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# Visions and Reflections (Minireview)

# Cyclic AMP: swing that message!

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Cells constantly receive a variety of environmental stimuli that are relayed to intracellular effectors to generate unique and appropriate responses. A fundamental problem in signal transduction research is to understand how specificity is achieved when just a few messenger molecules are used to control multiple functions. Cyclic AMP is the classic second messenger regulating a vast number of functions in virtually all kinds of cells. The intracellular concentration of cAMP ($[cAMP]_i$) is determined by cAMP-generating adenylate cyclases (ACs) and degrading phosphodiesterases (PDEs). In metazoan cells, the effects of the messenger are mediated by cAMP-dependent protein kinases (PKAs) [1, 2], guanine nucleotide exchange factors (Epacs) [3] and cyclic nucleotide-gated ion channels (CNGs) [4].

It was recognized early on that subcellular compartmentalization of signalling components is important to obtain specificity in cAMP-regulated pathways. For example, adrenaline and prostaglandin E were found to activate different cellular pools of PKA in cardiomyocytes [5]. The concept of compartmentalization has now received substantial experimental support, including direct demonstration of intracellular gradients of $[cAMP]_i$ and PKA phosphorylation [6, 7]. In most cases, spatially confined cAMP signals are dependent on A-kinase-anchoring proteins (AKAPs), which represent a growing family of versatile scaffolding proteins that organize local signalling protein complexes around PKA. In addition to binding effector proteins and their substrates, AKAP complexes often contain ACs or PDEs and protein phosphatases that restrict the spread of the messenger and contribute to signal termination [8–10].

Less attention has been paid to the role of temporal aspects of cAMP signalling for obtaining specificity. In signalling based on $Ca^{2+}$, the other classic second messenger, it has been convincingly demonstrated that the signal pattern often affects the specificity of the response. Many stimuli trigger oscillations of the cytoplasmic $Ca^{2+}$ concentration ($[Ca^{2+}]_i$), and the activation of downstream effectors has been shown to depend on the amplitude and frequency of the oscillations [11–14]. In contrast to the extensively characterized $Ca^{2+}$ signals, little is known about cAMP kinetics. Until recently, only a few examples of oscillatory cAMP signalling have been described. More than 30 years ago it was found that $[cAMP]_i$ varied during the cardiac contraction cycle [15]. Several kinetic models have predicted the existence of $[cAMP]_i$ oscillations based on the known interactions between cAMP and $Ca^{2+}$ [16–20]. One study indeed reported $[cAMP]_i$ oscillations triggered by spontaneous bursts of electrical activity with $[Ca^{2+}]_i$ spiking in Xenopus embryonal neurons [18].

Progress in studies of cAMP dynamics has for a long time been hampered by the lack of suitable methods to measure $[cAMP]_i$ in individual living cells. However, the development of tools and technology over the last

few years has provided a set of biosensors, which allow examination of $[cAMP]_i$ signals in single cells with unprecedented spatiotemporal resolution [6, 21–25]. Engineered CNGs [25] and novel single-chain biosensors, based on changes of fluorescence resonance energy transfer (FRET) between fluorescent proteins fused to isolated cAMP-binding domains from PKA or Epac [21–23], respond fast and lack catalytic activity. Being expressed as single polypeptide chains, the latter reporters do not suffer from the drawbacks associated with the previous generation of probes relying on FRET between fluorescent proteins expressed on separate polypeptide chains, often at markedly different levels. The biosensor undergoing the largest signal change reported so far does not rely on FRET as readout. It consists of fluorescence-tagged PKA subunits engineered so that the regulatory subunit resides permanently in the plasma membrane and the catalytic subunit undergoes movements to and from the membrane upon changes in cAMP in the submembrane space [24]. Ratiometric evanescent wave microscopy detection of this translocation results in severalfold changes of the signal after stimulation with physiological receptor agonists [24].

The available tool box of cAMP biosensors has enabled the demonstration of transients and complex spatiotemporal patterns of $[cAMP]_i$ in many cell types. Two independent studies describe $[cAMP]_i$ oscillations in insulin-secreting β cells [24, 26]. Dyachok et al. [24] observed $[cAMP]_i$ oscillations induced by the insulinotropic gut hormone glucagon-like peptide-1. This finding is the first demonstration of $[cAMP]_i$ oscillations in response to a receptor agonist. Simultaneous recording of $[Ca^{2+}]_i$ revealed mutual reinforcement of cAMP and $Ca^{2+}$ signals in this cell type. Accordingly, stimulation with the $[cAMP]_i$-elevating agonist triggered subsequent synchronized oscillations of $[Ca^{2+}]_i$ and $[cAMP]_i$, disappearing when $Ca^{2+}$ was removed from the extracellular medium [24]. Landa et al. [26] reported that $[Ca^{2+}]_i$ oscillations evoked by combining an increase of glucose with the $K^+$ channel inhibitor tetraethylammonium triggered antisynchronous oscillations of $[cAMP]_i$, probably due to activation of a PDE by the high $[Ca^{2+}]_i$ transients obtained with this experimental protocol. There is also a tight relationship between $Ca^{2+}$ and cAMP oscillations in other types of cells. A recent study by Willoughby and Cooper [27] demonstrates that imposed oscillations of $[Ca^{2+}]_i$ are translated into cyclic changes of $[cAMP]_i$ in human embryonic kidney cells expressing $Ca^{2+}$-calmodulin-sensitive AC8 [27]. The mechanisms underlying the cAMP oscillations observed in mammalian cells remain poorly understood. As predicted by kinetic models, interplay with $Ca^{2+}$ seems important in most cases. $Ca^{2+}$ can either increase $[cAMP]_i$ via $Ca^{2+}$-calmodulin-stimulated ACs, or decrease $[cAMP]_i$ via $Ca^{2+}$ inhibition of ACs and $Ca^{2+}$-calmodulin activation of PDE1 family members. Whether the positive or negative influences will dominate can be expected to be context dependent and determined by the relative expression levels of the proteins, by the duration and amplitude of $[Ca^{2+}]_i$ and $[cAMP]_i$ reached during stimulation, and by additional regulatory influences from protein kinases and phosphatases. Interestingly, the $Ca^{2+}$/calmodulin-activated protein phosphatase PP2B (calcineurin) is associated with some AKAP complexes [28], where it dephosphorylates local substrates and thereby counteracts the effect of PKA and other kinases. Opposing actions of cAMP and $Ca^{2+}$ via co-anchored enzymes may thus be important in oscillatory signalling by generating cycles of phosphorylation and dephosphorylation. Additional clues for understanding the relationship between $Ca^{2+}$ and cAMP can be obtained from analysis of the regional distribution of the molecular components. For example, AC8 has been found to be activated preferentially by store-operated $Ca^{2+}$ influx in non-excitable cells, which can be explained by the co-localization of store-operated channels and AC8 in lipid rafts [29]. Future studies will clarify if there is a similar organization in excitable cells, like β cells, cardiomyocytes or neurons, and - to what extent PDEs are also functionally associated - with ion channels in the plasma membrane.

$Ca^{2+}$ oscillations occur over a wide range of frequencies with periods from milliseconds to tens of minutes. Are cAMP and $Ca^{2+}$ signals interdependent over the entire range of frequencies? In β cells, hormone-induced $[cAMP]_i$ oscillations had frequencies up to 1.5 $min^{-1}$, in good agreement with those of $[Ca^{2+}]_i$ [24]. Testing the influence of frequency of imposed $[Ca^{2+}]_i$ oscillations on $[cAMP]_i$ in HEK cells, Willoughby and Cooper [27] found that AC8 acted as a low-pass filter integrating the input to stable $[cAMP]_i$ elevation at $[Ca^{2+}]_i$ oscillation frequencies $>3$ $min^{-1}$. In a similar model system, Gerbino et al. [30] found that the cAMP signalling system was even more resistant to $[Ca^{2+}]_i$ oscillations, requiring $[Ca^{2+}]_i$ elevations of at least 1.5 min duration to affect $[cAMP]_i$. However, the latter studies [27, 30] measured global $[cAMP]_i$ changes and cAMP signals can be expected to be much more dynamic in subcellular microdomains, in particular in the vicinity of $Ca^{2+}$ channels in the plasma membrane.

Can cAMP oscillations occur without corresponding changes of $Ca^{2+}$? Although a direct demonstration of $Ca^{2+}$-independent $[cAMP]_i$ oscillations is still missing, recent work from the Scott and Cooper groups on the organization of AKAP signalling complexes suggests several different mechanisms for generating transient

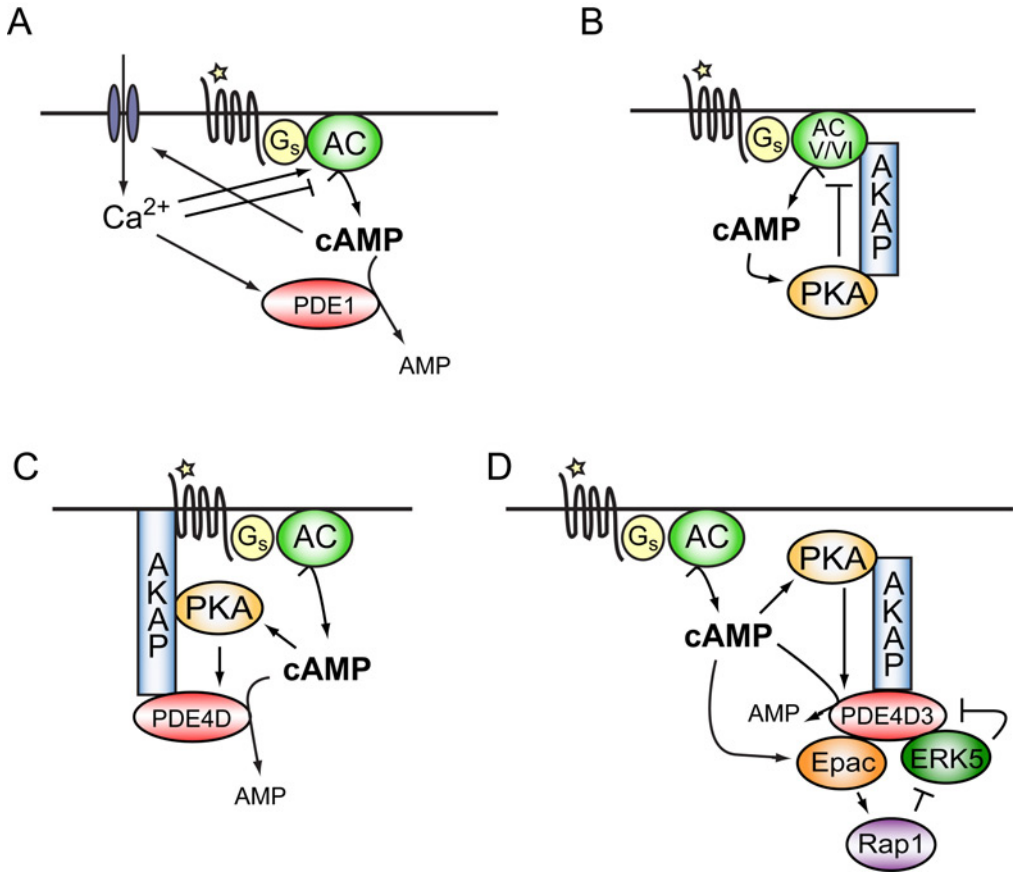


**Figure 1.** Different mechanisms for receptor-mediated generation of transient $[cAMP]_i$ elevations. (A) Interplay between $Ca^{2+}$ and cAMP can generate complex temporal $[cAMP]_i$ dynamics via $G_s$-protein activated ACs positively or negatively regulated by $Ca^{2+}$, via $Ca^{2+}$-activated PDE1 as well as via cAMP-triggered $Ca^{2+}$ signals. (B) Suppression of receptor-induced cAMP production by a minimal feedback loop involving PKA-mediated attenuation of $G_s$-stimulated ACV/VI activity facilitated by AKAP interaction with the AC [10]. (C) Stimulation of cAMP degradation by PKA-mediated activation of PDE4D facilitated by AKAP binding to the PDE and to the plasma membrane and/or receptor [31] (D). Stimulation of cAMP degradation by two coupled feedback loops. An AKAP anchors PKA-activated PDE4D3 and the PDE in turn anchors Epac1 and ERK5. ERK5 phosphorylation suppresses the PDE activity. A rise in cAMP leads to PKA phosphorylation and activation of PDE4D3 as well as to Epac1 activation of Rap1, which suppress ERK5 activity and relieves its inhibitory action on the PDE [32].

elevations of $[cAMP]_i$. Via anchoring of PKA to PKA-stimulated PDEs [31] or to PKA-inhibited ACs [10], rises of $[cAMP]_i$ in the submembrane space will quickly be interrupted by feedback activation of cAMP degradation or inhibition of cAMP production. A more complex arrangement with two coupled feedback loops was found in rat neonatal cardiac ventriculocytes [32]. In these cells, mAKAP anchored both PKA and the PKA-activated PDE4D3 and the PDE in turn served as a scaffold for Epac1 and ERK5. PDE activity is negatively regulated by ERK and the ERK activity is suppressed by Epac1. Thus, these signalling complexes provide means for generating discrete pulses of cAMP in restricted cell compartments (Fig. 1). It is possible to envisage a model, where signal termination results in dephosphorylation of the PKA and ERK substrates, which then will be ready for a new round of activation. Oscillations of $[cAMP]_i$ may then result if the stimulus persists.

What is the evidence that $[cAMP]_i$ oscillations contribute to specificity in the cAMP signalling pathways? A possible functional importance of $[cAMP]_i$ oscillations was recently suggested from studies of the effect of oscillatory and stable $[cAMP]_i$ elevations on the generation of $Ca^{2+}$ signals and the nuclear translocation of the PKA catalytic subunit in insulin-secreting cells [24, 33]. Whereas brief increases of $[cAMP]_i$ were found to trigger rapid on-off $Ca^{2+}$ signals, only sustained $[cAMP]_i$ elevations induced nuclear translocation of the PKA catalytic subunit. Thus, brief elevations of $[cAMP]_i$ can selectively regulate local cytoplasmic events without inducing transcriptional events in the nucleus, probably because of the relatively slow diffusion of PKA catalytic subunits through the nuclear pores [34]. Further studies are required to clarify how the duration and amplitude of cAMP signals affect the activation of different effectors. Since the different cAMP effectors

are activated by widely different concentrations of cAMP, it is possible that transient elevations help to maintain $[cAMP]_i$ at levels that favour activation of the higher-affinity cAMP-binding targets.

In summary, the development of new tools and technology finally allows tracking of the temporal aspects of cAMP signals in response to different stimuli in various types of cells. A complex picture is emerging where local signalling complexes and interactions between messengers tailor the pattern of the signal and thereby affect the downstream responses. Future challenges include exploration of the role of cAMP kinetics in disease states, such as cardiac arrhythmias, heart failure, neurological disorders and diabetes. Persistent efforts to measure quantitative dynamic parameters must be combined with traditional biochemistry, molecular perturbation strategies and mathematical modelling to develop comprehensive models of the generation of cAMP signals and how effector systems are tuned to sense the swinging rhythm of the message.


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