Crosstalk between G-protein and Ca\textsuperscript{2+} pathways switches intracellular cAMP levels

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Cyclic adenosine monophosphate and cyclic guanosine monophosphate are universal intracellular messengers whose concentrations are regulated by molecular networks comprised of different isoforms of the synthases adenylate cyclase or guanylate cyclase and the phosphodiesterases which degrade these compounds. In this paper, we employ a systems biology approach to develop mathematical models of these networks that, for the first time, take into account the different biochemical properties of the isoforms involved. To investigate the mechanisms underlying the joint regulation of cAMP and cGMP, we apply our models to analyse the regulation of cilia beat frequency in Paramecium by Ca\textsuperscript{2+}. Based on our analysis of these models, we propose that the diversity of isoform combinations that occurs in living cells provides an explanation for the huge variety of intracellular processes that are dependent on these networks. The inclusion of both G-protein receptor and Ca\textsuperscript{2+}-dependent regulation of AC in our models allows us to propose a new explanation for the switching properties of G-protein subunits involved in nucleotide regulation. Analysis of the models suggests that, depending on whether the G-protein subunit is bound to AC, Ca\textsuperscript{2+} can either activate or inhibit AC in a concentration-dependent manner. The resulting analysis provides an explanation for previous experimental results that showed that alterations in Ca\textsuperscript{2+} concentrations can either increase or decrease cilia beat frequency over particular Ca\textsuperscript{2+} concentration ranges.

Introduction

Cyclic 3',5' adenosine monophosphate (cAMP) and cyclic 3',5' guanosine monophosphate (cGMP) were first identified as intracellular mediators in the 1950s.\textsuperscript{1} Intracellular signalling via the cAMP and cGMP nucleotides is involved in many areas of animal cell biology and is an important area of research. Conceptual models of cAMP concentration regulation have undergone significant changes in recent years as more details of the role of adenylate cyclases (ACs) and cyclic nucleotide phosphodiesterase (PDE) in cAMP regulation have been reported.\textsuperscript{2-4} Both these enzymes exhibit a continuum of isoforms with unique biochemical properties, which are expressed in various combinations within both single and multicellular organisms. Such isoforms are also sometimes referred to as gene products,\textsuperscript{5} and are expressed by somewhat different gene groups or may arise from the same gene by alternative splicing.

Dictyostelium cells contain three distinct AC isoforms\textsuperscript{6} and mammalian cells can express ten different major AC gene isoforms.\textsuperscript{5} As well as having different structures and biochemical properties, it is now well-established that different AC gene products can be either activated or inhibited by G-protein\textsuperscript{7} and Ca\textsuperscript{2+} pathways.\textsuperscript{8} The ten AC genes can be divided into five families according to their structural and activation properties.\textsuperscript{5} The Ca\textsuperscript{2+}–CaM activated isoforms are ACI, ACIII and ACVIII. ACII, ACIV and ACVII are activated by G\textsubscript{i2,3} and G\textsubscript{i1,2}. ACV and ACVI are inhibited by G\textsubscript{i2} isozymes (G\textsubscript{i2,6}, G\textsubscript{i2,7}, G\textsubscript{i2,12}, G\textsubscript{i2,13}, and G\textsubscript{i2,21}) and Ca\textsuperscript{2+} ions. There is an ongoing debate as to whether the ACV and ACVI isoforms are inhibited by Ca\textsuperscript{2+} directly or via CaM.\textsuperscript{9} The last membrane-bound ACIX isoform is the most divergent from the other eight membrane-bound isoforms. The last soluble isoform is similar to the AC found in Anabaena.\textsuperscript{10}

In combination with synthesis by AC and guanylate cyclase, PDEs regulate the levels of cAMP and cGMP by degradation of the cyclic molecules, and these enzymes are expressed in a wide range of organisms: Trypanosoma,\textsuperscript{11,12} Dictyostelium,\textsuperscript{13} Drosophila\textsuperscript{14} and Homo sapiens.\textsuperscript{15,16} Eleven families of PDE in various species, each containing several isoforms with diverse structural and biochemical properties, have now been identified in the literature.\textsuperscript{17} The calcium and calmodulin-dependent PDEI family was one of the first to be identified.\textsuperscript{18} The specific property of PDEII enzymes is that they are activated by cGMP binding.\textsuperscript{19} cAMP hydrolysis by the PDEIII family, unlike in the case of PDEII, is inhibited by cGMP. PDEIV, PDEVII and PDEVIII are highly sensitive to cAMP as a substrate, with Km being of the order of magnitude of 1μM and lower.\textsuperscript{20-22} PDEV was originally identified in platelets.\textsuperscript{23}
and later become a target for a number of drugs regulating vascular smooth muscle contraction.\textsuperscript{24,25} PDEV is characterized by relative specificity to cGMP rather than to cAMP. PDEV is also known as a photoreceptor phosphodiesterase due to its expression in mammalian retina. PDEIX has the highest affinity for cGMP and has been proposed as a regulator of cGMP signaling.\textsuperscript{26,27} PDEX is not as well characterized as some other PDE families. However, it has been reported that PDEX is more cAMP rather than cGMP specific.\textsuperscript{28} PDEXI is the most recently discovered phosphodiesterase and has been reported to hydrolyse both cAMP and cGMP without any preference for either nucleotide.

It is now well established that many of the intracellular effects of cAMP are mediated by cAMP-dependent kinase (PKA). PKA catalyzes the transfer of the terminal phosphate group from ATP to serines and threonines of target proteins, hence modulating their activity. The phosphorylation of different substrates by PKA in various cells certainly provides a partial explanation for the variety of cAMP effects in different cellular systems. In this paper, we use a structural systems biology approach to further investigate this issue, and propose that the multitude of AC, GC and PDE gene products which may occur in living cells provides another important mechanism for cAMP concentration-dependent differential regulation of intracellular events. The proposed approach can therefore be regarded as a natural extension of previous approaches to modelling cAMP regulation,\textsuperscript{30,31} which allows us to generate isoform specific cAMP concentration profiles and study isoform specific regulatory effects.

An important, but perhaps insufficiently recognized, problem in nucleotide signalling is the convergence and mutual influence of Ca\textsuperscript{2+} and G-protein pathways on cAMP production. It is not clear to what extent each of these pathways contributes to the shaping of the output AC response and subsequent modulation of the cAMP-dependent effects across the cell. Previous mathematical modelling studies have largely focused on the separate effects of either G-protein or Ca\textsuperscript{2+} signals on cAMP production.\textsuperscript{4,31–36} We aimed here to develop a model to include the effects of AC regulation by both Ca\textsuperscript{2+} and G-protein dependent signals and thus to elucidate the interplay between these two major pathways modulating AC, and hence cAMP, concentrations within the framework of the network shown in Fig. 1. By analysing this model, we are able to predict various potential outputs for cAMP dependence on Ca\textsuperscript{2+} and G-protein signals and we can thus compare the model predictions for nucleotide signalling with a number of experimental studies, in order to identify potential cellular mechanisms governing the interplay between Ca\textsuperscript{2+} and G-protein pathways. In this study, we decided to focus on the number of AC isoforms that have been elucidated experimentally and represent different types of concentration profiles: activation, inhibition, bell-shaped dependence and reverse bell shaped dependence.

cGMP acts as a second messenger in a similar way to cyclic AMP, but exhibits several notable differences. In particular, significant activity of cGMP is observed at higher Ca\textsuperscript{2+} concentrations.\textsuperscript{37} cGMP synthesis is catalyzed by GC, which converts GTP to cGMP, and is hydrolysed by cyclic nucleotide phosphodiesterases (PDE 1–IV) into 5\textsuperscript{‘}-GMP. Both cGMP and cAMP have been reported to be involved in cell migration and chemotaxis mechanisms in unicellular organisms such as \textit{Dictyostelium} and \textit{Paramecium}.\textsuperscript{41–43} Currently, however, there is limited understanding of the nature and extent of crosstalk between these two pathways. To investigate this issue, we aimed to develop a model incorporating Ca\textsuperscript{2+} dependent alterations of both cAMP and cGMP. The model predictions are tested against multiple sets of experimental data on the complementary roles of cAMP and cGMP-responsive cilia beat in \textit{Paramecium}, and allow us to propose a new explanation for how intracilia Ca\textsuperscript{2+} and nucleotide concentration alterations translate to cilia beat frequency and the movement that defines the trajectory of \textit{Paramecium} motion.

Results

Isoform-specific modelling reveals the diversity of cAMP concentration profiles

Our isoform-based model predicts that the differences in cAMP profiles among various cell types are at least partly due to the many different combinations of AC and PDE isoforms expressed in particular types of cells and tissues. Various combinations of AC and PDE isoforms, each with their own unique biochemical properties, can provide a multitude of cell specific concentration profiles. In this section, we demonstrate how the AC and PDE pairs regulated by Ca\textsuperscript{2+} and G-protein subunits form cAMP concentration profiles (Fig. 1) and tune physiological effects. Model predictions for ACI, ACII and PDEI isoform activation (Fig. 2A) by Ca\textsuperscript{2+} are compared against experimental data from the literature\textsuperscript{44,45} in Fig. 2B. The combination of ACI and ACII with PDEI in our model allows quantitative predictions concerning...
intracellular cAMP concentrations to be made. Eqn (6) and (7) (see Experimental) have been employed to combine the Ca\(^{2+}\)-CaM-dependent PDEI isoform with the Ca\(^{2+}\)-dependent AC isoforms. Fig. 2C–H show the resulting intracellular cAMP concentration profiles predicted by the model. In addition to the clear dependence of cAMP levels on the unique biochemical properties of the AC and PDE isoforms, the shape of the cAMP concentration profile is seen to be highly sensitive to the values of the CaM-AC dissociation constants. Indeed, variation of these parameters in the model resulted in dramatic alterations in cAMP profiles even for the same AC and PDE isoforms (Fig. 2C, 2D, and 2E). Similarly, the same variability of the CaM–PDEI equilibrium dissociation constants produced qualitatively different cAMP concentration profiles for the ACII and PDEI enzymes on Fig. 2F, 2G, and 2H.

Some AC isoforms interact with G-protein subunits that transmit extracellular signals via G-protein coupled receptors (Fig. 3A). We used our model to analyse the intracellular cAMP concentration dependence as a function of these G-protein signals. Fig. 3B compares the model responses against experimental data for the ACI and ACII regulation curves by G-protein subunits. Since the G-protein mediated signals do not always assume simultaneous Ca\(^{2+}\) stimulation, we have chosen to use the steady-state level of Ca\(^{2+}\)-dependent PDEI activity in combination with G-protein activated AC isoforms shown in Fig. 3B. Fig. 3C–E shows the range of intracellular cAMP concentration profiles achieved by the G-protein mediated signals. The data suggests that G-protein mediated signals can cause intracellular cAMP concentration to decrease (Fig. 3D), increase (Fig. 3E), or even exhibit a reversed bell-shaped dependence (Fig. 3C).

Interactions between G-protein and Ca\(^{2+}\)/CaM signalling pathways switch cAMP concentrations

We next employed our model to investigate the level of interaction between the G-protein and Ca\(^{2+}\) signalling pathways. As discussed above, AC can be activated by Ca\(^{2+}\)-CaM complexes as well as by G-protein subunits. While the dose-dependent responses have been characterized for both Ca\(^{2+}\) and G-protein pathways, it is not always clear if and how interactions between these two different pathways occur. Since both Ca\(^{2+}\) and G-protein mediated signals can activate or inactivate AC, it is of interest to establish what state the AC is
likely to be at if there is an inhibition signal through the G-protein and activation signal via the Ca\(^{2+}\) pathway.

Building on work on nucleotide signalling showing that AC was activated by G-protein subunits,\(^{35,46,47}\) it was found that there are many other regulators of AC with various activation patterns. In addition to regulation by G-protein subunits, the activities of AC are also modulated by Ca\(^{2+}\), phosphorylation, glycosylation and many other regulatory phenomena.\(^{48}\) An interesting example of a molecular system with joint Ca\(^{2+}\)–CaM and G-protein dependent AC regulation is the network underlying cAMP production in *Dictyostelium*.\(^{49}\) Indeed, in these cells the interplay between Ca\(^{2+}\) and GPCR induced signals appears to govern the direction and the rate of migration in chemotaxis.\(^{50}\) Fig. 4A shows a schematic diagram for AC interactions with Ca\(^{2+}\), Ca\(^{2+}\)/CaM and with G-protein subunits. Although AC has two ATP binding domains, it has been shown that these domains are inactive separately and require dimerization for AC activity.\(^{51}\) This fact allows us to introduce a simplification and consider a model of AC with one catalytic centre that describes the activity of the two domains. The multiple G-protein subunits (15α, 5β and 13γ) together with the nine membrane bound AC isoforms raise the issue of selectivity between the interacting partners.\(^{52}\) We showed previously that structural selectivity can be complemented by ligand concentration-based selective target activation.\(^{53}\) In this case, however, the selectivity of the regulation achieved appears to be due to the combination of signals from separate pathways. For example, G-protein subunit binding may inhibit or activate AC, depending on the AC isoform and the subunit. At the same time, the binding of the same subunit, in combination with AC phosphorylation or by binding to a Ca\(^{2+}\)–CaM pair, may cause a different degree of AC activity, produce more or less cAMP and as a result induce a different intracellular effect. In order to investigate the relationship and the influence of the two regulatory pathways, we analysed an example of the Ca\(^{2+}\)–CaM and G-protein subunit interactions with AC. In the most general case there is no strong reason to assume that the actual activity of AC is simply the sum or multiplication of activities mediated by the G-protein subunits and Ca\(^{2+}\)–CaM separately. In other words, binding of one of the modulators to the AC is likely to have an impact on the overall molecular conformation and may thus lead to alterations in its affinity to another modulator. Since the AC activity may not be just a linear combination of the G-protein and Ca\(^{2+}\) pathway induced signals, we considered the overall state of the AC molecules and employed mathematical modelling to analyse a range of potential outcomes.

Fig. 4 shows model predictions for the variety of AC activation modes by Ca\(^{2+}\) in different conformational states. The results of this analysis suggest that Ca\(^{2+}\) can be either an activator or inhibitor of AC, depending on the AC conformation in the complex with G-protein subunits. The binding of G-proteins alters the conformation of the molecule, changes the Ca\(^{2+}\), Ca\(^{2+}\)–CaM and substrate interaction constants with AC and thereby modulates the activity state. The predicted probabilities for AC molecules to be in complexes with G-protein subunits and Ca\(^{2+}\)–CaM complexes are shown in Fig. 4B as a function of Ca\(^{2+}\) concentration. As shown in Fig. 4, the proportion of non Ca\(^{2+}\)/CaM bound AC species decreases with the elevation of Ca\(^{2+}\) concentration, whereas the proportion of other species gradually increases. The sum of the activities for individual AC species is represented in Fig. 4C with low and high concentrations of G-protein subunits. The model predicts that the switch between Ca\(^{2+}\)-dependent activation and inhibition of AC is due to the interaction with G-proteins. G-protein subunits shift the balance between the number of AC conformations which increase with Ca\(^{2+}\) and the number of those that decrease with Ca\(^{2+}\). Thus, Ca\(^{2+}\) signals can both activate or inhibit AC depending on the presence or absence of specific G-protein subunits. The difference between the AC isoforms in terms of their Ca\(^{2+}\) and G-protein mediated properties appears to be in the variability of affinity constant pairs to the substrate and to the G-protein subunits. Fig. 4B, 4C, 4D and 4E show alternative combinations of dissociation constants of AC molecules. At the same time, the G-protein mediated signals modulate the amplitude of the Ca\(^{2+}\)-dependent responses. Effectively, G-protein subunits define the state of the AC, depending on which cAMP production can be
either Ca\(^{2+}\) inhibited (Fig. 4C), Ca\(^{2+}\) independent (Fig. 4D) or Ca\(^{2+}\) activated (Fig. 4E).

**Complementary regulation of cilia beat frequency by cAMP and cGMP**

The steady-state concentration of cGMP as a function of Ca\(^{2+}\) concentration predicted by eqn (8) in our model (see Experimental) has been validated against the experimental data from.\(^{37}\) It is clear from the experimental data that some GC and PDE isoform pairs also produce a bell-shaped dependence of cGMP on Ca\(^{2+}\) concentration, in a similar way as AC and PDE do with cAMP. However, the peak cGMP concentration is reached at a significantly higher value of Ca\(^{2+}\) compared to the maximum cAMP concentration (Fig. 5B). This similarity in the shape of the concentration-dependent curves and at the same time the difference in the Ca\(^{2+}\) concentration corresponding to the highest nucleotide levels is rather intriguing, and may shed light on the mechanisms involved in synchronised intracellular nucleotide signalling.

To investigate the issue of joint regulation by cAMP and cGMP, we considered an experimental system that requires the alteration of both cAMP and cGMP nucleotides in a Ca\(^{2+}\)-dependent manner. Multiple studies have found that cilia beat frequency (CBF) is regulated by cAMP phosphorylation of dynein subunits.\(^{41,54,55}\) cGMP-dependent kinase has also been shown to be involved in CBF regulation.\(^{56–58}\) Ca\(^{2+}\) shapes both cAMP and cGMP intracilia concentrations and it is well established that it controls the cilia beat.\(^{59}\) In Paramecium, elevation of Ca\(^{2+}\) is reported to decrease cilia beat to the point at which the direction of beat is reversed.\(^{60}\) However, a further rise in Ca\(^{2+}\) leads to increased CBF,\(^{61}\) and the results of this study generally revealed a highly complex

**Fig. 4** AC regulation by Ca\(^{2+}\) and G-protein pathways: (A) Schematic representation of the AC activity regulation network. Various AC isoforms are affected by Ca\(^{2+}\) and G-protein pathway signals according to their unique structures and corresponding biochemical properties. The model elucidates the link between the effect of Ca\(^{2+}\) and G-proteins on cAMP production via the regulation of AC. The model predicts four different potential scenarios for AC regulation by Ca\(^{2+}\) in the presence of G-protein subunits. (B) G-proteins may possess switching functionality. The model predicts that AC is inhibited by Ca\(^{2+}\) in the absence of G-proteins but can become insensitive to Ca\(^{2+}\) alterations when the number of G-protein subunits increases. Further increase of G-protein subunits produces a switching effect so that AC is now activated by Ca\(^{2+}\). The analysis of other potential scenarios for the interactions of G-protein and Ca\(^{2+}\) pathways reveals that AC can be completely insensitive to Ca\(^{2+}\) (D) or Ca\(^{2+}\) can inhibit (C) or activate (E) AC, over the full physiological range of G-protein subunit concentrations.

**Fig. 5** Cilia beat frequency dependence on Ca\(^{2+}\) concentration in Paramecium: (A) Schematic diagram representing the molecular network regulating CBF. Dynein subunits responsible for creating cilia movement are phosphorylated by cAMP and cGMP-dependent kinases. The levels of cAMP and cGMP are regulated by AC, GC, and PDE. The activities of these enzymes are regulated by CaM in a Ca\(^{2+}\)-dependent manner. Mg\(^{2+}\) binds competitively to the Ca\(^{2+}\) binding sites on CaM. (B) The comparison of the nucleotide concentrations and the CBF dependence on Ca\(^{2+}\) concentrations. The data for CBF,\(^{61}\) AC\(^{69}\) and GC\(^{37}\) is shown as circles, squares, and pentagons, respectively. Both experimental data and the model predictions suggest that CBF almost superimposes on the sum of AC and GC steady-state curves. The only difference is the shift along the Ca\(^{2+}\) concentration, which can be explained by the difference in Mg\(^{2+}\) concentration at which the experiments took place.
CBF dependence on the Ca\(^{2+}\) concentration. In mammals, increases in Ca\(^{2+}\) concentration almost always intensify cilia beat\(^{65,66}\) and reduced Ca\(^{2+}\) appears to diminish CBF.\(^{64}\) In spite of these and other experimental studies, there still appears to be considerable disagreement on the precise mechanisms of Ca\(^{2+}\) action in this system.\(^{56,66}\)

We have used the mathematical model for Ca\(^{2+}\)-dependent cAMP and cGMP metabolism developed in this study to investigate the underlying molecular mechanism of CBF regulation in *Paramecium*. The model predictions for cAMP and cGMP concentration (Fig. 5B) according to eqn (6) and (8) were superimposed on one graph (Fig. 5B) and compared with the experimentally measured dependence of CBF on Ca\(^{2+}\) concentration in *Paramecium* cells. Previous studies have already demonstrated how phosphorylation of dynein subunits by cAMP- and cGMP-dependent enzymes translates into cilia movement.\(^{67,68}\) Here we compare the nucleotide concentrations in comparison with CBF, presuming that the degree of cilia movement is proportional to the nucleotide concentration, *via* the phosphorylation of dynein protein by protein kinases.

The superimposition of the sum of the cAMP and cGMP concentration profiles predicted by the model and the CBF as a function of Ca\(^{2+}\) reveals a striking overlay of the two pieces of data. The only difference is the shift along the Ca\(^{2+}\) concentration. This difference can be explained by the fact that the Mg\(^{2+}\) concentrations used in the sets of experiments were significantly different. It has been shown before that Mg\(^{2+}\) ions can competitively bind to the same Ca\(^{2+}\) binding sites of CaM protein and make it require higher Ca\(^{2+}\) concentrations to achieve the same effects. With the amendment to Mg\(^{2+}\) concentration, the sum of the nucleotides closely reproduces the CBF dependence on intracilia Ca\(^{2+}\) concentration. This observation, emerging from the combination of the nucleotide model predictions with experimental data, provides an alternative to the previously discussed roles of cAMP and cGMP in cilia beat. First, the model clarifies how and why the alterations of Ca\(^{2+}\) concentrations can both increase or decrease CBF at specific Ca\(^{2+}\) concentration ranges. Second, it is reasonable to suggest that cAMP and cGMP operate in combination, rather than having unique and separate roles in CBF.

**Discussion**

This study elucidates some fundamental properties of the cAMP and cGMP regulatory systems using a structural systems biology approach. Our model allows a detailed analysis of the AC, GC and PDE isofoms specific nucleotide distribution as a function of intracellular Ca\(^{2+}\) concentration and G-protein subunits. This analysis in turn allows us to distinguish the diverse modes of cAMP concentration regulation emerging from combinations of AC and PDE isoforms with unique structural properties (Fig. 2 and 3). The possibility of inhibition, bell-shaped, reverse bell-shaped and sigmoidal saturation of cAMP as a function of intracellular Ca\(^{2+}\) concentration suggests a potential explanation for mechanisms of intracellular selective cAMP-dependent signalling. In a previous work, we demonstrated that conformation-specific target activation is a mechanism for selective activation of multiple targets by multisite proteins.\(^{53}\) The non-bound, intermediate and fully saturated multisite protein conformations are mostly present at different ranges of Ca\(^{2+}\) concentration. In the case of nucleotide signalling, the predicted and observed concentration profiles as a function of Ca\(^{2+}\) and G-protein subunits (Fig. 4) can be interpreted as a mechanism for selective nucleotide signalling. Given that the cAMP-dependent PKA kinase phosphorylates so many different targets, a fundamental question arises as to how it targets its phosphorylation targets selectively. Structural studies demonstrate complementary protein surfaces are responsible for docking and phosphorylation but do not explain why different proteins or phosphorylation sites would be phosphorylated separately by the same cAMP-dependent PKA kinase. PKA has two cAMP binding sites and according to our previous work it could be selectively active to its phosphorylation targets in a complex with variable numbers of cAMP molecules bound. The diversity of possible cAMP concentration profiles demonstrated by this study provides additional potential mechanisms for selective regulation.

The model predictions for simultaneous AC activation by both Ca\(^{2+}\) and G-proteins illuminate the impact of these two signals on cAMP concentration. We investigated potential scenarios of Ca\(^{2+}\) influence on the AC enzymatic activity in the presence and absence of G-protein subunits. The experimental data suggest that both Ca\(^{2+}\) and G-protein subunits may act as either inhibitors or activators of AC under different conditions (Fig. 4). Our mathematical modelling-based analysis shows that one potential possibility is that the state of AC changes when G-protein subunits bind to the molecule. Fig. 3B and 3C show that Ca\(^{2+}\) can modulate AC enzymatic activity in activatory or inhibitory fashion depending on whether G-protein subunits are bound to the AC protein. It has been proposed that multiple regulators of AC form patterns resulting in variability of AC activity.\(^{3,48}\) Here we demonstrate the underlying mechanism and effects of Ca\(^{2+}\) and G-proteins on cAMP concentration *via* the AC regulation. The resulting analysis allows us to propose a new explanation for the switching properties of G-protein subunits involved in nucleotide regulation. In particular, we show that, depending on whether the G-protein subunit is bound to AC, Ca\(^{2+}\) can either activate or inhibit AC in a concentration-dependent manner, in agreement with recent experimental results.\(^{3,4}\)

The nature of the complementary regulation of cilia beat frequency in *Paramecium* cells by cAMP and cGMP has also been clarified by our study. While it has been known for some time that both nucleotides are required for cilia beat, little has been known about the level and nature of interaction between them. Our mathematical model allowed us to link both the nucleotide alterations with recorded CBF as a function of Ca\(^{2+}\) concentration (Fig. 5). The action of Ca\(^{2+}\) is translated into cilia movement *via* the sum of cAMP and cGMP bell-shaped concentration profiles with maximum concentrations. The trick here is that physiological intracilia concentration varies mostly between the peaks of cAMP and cGMP, effectively forming the reversed bell-shaped dependence of CBF on Ca\(^{2+}\) concentration. This result resolves the seeming inconsistency between reports suggesting that increasing Ca\(^{2+}\) may
increase or decrease CBF. Thus, the underlying mechanism of cilia movement in *Paramecium* represents an interesting example of complementary roles in nucleotide-dependent regulation, and also proposes an explanation for how intracellular Ca\(^{2+}\) and nucleotide concentration alterations translate to cilia movement, hence defining the trajectory of *Paramecium* motion.

The models presented suggest how the diversity of isoforms of PDE, AC and GC within a single organism can give rise to precise regulation of cellular activity and address the paradox that alterations in Ca\(^{2+}\) concentrations can either increase or decrease outputs over similar Ca\(^{2+}\) concentration ranges depending on the cellular state. The model should be able to help interpret the extraordinary evolutionary diversity, including the absence, of PDE, AC and GC proteins, and the complementary functions of different isoforms. It may also be valuable in revealing adverse or unexpected cellular responses to drugs or other inhibitors which, because of the multiple isoforms and complex interactions of these proteins related to signal-response proteins, may be otherwise unpredictable and hard to measure experimentally.

**Experimental**

**The model for intracellular cAMP metabolism**

Fig. 1 provides a schematic outline of the network regulating cAMP concentration that is considered in our model. The cAMP production rate is proportional to the concentration of AC molecules in the active state that form complexes with adenosine triphosphate (ATP) and hydrolyse ATP to cAMP. At the same time, PDE molecules constantly bind to cAMP and degrade the phosphodiester bond, thereby converting cAMP into inactive 5’AMP. The intracellular level of cAMP is defined by the concentrations of AC and PDE isoforms in the active state and their enzymatic activities. The major regulators of AC are Ca\(^{2+}\) and signals mediated via G-protein coupled receptors. Various AC isoforms have unique characteristics in terms of their dependence on both Ca\(^{2+}\) and G-proteins. Some of them are directly inhibited by Ca\(^{2+}\), whereas other AC isoform activities are regulated by Ca\(^{2+}\) via calmodulin (CaM). The main activity regulator for cAMP hydrolysing PDE isoform I is a Ca\(^{2+}\)...CaM pair. In our model, the law of mass action has been applied to derive a model based on measurable parameters for the dependence of cAMP levels on the AC and PDE enzymes. The chemical reactions underlying cAMP production and further hydrolysis are given by:

\[
\text{ATP} + \text{AC} \stackrel{k_1}{\Rightarrow} \text{ATPAC} \stackrel{k_1}{\Rightarrow} \text{cAMP} + \text{AC} + \text{P} \\
\text{cAMP} + \text{PDE} \stackrel{k_2}{\Rightarrow} \text{cAMPPDE} \Rightarrow 5’\text{AMP} + \text{PDE}
\]

where ATP is adenosine triphosphate, AC is one isoform or a combination of isoforms of AC, ATPAC is an ATP and AC complex, PDE is one or a combination of isoforms of PDE, cAMPPDE is a cAMP and PDE complex, 5’AMP is physiologically inactive compound and P is pyrophosphate.

The reduced system of kinetic equations for the chemical reactions in eqn (1) is given by:

\[
\frac{d[\text{cAMP}]}{dt} = k_3 \cdot [\text{ATPAC}] - k_2 \cdot [\text{cAMP}] \cdot [\text{PDE}] + k_{-2} \cdot [\text{cAMPPDE}]
\]

\[
\frac{d[\text{ATPAC}]}{dt} = k_1 \cdot [\text{ATP}] \cdot [\text{AC}] - (k_{-1} + k_3) \cdot [\text{ATPAC}]
\]

\[
\frac{d[\text{cAMPPDE}]}{dt} = k_2 \cdot [\text{cAMP}] \cdot [\text{PDE}] - (k_{-2} + k_4) \cdot [\text{cAMPPDE}]
\]

The law of total mass conservation gives:

\[
[\text{PDE}] + [\text{cAMPPDE}] = [\text{PDE}^*] [\text{AC}] + [\text{ATPAC}] = [\text{AC}^*],
\]

where [PDE\(^*\)] and [AC\(^*\)] are the total numbers of active molecules of PDE and AC, respectively. Assuming that the alterations of [ATPAC] and [cAMPPDE] complex concentrations are relatively small, and applying the quasi-stationary approximation to eqn (2), we derive:

\[
\frac{d[\text{cAMP}]}{dt} = k_3 \cdot \left( \frac{[\text{ATP}] \cdot [\text{AC}^*]}{k_1 + [\text{ATP}]} \right) - k_4 \cdot [\text{cAMP}] \cdot \frac{[\text{PDE}^*]}{k_3 + [\text{cAMP}]}
\]

where

\[
K_1 = \frac{k_{-1} + k_3}{k_1}, \quad K_2 = \frac{k_{-2} + k_4}{k_2}
\]

Given that [ATP] >> K\(_1\) in real cells (the ATP concentration is usually of the order of mM, whereas K\(_1\) is of the order of uM), eqn (4) can be transformed to the following form:

\[
\frac{d[\text{cAMP}]}{dt} = k_3 \cdot [\text{AC}^*] - k_4 \cdot [\text{cAMP}] \cdot \frac{[\text{PDE}^*]}{k_3 + [\text{cAMP}]}
\]

Eqn (5) has been solved under the steady-state assumption to give:

\[
\text{cAMP} = \frac{K_3 \cdot k \cdot [\text{AC}^*]}{[\text{PDE}^*] - k \cdot [\text{AC}^*]}
\]

In order to account for the different biochemical properties of the AC and PDE isoforms, we incorporate multiple AC and PDE isoforms into eqn (6) as follows:

\[
[\text{AC}^*] = [\text{AC}0] \cdot \sum_z \eta_z \cdot f_z(\text{Ca}^{2+}, m)
\]

\[
[\text{PDE}^*] = [\text{PDE}0] \cdot \sum_z \mu_z \cdot g_z(\text{Ca}^{2+}, n)
\]

where [PDE\(^*\)] and [AC\(^*\)] are the total number of PDE and AC molecules respectively, [AC0] and [PDE0] are the total concentrations of each AC and PDE isoform respectively, and

\[
\eta_z = \frac{[\text{AC}0_z]}{[\text{AC}0]}
\]
and

\[ \mu_z = \frac{[\text{PDE0}]}{[\text{PDE0}]} \]

are the fractions of each isoform of AC and PDE. The dependence of the AC and PDE activities on Ca\(^{2+}\) is separated from the G-protein dependence, which is denoted in the above equations by \(m\) and \(n\) for AC and PDE, respectively.

Similarly, in steady-state, cGMP concentration can be represented explicitly as a function of GC and PDE isoform concentrations and activities by:

\[ [\text{cGMP}] = \frac{K_z \cdot k \cdot [\text{GC}]}{[\text{PDE}^*] - k \cdot [\text{GC}^*]} \]  \(8\)

In order to account for the different biochemical properties of the GC and PDE isoforms, we incorporate multiple GC and PDE isoforms into eqn (8) as follows:

\[
[\text{GC}^*] = [\text{GC0}] \cdot \sum_z \lambda_z \cdot f_z(\text{Ca}^{2+}, l)
\]

\[
[\text{PDE}^*] = [\text{PDE0}] \cdot \sum_z \nu_z \cdot g_z(\text{Ca}^{2+}, n)
\]  \(9\)

where \([\text{PDE}^*]\) and \([\text{G}^*]\) are the total number of PDE and GC molecules respectively, \([\text{GC0}]\) and \([\text{PDE0}]\) are the total concentrations of each GC and PDE isoform respectively, and

\[
\lambda_z = \frac{[\text{GC0}_z]}{[\text{GC0}]} \]

and

\[
\nu_z = \frac{[\text{PDE0}_z]}{[\text{PDE0}]} \]

are the fractions of each isoform of GC and PDE. The dependence of the GC and PDE activities on Ca\(^{2+}\) is separated from the G-protein dependence, which is denoted in the above equations by \(l\) and \(n\) for GC and PDE, respectively.

The model for interactions between G-protein and Ca\(^{2+}\)/CaM signalling pathways

The “state” or activity of each AC molecule has been represented in our mathematical model as follows. In order to cover all potential combinations, we assume that the molecule can be in four different states with diverse ratios of dissociation constants:

1. \(v_0 = 0.1, k_0 = 1, 2, v_1 = 100, k_1 = 1, 3, v_2 = 1, k_2 = 10, 4, v_3 = 10, k_3 = 0.1.\)  \(10\)

In the above expression \(v_0, v_1, v_2, v_3\) are the enzymatic activity rates and \(k_0, k_1, k_2, k_3\) are the Michaelis–Menten constants for AC-substrate interactions. The probabilities for an AC molecule to be in each of these four different states are given by:

\[
\begin{align*}
    p_{0,0} &= \frac{K_{m1} \cdot K_{m2}}{(K_{m1} + a) \cdot (K_{m2} + b)} \\
    p_{0,b} &= \frac{a \cdot K_{m2}}{(K_{m1} + a) \cdot (K_{m2} + b)} \\
    p_{0,b} &= \frac{K_{m1} \cdot b}{(K_{m1} + a) \cdot (K_{m2} + b)} \\
    p_{a,b} &= \frac{a \cdot b}{(K_{m1} + a) \cdot (K_{m1} + a)}
\end{align*}
\]  \(11\)

where \(a\) and \(b\) are the concentrations of the G-protein subunits and Ca\(^{2+}\)-CaM complexes, respectively. \(K_{m1}\) and \(K_{m2}\) are the Michaelis–Menten constants for AC interactions with G-protein subunits and Ca\(^{2+}\)-CaM complexes, respectively. \(P_{0,0}, P_{a,0}, P_{0,b,0}, P_{a,b,0}\) are the probabilities for AC to be free, in a complex with a G-protein subunit, bound to a Ca\(^{2+}\)-CaM complex, or bound to both a G-protein subunit and a Ca\(^{2+}\)-CaM complex, respectively.

The rate of cAMP production by AC in each of the different states is given by:

\[
\begin{align*}
    \mu_0 &= \frac{v_0 \cdot p_0 \cdot S}{k_0 + S} \\
    \mu_1 &= \frac{v_1 \cdot p_1 \cdot S}{k_1 + S} \\
    \mu_2 &= \frac{v_2 \cdot p_2 \cdot S}{k_2 + S} \\
    \mu_3 &= \frac{v_3 \cdot p_3 \cdot S}{k_3 + S}
\end{align*}
\]  \(12\)

where \(S\) is substrate (ATP), \(p_0, p_1, p_2, p_3\) are the enzymatic activity rates, \(k_0, k_1, k_2, k_3\) are the Michaelis–Menten constants for AC-substrate interactions, and \(p_{0,0}, p_{1,0}, p_{2,0}, p_{3,0}\) are the probabilities in eqn (12).

The full activity of the molecule is given by:

\[
M = \mu_0 + \mu_1 + \mu_2 + \mu_3
\]  \(13\)

where \(\mu_0, \mu_1, \mu_2\) and \(\mu_3\) are the activities of individual AC conformations in the states defined by eqn (12).

Abbreviations

cAMP cyclic 3',5' adenosine monophosphate
cGMP cyclic 3',5' guanosine monophosphate
GC guanylate cyclase
PKA cAMP-dependent kinase
CBF cilia beat frequency
AC adenylate cyclase
PDE phosphodiesterase
ATP adenosine triphosphate

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References
