 cells by an entirely novel mechanism involving phosphoinositide-3 kinase, which will require further investigation (Shen *et al.*, 2011). Whether or not this is relevant in airway cells remains unclear, although these cells certainly express TRPC6, which may play a role in agonist induced calcium entry (Corteling *et al.*, 2004).

Epac-mediated signalling

Although assumed for many decades that PKA was the sole downstream target of cAMP, it was revealed in 1998 that cAMP also activates a family of proteins namely Epac (de Rooij *et al.*, 1998; Kawasaki *et al.*, 1998). To date, two isoforms of Epac have been characterized: Epac1 and Epac2, the latter of which has been found to exist as two splice variants. (For recent reviews, please refer to Holz *et al.*, 2008; Borland *et al.*, 2009; Gloerich and Bos, 2010).

Epac family members are guanine nucleotide exchange factors (GEFs) that catalyse the exchange of G-protein-bound GDP for GTP rendering the G-protein active. The major targets for Epac are the Ras-like GTPases Rap1 and Rap2 and upon the binding of cAMP, a conformational change occurs in Epac, making the catalytic region available for the binding of Rap (Gloerich and Bos, 2010). In addition to signalling via Rap, Rap-independent signalling has also been reported as well as the involvement of Epac with other signalling pathways including Ras, Akt, PLC, PLD and ERK (Grandoch *et al.*, 2010; Roscioni *et al.*, 2011). For additional detail, the reader is directed to an excellent recent review by Gloerich and Bos (2010).

In addition to defining the cellular role of Epac, work on this protein has also given rise to an impressive set of tools capable of providing pharmacological, spatial and temporal information regarding cAMP activity. Those mentioned repeatedly in this review take advantage of FRET and following transfection of the construct induce cells to express the Epac protein with a YFP fused to the N-terminus and a CFP at the C-terminus. Under basal (low cAMP) conditions, the termini are close together and FRET occurs; however, as mentioned above, increased cAMP induces a conformational change that results in a loss of FRET. Coding modifications (e.g. those directing the probe to diverse cellular locations) and improvements to the fluorescent profiles of the fluorophores (e.g. in terms of stability, brightness) have further enhanced the usefulness of these tools (Klarenbeek *et al.*, 2011; also see review by Hill *et al.*, 2010).

To date, it has become clear that Epac has some diverse functions when compared with PKA; indeed, in cardiac fibroblasts, Epac and PKA have polar opposite effects on cell migration (Epac: pro-migratory, PKA: anti-migratory) (Yokoyama *et al.*, 2008). Within the context of the airways, direct activation of Epac has been observed to induce hASM relaxation in a largely PKA-independent manner, and in two recent studies, the Epac-mediated downregulation of Rho was identified as the mechanism (Roscioni *et al.*, 2011; Zieba *et al.*, 2011). This study, as the majority of studies aiming to stratify Epac- versus PKA-mediated signals, utilized the Epac-selective cAMP analogue, 8-pCPT-2′-O-Me-cAMP or '007' (Christensen *et al.*, 2003; Rehmann *et al.*, 2003; and reviewed in Holz *et al.*, 2008). Teasing out PKA- versus Epac-mediated events in ASM has revealed that the anti-mitogenic effects of β_2-adrenoceptor agonists are likely PKA-mediated (Yan *et al.*, 2011). A further challenge will lie in dissecting out Epac1- versus Epac2-mediated effects.

As the affinity of cAMP for PKA and Epac has been reported to be within a similar range, it has been hypothesized that cellular specificity between the two pathways could be achieved by the relative abundance of each in cAMP microdomains (Dao *et al.*, 2006), the existence of which is discussed next.

Spatiotemporal control of cAMP

In addition to the magnitude of cAMP response, it is important to consider the cellular location and temporal organisation of signalling. Although not a novel concept, the ability to provide evidence for and even visualize cAMP microdomains in cells has changed significantly in the last 10 years. Zaccolo and Pozzan's (2002) pioneering research into microdomains, published in *Science* in 2002, provided detailed images of cAMP signalling 'hotspots' located in the sarcomere regions of rat neonatal cardiac myocytes. Since then, perhaps the real advance has been from confirming the existence of cAMP microdomains to comprehending how finely tuned individual microdomains are within the same cell in terms of scaffolding participants and hence signalling specificity. In elegant experiments in HEK293 cells, Cooper and colleagues compared the cAMP signals picked up by (i) a cytosolic cAMP probe, (ii) a cAMP probe targeted to the plasma membrane and (iii) a probe targeted to a specific AC isoform, AC8 (Wachten *et al.*, 2010). These studies revealed a startling disparity between the cAMP signals recorded by the probes in response to the same ligand; thyrotropin-releasing hormone (TRH) exposure induced a significant increase in cAMP as recorded by the AC8-specific probes, whereas under the same conditions, the cytosolic and plasma membrane-targeted probes registered a decrease in cAMP.

It is important to consider the array of molecular combinations that can constitute a cAMP microdomain – particularly when the range of isoforms of each is taken into account. These domains are associated with lipid rafts of the plasma membrane and in addition to diverse AC isoforms, active participants in cAMP microdomains include combina-

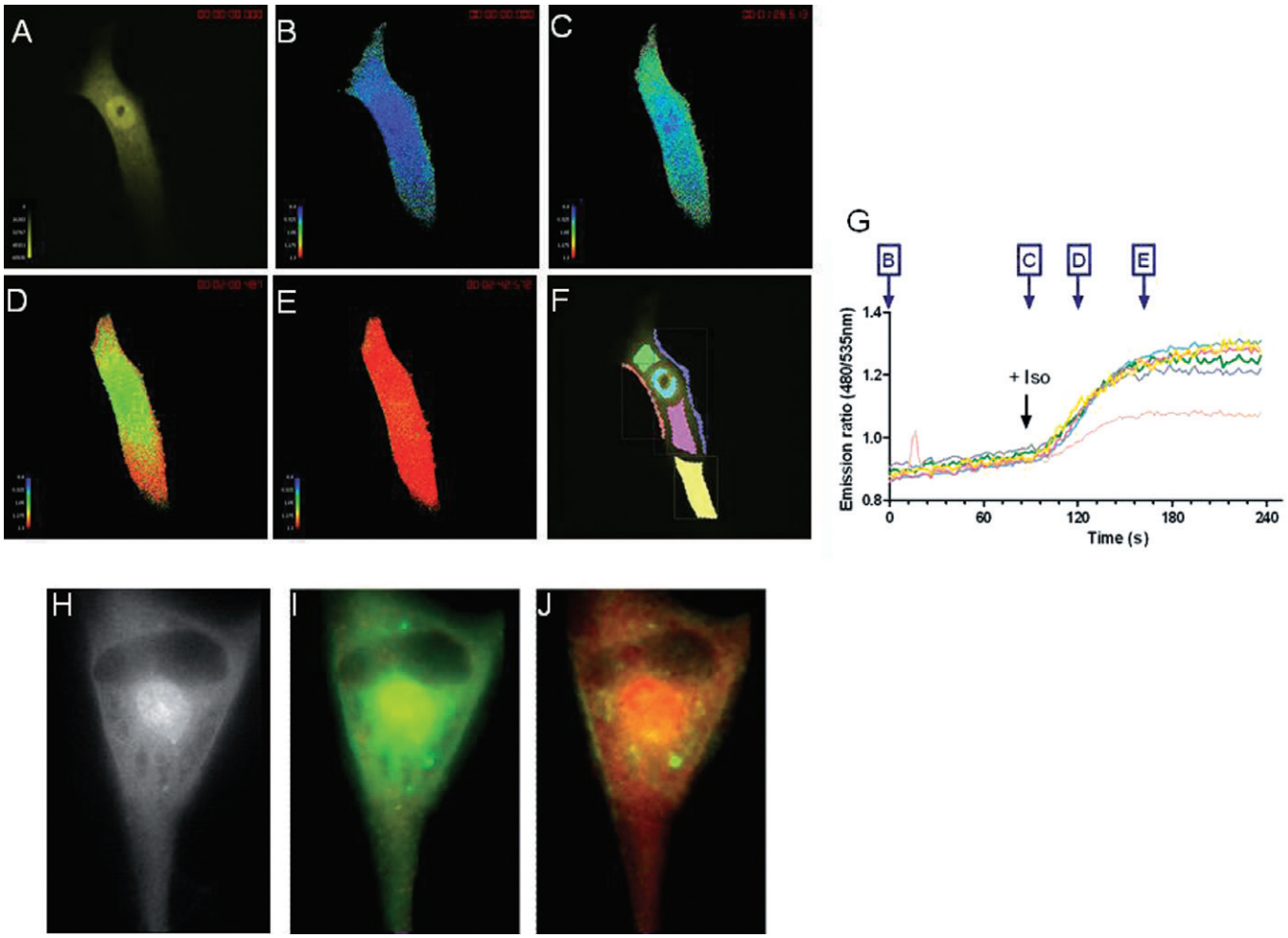

Figure 2

Distribution of PKA-based and Epac-based probes in cultured human airway smooth muscle cells under basal conditions and following exposure to 10 μM Isoprenaline. Panel A shows the distribution of CFP-Epac(dDEP,CD)-VENUS in untreated human airway smooth muscle cells. Panels B through E utilize a pseudocolour scale to demonstrate the emission ratio from CFP/YFP with blue corresponding to probe activity observed under basal conditions (B) and red corresponding to maximal probe activity following stimulation with isoprenaline (E). Panels C and D show probe activation at timepoints intermediate to these. Panel F shows six regions of interest selected from the same cell, and the plots of emission ratio for each of these are shown in panel G. The time point at which 10 μM isoprenaline added is marked (+Iso). Panels H through J show a human airway smooth muscle cell expressing both the catalytic subunits of PKA fused to YFP and the regulatory regions of PKA fused to CFP. Panel H simply shows the location of the probe under basal conditions (the YFP emission is shown but no difference was observed between this and the distribution of CFP). Again utilizing a pseudocolour range, panel I shows the cell under basal conditions, whilst panel J is the cell following exposure to 10 μM isoprenaline.

tions of the following; isoforms of PDEs (see review Houslay, 2010), PKA and its tether, AKAP, calmodulin, G-proteins, caveolin, phosphatases, sarco/endoplasmic reticulum $Ca^{2\pm}$ATPase (SERCA), dynein, NHE1, TRPC channels, Epac, Stim, Orai and the actin cytoskeleton (see reviews Willoughby and Cooper, 2007; Grandoch *et al.*, 2010). The discovery of small molecules that have been shown to inhibit AKAP/PKA interactions in cardiac myocytes constitute intriguing tools for future research; Christian *et al.* (2011) report these molecules to induce selective interference with compartmentalized cAMP signalling.

That the same cell is likely to contain myriad variations in microdomains with the variety of participants, relative abundance of each and presumably the spatial configuration of scaffold participants determining the signalling properties of that complex is an intriguing avenue for future research. Furthermore, determining how these discrete microdomains operate and interplay under basal, stimulated and diseased conditions will be an interesting challenge.

Specifically in the airway, there is much suggestive evidence for the existence of microdomains particularly in terms of the lack of linear correlation between levels of cAMP production and airway relaxation. Kume *et al.* (1994) highlighted this disparity in 1994 by quantifying both airway relaxation and cAMP production in equine tracheal strips in response to either forskolin, a direct activator of AC, or the non-selective β-adrenoceptor agonist, isoprenaline. Whilst each agent was comparable at inducing airway relaxation, forskolin induced

twice as much cAMP production as isoprenaline. Further studies demonstrated further disparity between whole cell cAMP production and cAMP-driven gene expression, whereby salbutamol and salmeterol behaved as partial agonists as regards whole-cell cyclic AMP production but as full agonists when expression of cAMP Response Element (CRE)-driven luciferase was used as a readout (Scott *et al.*, 1999).

Whilst we have attempted to visualize cAMP microdomains in hASM cells utilizing both the PKA-based probe generated by Zaccolo *et al.*, and the Epac-based probe generated by Jalink and colleagues, there is no clear evidence for microdomains in these cells at this juncture (see Figure 2). However, given the additional tools available including the AC-specific targeted cAMP probe (Wachten *et al.*, 2010) and a recently generated and improved Epac probe (Klarenbeek *et al.*, 2011), this requires further investigation.

Genetic considerations

Given the key role for cAMP in regulation of ASM tone, the possibility that inter-individual differences in responsiveness in the major components of the relevant signalling pathways might underlie either disease risk or treatment response has received much attention. Initial studies concentrated on candidate gene approaches and, in particular, focused on variability in the β_2 adrenoceptor gene, *ADRB2*. This receptor is known to be polymorphic, with three coding region variants ($Arg^{16}Gly$, $Gln^{27}Glu$ and $Ile^{164}Thr$) being known to have functional effects, the former two on receptor downregulation profiles, and the latter on agonist coupling. There is a large literature on the potential clinical relevance of these variants (reviewed by Chung *et al.*, 2011). In summary, variants in *ADRB2* (in particular, the Arg^{16} variant that in Caucasian populations has an allele frequency around 35%) have been suggested to alter risk of disease progression in asthma (Hall *et al.*, 2006) and to alter response to regular short acting β_2-adrenoceptor agonist therapy (Israel *et al.*, 2004) but do not appear to alter the risk of developing airway disease *per se* (Hall *et al.*, 2006).

There are a number of described variants in other components in downstream pathways, but little good evidence that these alter airway responses except for variants in the PDE4D gene. In a recent look up of potential candidate genes in the Spirometa study, rs298028 in *PDE4D* predicted FEV1 in current smokers suggesting a potential role for *PDE4D* in lung function determination (Obeidat and Hall, 2011). Interestingly, the potential importance of this gene is also suggested by the PDE4D knock-out mouse model that has altered airway responsiveness in terms of a loss of response to cholinergic stimulation and a lack of airway hyperreactivity following antigen exposure (Hansen *et al.*, 2000). It is worth noting that the PDE4D gene has also been linked to stroke (Gretarsdottir *et al.*, 2003; Munshi and Kaul, 2008; Meschia, 2011).

Summary and therapeutic implications

Classically, cAMP has been considered a universal second messenger within the cell, and it had been thought until recently that whole cell cAMP levels in general regulate downstream pathways. However, as depicted in Figure 3, over the last few years, it has become increasingly clear that signalling through AC-coupled pathways is considerably more complex and sophisticated than was previously considered. Spatial and temporal regulation within individual cells underlie this complexity, and a wide range of molecules have been identified, which help confer this specificity often by localizing key signalling components to specific intracellular locations. Relatively little is known regarding the regulation of the newly identified components of these pathways in airway cells and their potential contribution to disease, but the availability of new tools to explore these issues will help further our knowledge in the next few years. It is possible that components of these signalling cascades other than the traditionally recognized ones (i.e. cognate receptors and PDEs) may be useful therapeutic targets in their own right. As the potential exists to specifically target the airways using inhaled

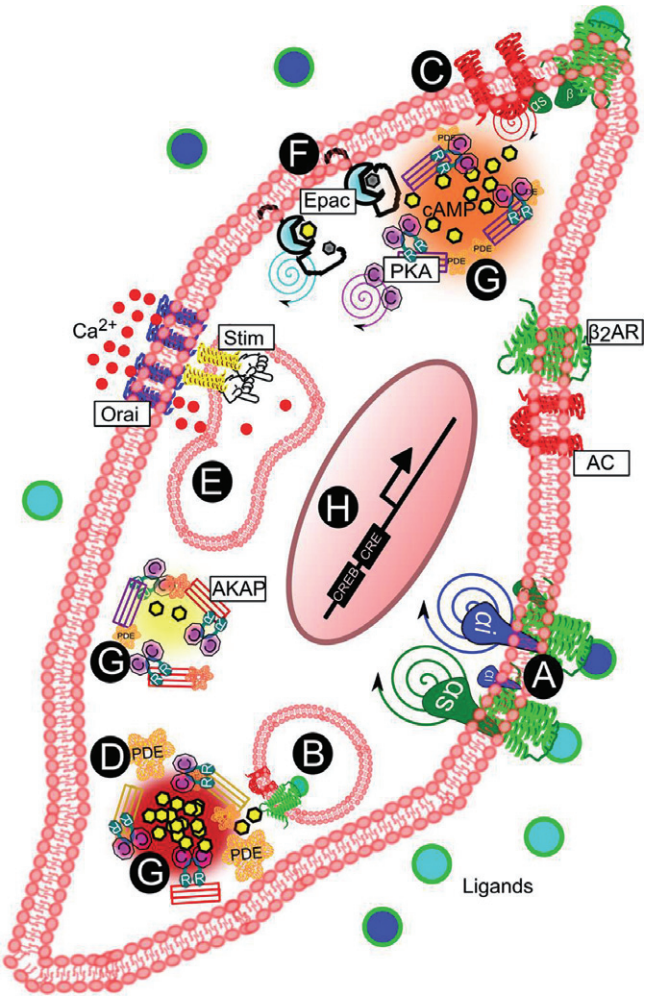

Figure 3

Novel cAMP signalling paradigms. (A) Biased agonism; (B) continued signalling following internalization; (C) modulation of cAMP by AC; (D) control of cAMP degradation; (E) cAMP and calcium crosstalk; (F) Epac-mediated signalling; (G) spatiotemporal control of cAMP; (H) genetic considerations.

approaches, unwanted systemic effects of such agents may be avoidable. This approach, however, requires knowledge of the effects of agents at both the target cell (e.g. ASM) and other cell types in the airways exposed to drug, in particular epithelial cells, which are the cell type with the initial exposure to inhaled agents.

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Conflicts of interest

The authors have no conflicts of interest.

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