Computational modelling suggests dynamic interactions between Ca\(^{2+}\), IP\(_3\) and G protein-coupled modules are key to robust Dictyostelium aggregation

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Under conditions of starvation, Dictyostelium cells begin a programme of development during which they aggregate to form a multicellular structure by chemotaxis, guided by propagating waves of cyclic AMP that are relayed robustly from cell to cell. In this paper, we develop and analyse a new model for the intracellular and extracellular cAMP dependent processes that regulate Dictyostelium migration. The model allows, for the first time, a quantitative analysis of the dynamic interactions between calcium, IP\(_3\) and G protein-dependent modules that are shown to be key to the generation of robust cAMP oscillations in Dictyostelium cells. The model provides a mechanistic explanation for the transient increase in cytosolic free Ca\(^{2+}\) concentration seen in recent experiments with the application of the calmodulin inhibitor calmidazolium (R24571) to Dictyostelium cells, and also allows elucidation of the effects of varying both the conductivity of stretch-activated channels and the concentration of external phosphodiesterase on the oscillatory regime of an individual cell. A rigorous analysis of the robustness of the new model shows that interactions between the different modules significantly reduce the sensitivity of the resulting cAMP oscillations to variations in the kinetics of different Dictyostelium cells, an essential requirement for the generation of the spatially and temporally synchronised chemoattractant cAMP waves that guide Dictyostelium aggregation.

Introduction

The social ameba Dictyostelium discoideum is widely recognised as an important biological model for the study of cell motility\(^1,2\) and human disease,\(^3,4\) as many of the core molecular signalling pathways governing Dictyostelium chemotaxis and migration appear to be conserved in higher organisms, and impaired chemotaxis is associated with a range of diseases including asthma, arthritis, atherosclerosis and a number of cancers.\(^5,6\) The diverse modes of Dictyostelium behaviour range from single cell states to the formation of functionally different multicellular structures such as moulds, slugs and fruiting bodies.\(^9\)

Previous computational studies have provided significant insight into the molecular mechanisms underlying Dictyostelium chemotaxis.\(^10\) One of the first models developed was based on a set of rules for cAMP metabolism, release and cAMP diffusion-based cell migration.\(^11,12\) The rule-based modelling of Dictyostelium movement was further developed into computer models of Dictyostelium aggregation in refs. 13–15. Other early models, which were developed before the role of G proteins was discovered, focussed on oscillations in ATP and cAMP concentrations\(^16,17\) or on interactions between Ca\(^{2+}\) and camp.\(^18\) A third set of models employed simplified reaction-diffusion equations.\(^19,20,21\) A detailed explanation of how cAMP oscillations can arise in Dictyostelium based on a network involving G protein-coupled receptors was developed more recently in ref. 22 and 23.

While the above computational studies have significantly advanced our understanding of various aspects of Dictyostelium behaviour, there are a range of questions that still require further elucidation. It has been shown experimentally that the levels of intracellular Ca\(^{2+}\) and cAMP in Dictyostelium are tightly interconnected\(^24–27\) and a number of experiments involving the application of calmidazolium (R24571), a calmodulin (CaM) inhibitor, to Dictyostelium have demonstrated a dramatic impact on the light scattering oscillations\(^26,28\) which are one of the major characteristics of aggregation.\(^29\) Ca\(^{2+}\) has also been shown to be directly involved in cell migration via the stretch-activated Ca\(^{2+}\) channels (SACs).\(^1\)

Previously developed models for Dictyostelium signalling and aggregation do not allow these phenomena to be investigated, however, as they all omit one or more of the networks involved. To address this issue, we have employed a modular approach to develop a new model for cAMP oscillations in Dictyostelium that explicitly incorporates networks involving intracellular calcium (Ca\(^{2+}\)), inositol 1,4,5-triphosphate (IP\(_3\))
and the G protein coupled receptor cAR1. The proposed model provides a new understanding of how Ca\(^{2+}\) alterations are translated into cAMP oscillations and of the observed effects on light scattering oscillations of CaM inhibitors. We also use our model to study the effects of SACs conductivity and external phosphodiesterase (PDE) concentration on the oscillatory regime of an individual cell. Finally, we show that our model provides a new example of what has become a key theme in recent Systems Biology research—apparent structural redundancy in the proposed model (since cAMP oscillations can be generated in silico using just the G protein-coupled module on its own,\(^{22,23}\)) appears to have been deliberately engineered by nature to ensure greater levels of robustness to uncertainty in the elements of the overall system. In particular, inclusion of interactions between the different modules in the proposed model is shown to result in significantly improved robustness to variations in the kinetics of the extracellular cAMP feedback loop, a critical requirement in the very early stages of aggregation when the levels of extracellular cAMP are likely to be very low.

Results

A new model for cAMP oscillations in Dictyostelium exhibits spontaneous oscillations matching experimentally verified results and is entrained by surrounding cells

A new model for cAMP oscillations in Dictyostelium has been developed that explicitly incorporates multiple networks of proteins governing the directed migration of Dictyostelium cells during the transition from single cell to multicellular organism.\(^{30,31}\) In particular, the molecular circuit regulating Dictyostelium chemotaxis along cAMP gradients has been modelled as three interconnected modules involving intracellular calcium (Ca\(^{2+}\)), inositol 1,4,5-triphosphate (IP\(_3\)) and the G protein-coupled receptor cAR1,\(^{24,26,32,35}\) as shown in Fig. 1. When external cAMP binds to cAR1, the G-protein cascade activates intracellular adenylyl cyclase (ACA).\(^{36}\) Transiently activated cAR1 can also lead to the activation of adenylyl cyclases via the MAP kinase (ERK2). Intracellular cAMP is produced by ACA and degraded by intracellular phosphodiesterase (RegA). ERK2 is inhibited by cAMP-dependent protein kinase A (PKA).\(^{22,37}\) Inactivated ERK2 loses its ability to phosphorylate RegA, in turn boosting the level of RegA activity. cAMP release creates an extracellular feedback loop for the cell, and also provides a source of additional cAMP signals for other cells in the vicinity. In addition to diffusion, the external cAMP feedback loop is also diminished by the external PDE.

Based on the experimental results reported in ref. 24–27, we have considered cAMP production and degradation to be dependent on the level of intracellular Ca\(^{2+}\), which in turn is determined by a balance of fluxes into the cytoplasm from extracellular medium and endoplasmic reticulum (ER) and fluxes mostly generated by the membrane pumps compensating Ca\(^{2+}\) leaks across the plasma and ER membranes. IP\(_3\) is synthesised by Ca\(^{2+}\)-dependent and G protein-dependent phospholipase C (PLC). IP\(_3\) concentration also has a major impact on the Ca\(^{2+}\)-dependent inositol triphosphate receptor (IP\(_3\)R) located on the ER membrane.

The network responsible for Ca\(^{2+}\) oscillations in Dictyostelium constitutes two feedback mechanisms. The first feedback loop is based on the movement of Ca\(^{2+}\) ions between the ER and cytoplasm. Intracellular Ca\(^{2+}\) is sequestered into the ER by Sarco/Endoplasmic Reticulum Ca\(^{2+}\)-ATPase (SERCA). It is released from the ER back into the cytoplasm via the IP\(_3\)R (which has both a Ca\(^{2+}\)- and IP\(_3\) dependence\(^{38}\)), as well as by a direct leak through the ER membrane. Another feedback mechanism involves the Ca\(^{2+}\) release from the intracellular compartment into the extracellular space by a plasma membrane Ca\(^{2+}\) pump (PMCA). PMCA compensates for the constant Ca\(^{2+}\) leak throughout the surface of the plasma membrane into the intracellular space. Other routes of Ca\(^{2+}\) into the cytoplasm include a range of Ca\(^{2+}\) channels, including the stretch-activated Ca\(^{2+}\) channels which play a particularly important role in Dictyostelium chemotaxis\(^{39}\) via the directed migration mechanism.\(^{40,41}\) IP\(_3\) is produced by the only PLC isoform found in Dictyostelium which is structurally similar to the mammalian PLC\(_8\) isoform and regulated by both Ca\(^{2+}\) and G-protein pathways.\(^{32,44}\) IP\(_3\) is further converted into IP\(_4\) by IP\(_3\) kinase (IP\(_3\)K).

The intracellular Ca\(^{2+}\) regulation network described above is directly connected in our model with the G protein coupled pathways included in some previously published models.\(^{10,22,23}\) Intracellular cAMP in Dictyostelium can be produced by ACA, ACB and ACG adenylate cyclase isoforms. ACA is related to the mammalian and Drosophila G protein-coupled adenylcyclase isozymes, reported to be expressed during aggregation.\(^{45}\) This isoform is responsible for the synthesis of cAMP in early development and plays a role in cell-cell signalling. The other two isoforms, ACB and ACG, are involved in terminal differentiation and spore germination, respectively. Given the focus of this study on elucidating the molecular mechanisms controlling cellular aggregation, our model includes the ACA isoform only. Since the (direct or indirect) dependence of ACA on the G-protein and Ca\(^{2+}\) pathways in Dictyostelium has not been as completely characterized experimentally as in the case of some mammalian adenylcyclase isozymes, we computationally tested a number of different mechanisms for the ACA activation and chose the one that led to stable robust oscillations displaying the correct relationship between the Ca\(^{2+}\) and cAMP waveforms. In the model, the cAMP release from the cell incorporates two Ca\(^{2+}\) and cAMP-dependent terms. The released cAMP diffuses in all directions and influences both the cell that pumped it, and any surrounding cells present in the vicinity. It is also degraded by extracellular PDE which is released with cAMP and by a PDE isoform expressed on the surface of Dictyostelium. The cAMP-cAR1 interactions lead to ACA, ERK2 and PLC activation via the G-protein pathways. ERK2 when activated through the cAR1 G protein-coupled pathway can inhibit RegA activity and at the same time stimulate ACA. PKA phosphorylates and thereby inhibits ERK2 and cAR1 in a G-protein manner.
oscillations in the modelled variables all show good agreement with experimentally observed results. The amplitude of predicted Ca\(^{2+}\) oscillations is in the physiological range of several hundreds of nM. The period of predicted oscillations is of the order of 6–7 min which also corresponds closely to the experimentally observed values. The relationship between the phases of the Ca\(^{2+}\) and cAMP oscillations is defined by the properties of ACA. It has been shown previously that Ca\(^{2+}\)-CaM-dependent protein regulation is dependent on the number of Ca\(^{2+}\) ions bound to CaM and the complex interplay between the Ca\(^{2+}\) and G-protein pathways thus leads to a diversity of cAMP activation patterns. We computationally elucidated three possibilities for hypothetical direct or indirect ACA activation by CaM in complex with zero, one and two Ca\(^{2+}\) ions, leading to three potential scenarios for the interplay between Ca\(^{2+}\) and G-protein signals. In one case (Fig. 3A) Ca\(^{2+}\) inhibits the ACA activity and any activation by G-protein coupled receptors is only possible when there is no Ca\(^{2+}\) signal. If ACA is regulated by Ca\(^{2+}\)-CaM complexes with one or two Ca\(^{2+}\) ions bound to CaM however, (Fig. 3B and C, respectively), the dependence on Ca\(^{2+}\) becomes bell shaped with an “optimal” Ca\(^{2+}\) concentration. In these cases, any signal from the G-protein pathways further amplifies the ACA activity. The mechanism for ACA activation shown in Fig. 3C has been used in our model, since it corresponds to the most likely situation corresponding to the correct phase relationship between the cAMP and Ca\(^{2+}\) oscillations.

We note three distinct situations on the graph shown in Fig. 3C, labelled as cases 1, 2 and 3. When Ca\(^{2+}\) is very low (case 1) or very high (case 3) the system largely depends on the G-protein pathway only, in a sigmoid dependent manner. In the intermediate Ca\(^{2+}\) concentrations, the G-protein signalling is magnified, the sigmoid type-dependence on G-pathway signals is still present, but there is a strong signal even in response to a low G-protein pathway incoming dynamics. The ACA dependence on Ca\(^{2+}\), as can be seen from the ACA versus Ca\(^{2+}\) projection without considering the G-protein signals, represents a bell shaped curve, which leads to the full ACA activation in the intermediate intracellular Ca\(^{2+}\) concentration range and inhibition at high and low Ca\(^{2+}\) levels. An in-depth analysis of the Ca\(^{2+}\) and G-protein cross-talk mechanisms is presented in ref. 48. The Erk2
dependence is not shown on this graph, as it is incorporated in the model by utilising the law of mass action reaction as described in the Methods section. The ACA regulation is undoubtedly one of the key mechanisms participating in the interplay of the three modules considered in this manuscript. The computational results shown in Fig. 3C provide an example of how experimental data (summarized in Fig. 1) may be augmented by Systems Biology analysis to provide new interpretations which are not obvious from a straightforward analysis of the biological circuit diagram.

We next investigated the ability of the proposed model to replicate the experimentally observed levels of entrainment between separate oscillating Dictyostelium cells. Specifically, we were interested in the relationship between the oscillations of intracellular cAMP in one cell and the alterations of extracellular cAMP concentration caused by multiple surrounding cells. Fig. 4 shows the effects in our model of synchronization between the extracellular oscillations mediated by a number of cells and the oscillations initiated independently by a single Dictyostelium cell. It is clear from Fig. 4A that the extracellular cAMP alterations, even when applied at different time points (data not shown) with respect to the internal oscillations, have a modulatory effect on a cell, and quickly achieve absolute synchronization. In other words, external oscillations of higher amplitude can always entrain the oscillations in an individual cell regardless of the initial differences in phase. As expected, reducing the amplitude of cAMP oscillations in the extracellular medium results in a corresponding reduction in the level of entrainment (Fig. 4B), while the ability of external cAMP oscillations to change the frequency of the oscillations within a single cell is clearly shown in Fig. 4C and D.

The proposed model provides a mechanistic explanation of the experimentally observed effects of calmidazolium on cytosolic free Ca²⁺ concentration

It has been shown experimentally that the wavelike pulses of cAMP released in suspensions of Dictyostelium cells are coupled with oscillations of light scattering. The oscillations of light scattering, in turn, have been reported to depend on Ca²⁺ mechanisms, including the release and re-uptake of Ca²⁺ from the intracellular compartment into the extracellular space, and feedback loops between the intracellular compartment and intracellular Ca²⁺ stores. In order to investigate the above issues quantitatively, we included the networks regulating the Ca²⁺ flows through the extracellular space, intracellular compartment and ER in the proposed mathematical model. We also incorporated a simple dependence of the cell shape alterations on the intracellular Ca²⁺ concentration. We then used the model to provide a mechanistic interpretation of the results of experiments with the application of the CaM inhibitor, calmidazolium (R24571), which causes a transient surge in cytosolic free Ca²⁺ concentration.

It has been shown that one of the targets for the R24571 action is the CaM that regulates PMCA. PMCA pumps Ca²⁺ out of the cytoplasm and compensates for the constant Ca²⁺ leak through the plasma membrane. In order to evaluate the action of calmidazolium, we incorporated two potential time courses of the compound action on the CaM activity (Fig. 5A). In one case, CaM activity was inhibited and subsequently kept at that level. In this case, the model predicted the disappearance of cellular oscillations (Scenario 1 in Fig. 5A). In another scenario, the effect of R24571 on CaM activity was modelled as a temporary inhibition, with a subsequent complete recovery in the level of CaM activity. Under this assumption, the application of calmidazolium caused a surge of Ca²⁺, after which the oscillations returned to their previous levels (Scenario 2 in Fig. 5A). At the same time the temporal Ca²⁺ surge is observed in both scenarios. Since this second response is consistent with the R24571 application experiments, the results of our computational analysis lead us suggest that CaM is
inhibited temporally (Fig. 5B) and that the Ca\(^{2+}\)-CaM-dependent regulation of PMCA appears to be one of the main targets for calmidazolium action in the light scattering experiments.\(^{26,57}\) More generally, the precise nature of the relationship between cellular oscillations of cAMP, Ca\(^{2+}\) and other intracellular enzymatic activities in an individual Dictyostelium cell and the oscillations of optical density observed in a number of cells remains somewhat unclear. The changes in optical density may be due to either an increased number of cells or to cell shape alterations. Both these factors depend on the lamellipodia formation and disassembly turnover as cells migrate during aggregation. Amongst many other factors, intracellular Ca\(^{2+}\) has been shown to modulate both the speed and direction of Dictyostelium motility.\(^{1}\) In an attempt to shed light on the above issues, we employed our model to develop the following testable hypothesis. We sought to investigate whether or not the Ca\(^{2+}\) surges caused by the application of calmidazolium added at different time points of the oscillatory cycle would result in similar phase delays in optical density oscillations to those observed experimentally in ref. 26. Fig. 5C shows the various perturbations caused to the oscillations of intracellular Ca\(^{2+}\) by application of R24571 at different time points of the oscillatory cycle. The corresponding normalized phase shifts, as a function of the time point of the R24571 application, are shown on Fig. 5D. The strong agreement between these values and the experimental data points in the phase diagram depicting the magnitude of the phase shifts induced by R24571 reported in ref. 26 reinforces the notion that strong interactions between Ca\(^{2+}\) and cAMP oscillations, as specified in our model, are crucial to the regulation of Dictyostelium migration during aggregation.

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**Fig. 3** ACA activity dependence on the Ca\(^{2+}\) and G-protein mediated signals. The dependence of ACA activity (as a percentage of full activation) is shown according to the following assumptions: (A) ACA is regulated by CaM in the apo-state, (B) ACA depends on CaM with one Ca\(^{2+}\) ion bound and (C) ACA is governed by CaM in complex with two bound ions of Ca\(^{2+}\). The last possibility has been employed in the present model as being the most realistic case in the light of recent structural studies. The three situations discussed in the text are denoted as cases 1, 2 and 3 in the Figure.

**Fig. 4** Entrainment of an individual Dictyostelium cell by surrounding cells in our model. (A) The extracellular cAMP oscillations generated by aggregating Dictyostelium cells cause an individual cell to oscillate with the same phase even when the extracellular oscillations are applied at a random time point in the oscillatory cycle. (B) Extracellular cAMP oscillations of smaller amplitude have a lesser effect on an individual cell. (C) and (D) Extracellular cAMP oscillations of variable frequency entrain oscillations of a single Dictyostelium cell.
The proposed model highlights the role of stretch-activated Ca\(^{2+}\) channels and extracellular PDE in Dictyostelium aggregation

The two key factors characterizing cell motion during Dictyostelium aggregation are the direction and velocity of migration.\(^{39}\) It has been shown that in the absence of an extracellular cAMP gradient, Dictyostelium cells undergo a random motion, while they will move up the cAMP gradient when it exists. We employed the proposed model in order to seek new insights into the potential relationship between the cellular oscillations in Dictyostelium and migration in chemotaxis by varying two critical extracellular parameters which are very likely to influence cellular oscillations.

First we varied the level of extracellular PDE, because it is released together with cAMP during the periodic oscillations and has a strong influence on the extracellular feedback loop. Recent experimental results have also shown that a PDE isoform is expressed on the outer surface of the Dictyostelium cell membrane.\(^{58}\) Fig. 6A reveals the emergence followed by the disappearance of ACA oscillations predicted by our model in response to a gradual increase and decrease in the extracellular PDE concentration (Fig. 6B). The observed pattern of oscillations closely matches the experimentally measured periodic oscillations in Dictyostelium that have been shown to spontaneously appear during development.\(^{22}\)

A second factor that was investigated using the proposed model was the overall conductivity of SACs, which have been shown to be one of the major players in governing cell motion. Fig. 6C shows model predictions for the impact of two crucial parameters for cell migration on the cellular oscillations in Dictyostelium. ACA oscillations (A) and (C) are shown as a function of the extracellular PDE activity (B) and overall conductance of stretch-activated Ca\(^{2+}\) channels (D). The lines showing the extracellular PDE activity (B) and overall conductance of stretch-activated Ca\(^{2+}\) channels (D) represent the hypothetical PDE activity and SACs conductivity levels that can be controlled by the intracellular PDE and extracellular SACs inhibitors, respectively. Variations of both stretch-activated Ca\(^{2+}\) channels (D) and extracellular PDE (B) predict that oscillations can exist only within the physiological domain shown.
migration. Since during migration all cells undergo membrane protrusion and retraction cycles, stretch-activated Ca\(^{2+}\) channels inevitably play a role in chemotaxis via the intracellular Ca\(^{2+}\) circuit. Fig. 6C reveals the emergence followed by the disappearance of ACA oscillations predicted by our model in response to a linear elevation of the number of SACs in the open state (Fig. 6D).

The above model predictions that the existence of stable oscillations depends on two “migration”-specific parameters, together with the corresponding experimental results now available in the literature, lead us to propose a central role for the intracellular Ca\(^{2+}\) circuit in ensuring chemotaxis-dependent aggregation in Dictyostelium. According to previous studies, intracellular Ca\(^{2+}\) oscillations are required for the turnover of focal adhesions (FAs) in migrating cells.\(^{40,59}\) It has also been reported that the Ca\(^{2+}\) concentration is lower in the leading lamellipodia\(^{60,61}\) of migrating cells. Taken together, these results lead us to suggest that Ca\(^{2+}\) plays a role not only in the locomotion mechanism, but also in defining the direction of migration. The Ca\(^{2+}\) network in Dictyostelium appears to be complementary to the G protein-coupled cAMP network, and their oscillations observed in aggregation appear to be the core cellular engine allowing movement in the direction defined by extracellular conditions such as cAMP gradient and the presence of nutrition. While the alterations in extracellular cAMP concentrations provide the external cues which guide the direction of migration, our model underlines the important role of Ca\(^{2+}\) in actually achieving directionality of motion. By protruding more lamella on one side of the cell and retracting it on the other, the intracellular machinery thus carries out the routine job of membrane protrusion according to the extracellular stimuli, focal adhesion assembly, tail retraction and focal adhesion disassembly in an intracellular Ca\(^{2+}\) and cAMP oscillation-dependent manner.

Interactions between calcium, IP\(_3\) and G protein-dependent networks significantly improve the robustness of cAMP oscillations

At the very beginning of the aggregation process, some spatially isolated Dictyostelium cells are able to independently generate stable oscillations in their levels of intracellular cAMP, even in the absence of strong extracellular cAMP waves. Previous computational studies have suggested that the release of cAMP by an individual cell with subsequent binding of some portion of extracellular cAMP to the cAR1 receptor constitutes the key feedback mechanism required for maintaining stable cAMP oscillations during this phase of the aggregation process.\(^{23,62}\) Although initial robustness analysis studies of the models proposed for this feedback loop showed it to be surprisingly fragile, with rather small variations in the system’s kinetics destabilising the resulting cAMP oscillations,\(^{23,62}\) subsequent analyses taking into account the effects of stochastic noise and extracellular synchronisation showed improved levels of robustness.\(^{23,62}\) In this section, we show that dynamic interactions between (apparently redundant) Ca\(^{2+}\), IP\(_3\) and G protein-dependent modules included in our model have the effect of further significantly improving the robustness of the overall system, allowing the maintenance of stable cAMP oscillations for an individual cell even in the absence of strong extracellular cAMP waves.

To quantify the effect of these interactions on the robustness of the generated cAMP oscillations, we compared the robustness properties of the proposed model with those of a

![Fig. 7 Robustness analysis of the period of the internal cAMP oscillations with respect to perturbations in model parameters. The figure shows the period distribution of the model including the G protein-coupled module only (first row) and the proposed model (second row) for one cell with 20%, 40%, and 60% perturbations in the four kinetic parameters determining the dynamics of the extracellular cAMP positive feedback loop. The bar on the extreme right represents the percentage of cells that are not oscillating. As the size of the perturbation increases, the proportion of non-oscillating cells increases from 0% to 32% for the model incorporating the G protein-coupled module only, whereas in the proposed model all cells display stable oscillations for all perturbations considered. The standard deviations of the periods are more than an order of magnitude smaller for the proposed model, even though the standard deviation is calculated only for the cells displaying stable oscillations in each case. Each plot is the result of 1000 simulations for different random samples of the model parameters using a uniform distribution about the nominal values.](618.png)
previous model for cAMP oscillations which included only the G protein-dependent module.\textsuperscript{23,62} We generated 1000 random samples of the 4 kinetic constants governing the dynamics of the extracellular cAMP feedback loop in each model from uniform distributions around the nominal values, for several different uncertainty ranges (see Methods). The period distributions of each model for three levels of uncertainty in the kinetic parameters, i.e., 20\%, 40\%, and 60\%, are shown in Fig. 7. In the figures, the bar on the extreme right denotes the total number of cases where stable oscillations were not observed and the trajectories converged to some steady state value. Note that the proportion of non-oscillatory trajectories is 23\% and 32\% for the G protein-coupled receptor model with a 40\% and 60\% level of uncertainty, respectively, while for the proposed model all cells continued to display stable oscillations even for the highest level of parameter uncertainty. Also, the standard deviation of the periods is more than an order of magnitude smaller for the proposed model, even though the standard deviation is calculated only for the cells displaying stable oscillations in each case.

Similar improvements in the robustness of the amplitude distributions are shown in Fig. 8—note that although the standard deviations of the amplitudes appear to be similar for both models, in reality there is a much greater level of variation in the amplitudes of the G protein-coupled module. This is because the standard deviations are calculated only for those cells displaying stable oscillations in each case, and it is precisely those cells which are no longer generating stable oscillations that are not included in the standard deviation calculation. The proportion of non-oscillatory trajectories increases from 0\% to 32\% for the model incorporating the G protein-coupled module only, whereas in the proposed model all cells display stable oscillations for all perturbations considered. Note that although the standard deviations of the amplitudes appear to be similar for both models, this does not accurately reflect the greater level of variation in the dynamics of the G protein-coupled module, as the standard deviations are calculated only for those cells displaying stable oscillations in each case. Each plot is the result of 1000 simulations for different random samples of the model parameters using a uniform distribution about the nominal values.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|}
\hline
& Laub Loomis model & Proposed model \\
\hline
\% perturbation & & \\
20\% & Mean: 11.12 & Mean: 0.62 \\
& STD: 0.50 & STD: 0.11 \\
40\% & Mean: 11.60 & Mean: 0.64 \\
& STD: 0.93 & STD: 0.12 \\
& 19.8\% no oscillation & & \\
60\% & Mean: 12.12 & Mean: 0.62 \\
& STD: 1.42 & STD: 0.13 \\
& 35.8\% no oscillation & & \\
80\% & Mean: 12.08 & Mean: 0.62 \\
& STD: 1.38 & STD: 0.13 \\
& 51.2\% no oscillation & & \\
\hline
\end{tabular}
\caption{Robustness analysis comparison}
\end{table}
cAMP oscillations which have the largest deviation from the nominal amplitude values. A summary of the robustness analysis comparisons with 20%, 40%, 60% (and 80%) perturbations is given in Table 1.

Discussion

This study presents a new computational model which balances a number of pieces of recent experimental data in an attempt to link molecular interactions at the cellular level with Dictyostelium behaviour during aggregation via chemotaxis. The mathematical model developed for this purpose incorporates the three major modules which are known to govern the relevant cellular events, and allows a direct quantitative analysis of how intracellular Ca2+ interacts with cAMP oscillations in Dictyostelium. In previous studies these modules have been modelled and analysed separately, however, recent experimental evidence suggests strong interactions between them, highlighting the need for an integrated model. The key coupling points between the networks include ACA and RegA that synthesize and hydrolyze intracellular cAMP, respectively. Another crucial piece of experimental evidence comes from the application of CaM blockers leading to the inhibition of light scattering oscillations during aggregation. Our model, which incorporates both cAMP and Ca2+ alterations, predicts intracellular and extracellular cAMP oscillations which are similar to those generated by previous models for cAMP regulation in Dictyostelium. However, unlike these models, it allows us to directly investigate how Ca2+ perturbations lead to cAMP-dependent effects. The proposed model also suggests a potential mechanism for Ca2+-dependent Dictyostelium migration, modulated by external cAMP waves during aggregation.

Under unfavourable environmental conditions, Dictyostelium cells start releasing extracellular cAMP and an area containing several cells becomes the centre of attraction to other cells. However, no aggregation would be possible if cells could not migrate up the cAMP gradient. Our model suggests that the intracellular Ca2+ network plays a key role in generating the internal motor for cell locomotion. Indeed, given that Ca2+ concentration alterations, observed in migrating cells, can be a key factor in FA disassembly, as well as the finding that lamellae formation is inversely proportional to cAMP concentration, it seems reasonable to propose the direct involvement of both the Ca2+ and cAMP networks in migration via the lamella protrusion and retraction mechanisms.

Although the extracellular cAMP feedback loop via cAR1 clearly plays an important role in generating cAMP oscillations, it is also somewhat surprising that such a loop can be maintained robustly solely by the self-release of cAMP by the same cell, as the amount of cAMP released in this manner is likely to be very small, and the diffusion and degradation mechanisms are likely to further diminish even that amount. In the light of our analysis, it seems reasonable to propose that in certain situations, a complementary oscillator functioning via the Ca2+-regulating proteins may initiate intracellular Ca2+ alterations, and in turn, cAMP oscillations, when necessary. However, the Ca2+ network is itself highly complex and is regulated by multiple mechanisms, including extracellular cAMP via the cAR1 receptor and the SACs located on the extracellular membrane. Our results suggest that all of these factors are crucial for the generation of oscillations. The necessity of both intracellular Ca2+ and cAMP oscillations for Dictyostelium migration during aggregation is supported by the model predictions for the application of CaM blocker. The inhibition by the CaM blocker of light scattering oscillations, modelled based on the assumption that filopodia and lamellipoda are being assembled and disassembled in a Ca2+ dependent manner, lends further supports to the necessity of Ca2+ oscillations in Dictyostelium aggregation. Indeed, a range of experimental reports from other types of cells confirm the role of Ca2+ as an intracellular engine for cellular locomotion. Ca2+ oscillations were observed in migrating neutrophils (See Fig. 1 of ref. 63) and were absent when cells were stationary. Chemotaxis studies in neutrophils revealed that there is less Ca2+ in the front than in the rear of migrating cells, while a detailed analysis of Ca2+ involvement in the direction and the speed of migration via the alteration of the cellular shape and FA adhesion dynamics as a function of various oscillation regimes is shown in ref. 40.

Conclusions

It has recently been argued that an important property that engineering and biological systems appear to have in common is the need for rather elaborate functional design strategies in order to generate robustly operational systems—while minimal designs are sufficient to generate nominal functionality, they often fail to provide crucial aspects of robustness and performance necessary for competitive survival in challenging environments. Examples of the use of complex designs to increase system robustness are abundant in the technological sciences, while examples from the natural sciences are still emerging. In the case of the system considered here, it appears that synchronisation effects between the different intracellular modules have a beneficial effect on overall systems robustness, a result which is consistent with our previous study of synchronisation effects between different Dictyostelium cells via the diffusion of external cAMP. The results of our analysis clearly show the improved robustness which is achieved in Dictyostelium cells by employing three coupled functional modules to generate cAMP oscillations, instead of a single network based on the G protein-coupled receptor pathway, and thus provide another concrete biological example of this type of “elaborate robust” design strategy.

Methods

The model equations

This model is designed to simulate and analyze the cAMP, Ca2+ and IP3 oscillations in an individual Dictyostelium cell when it is interacting with surrounding cells via extracellular cAMP. As shown in Fig. 1, the model has a modular structure, incorporating Ca2+, IP3 and G protein-coupled modules and the interactions between them. The time courses of the model
components are presented as a system of ordinary differential equations:

\[
V_i - V_{ER} \frac{d[Ca^{2+}]}{dt} = (S_i \cdot (j^{\mu}_i - j^{out}_i) + S_{ER} \cdot (j^{out}_i - j^{\mu}_i)),
\]

\[
V_{ER} \frac{d[Ca^{2+}]_{ER}}{dt} = S_{ER} \cdot (j^{\mu}_i - j^{out}_i).
\]

\[
(V_i - V_{ER}) \frac{d[IP_3]}{dt} = ((r_0 + r_1) \cdot S_i \cdot N_{PLC} \cdot P_{PLC} \cdot S_i \cdot [IP_3] - r_2 \cdot [IP_3] \cdot K).
\]

\[
V_i \frac{d(cAMP)_i}{dt} = k_1 \cdot ACA - k_2 \cdot RegA \frac{(cAMP)_i}{k_{RegA} + cAMP}.
\]

\[
V_{ER} \frac{d(cAMP)_{ER}}{dt} = S_{ER} \cdot J_{AMP} \cdot S_m \cdot J_{AMP} - k_3 \cdot PDE \cdot \frac{(cAMP)_{ER}}{K_{PDE} + (cAMP)_{ER}}.
\]

\[
V_i \frac{d(ERK2)}{dt} = -n_i \cdot ERK2 \cdot Phos + n_i \cdot (ERK2_{out} - ERK2) \cdot PKA.
\]

\[
V_{ER} \frac{d(ACA)}{dt} = -l_i \cdot ACA \cdot Phos + l_i \cdot (ACA_{out} - ACA) \cdot PKA.
\]

\( (1) \)

where \( V_i \) and \( V_{ER} \) are the cytoplasmic and ER volumes, respectively. \([Ca^{2+}]_i \) (mol \( L^{-1} \)) is the free \( Ca^{2+} \) concentration in the intracellular volume \((V_i - V_{ER})\). \([Ca^{2+}]_{ER} \) is the free \( Ca^{2+} \) in the ER volume \( V_{ER} \). \( j^{\mu}_i \) and \( j^{out}_i \) (mol \( sec^{-1} \mu m^{-2} \)) are the \( Ca^{2+} \) ion flows through the cytoplasmic membrane into the cytoplasm and out into the extracellular space, respectively. \( S_i \) and \( S_{ER} \) (\( \mu m^2 \)) are the surface areas of the cytoplasmic and ER membranes. \( r_0 (sec^{-1}) \) and \( r_1 (sec^{-1}) \) are the \( IP_3 \) hydrolisis rates by PLC, that reflect G-protein and \( Ca^{2+} \)-dependent pathway activation, respectively. \( r_2 (sec^{-1}) \) is an average \( IP_3 \) conversion rate by \( IP_3 \cdot K \) into \( IP_3 \). \( N_{PLC} \) (mol \( \mu m^{-2} \)) is the number of PLCs per \( \mu m^2 \), and \( P_{PLC} \) is the probability of PLCs being in the active form. \( cAMP_i \) (mol \( L^{-1} \)) is the free cAMP concentration in the intracellular volume \((V_i - V_{ER})\). \( ACA \) is the concentration of the intracellular \( ACA \), which in turn depends on the intracellular \( Ca^{2+} \) concentration, extracellular \( cAMP \), via the\( cA1 \) and G-proteins, and on the intracellular \( cAMP \), via phosphorylation by \( cAMP \)-dependent \( PKA \). \( RegA \) is the intracellular phosphodiesterase that hydrolyses intracellular \( cAMP \). \( k_1 (sec^{-1}) \) is the \( ACA \) enzymatic activity rate, \( k_2 (sec^{-1}) \) is the enzymatic \( RegA \) activity rate, and \( k_{RegA} \) (mol) is the \( cAMP - RegA \) dissociation constant. \( cAMP_i \) is the extracellular \( cAMP \) concentration in the extracellular volume \( V_i \). \( J_{AMP} \) (mol \( sec^{-1} \mu m^{-2} \)) are the \( cAMP \) flows into the extracellular space through the surface \( S_m (\mu m^2) \) of an individual cell and through the surface \( S_m (\mu m^2) \) of multiple surrounding cells, respectively. \( PDE \) is the extracellular phosphodiesterase, expressed on the surface of \( Dictyostelium \). \( k_3 \) is the enzymatic activity of the extracellular phosphodiesterase, \( k_{PDE} \) (mol) is the \( cAMP \)-PDE dissociation constant. \( ERK2 \) is the phosphorylated state of the \( ERK2 \) protein. \( n_i \) is the phosphorylation rate of \( ERK2 \) by \( PKA \). \( m_i \) is the dephosphorylation rate of \( ERK2 \) by protein phosphatase Phos. \( l_i \) is the phosphorylation rate by \( PKA \), and \( l_i \) is the dephosphorylation rate by the protein phosphatase Phos. \( ERK \) and \( ACA \) are the total concentrations of \( ERK2 \) and \( ACA \), respectively.

In order to present the model more conveniently, we have chosen the concentration units to be in \( \mu mol L^{-1} = d \) and time in seconds. Normalization of concentrations by \( d \) allows a transformation to new variables:

\[
\frac{[Ca^{2+}]}{d} = \frac{[Ca^{2+}]_i}{d}, \frac{[ERK2]}{d} = \frac{[ERK2]}{d}, \frac{[IP_3]}{d} = \frac{[IP_3]}{d}, \frac{[cAMP]}{d} = \frac{[cAMP]}{d}, \frac{[ACA]}{d} = \frac{[ACA]}{d}.
\]

All Michaelis constants are also dimensionalized by \( d \): \( k_m = \frac{k_m}{d} \).

The system (1) in the normalized form is transformed as follows:

\[
\frac{d(\frac{ca^{2+}}{d})}{dt} = a_1 \cdot \left( \psi_i \cdot 0.5 \cdot \ln \left( \frac{\frac{ip_3}{c^{out}}}{c^{out}} \right) \right) - a_2 \cdot \left( \frac{1}{p_{PM}} \cdot \frac{ca^{2+}}{ca^{2+}} \right)

+ a_3 \cdot \left( \frac{ca^{2+}}{d} \right) \cdot \left( \frac{m_{ER} - a_4 \cdot \frac{1}{P_{ER}} \cdot (ca^{2+}) \cdot \frac{p_{ER}}{P_{ER}} \cdot \frac{ca^{2+}}{d} \right)

\]

\[
\frac{d(\frac{ca^{2+}}{d})}{dt} = \frac{V_i - V_{ER}}{V_{ER}} \cdot (-a_1 \cdot \frac{ca^{2+}}{d} + a_4 \cdot \frac{1}{P_{ER}} \cdot (ca^{2+}) \cdot \frac{p_{ER}}{P_{ER}} \cdot \frac{ca^{2+}}{d})

\]

\[
\frac{d(\frac{ip_3}{d})}{dt} = m_0 \cdot \frac{cAMP}{K_{G} + cAMP} + m_1 \cdot \left( \frac{(ca^{2+})^2}{(k_{ca^{2+}} + ca^{2+})^2} \right)

+ a_4 \cdot \left( \frac{ca^{2+}}{d} \right) \cdot \left( \frac{m_{ER} - a_4 \cdot \frac{1}{P_{ER}} \cdot (ca^{2+}) \cdot \frac{p_{ER}}{P_{ER}} \cdot \frac{ca^{2+}}{d} \right)

\]

\[
\frac{d(\frac{cAMP}{d})}{dt} = D_{ACA} \cdot \frac{aca - j_{AMP} - \frac{RegA}{k_{RegA} + cAMP}}{K_{ER} + ca^{2+}}
\]

\[
\frac{d(\frac{cAMP}{d})}{dt} = D_{AMP} \cdot j_{AMP} - \frac{PDE}{K_{PDE} + cAMP},
\]

\[
\frac{d(\frac{ERK2}{d})}{dt} = d_1 \cdot \left( \frac{ka}{c^{out}} \cdot (1 - erk2) - erk2 \right).
\]

\[
\frac{d(\frac{aca}{d})}{dt} = d_2 \cdot \left( \frac{ka}{c^{out}} \cdot (1 - aca) - aca \right).
\]

(2)

where \( a_1 \) represents the sum of currents into the membrane, \( a_2 \) is the maximum \( Ca^{2+} \) current flow through the PMCs, \( a_3 \) is the maximum \( Ca^{2+} \) current flow through \( IP_3 \)Rs, and \( a_4 \) is the maximum \( Ca^{2+} \) current flow through the SERCs. \( m_0 \) and \( m_1 \) are the maximum PLC hydrolysis rates, mediated by the G-protein and \( Ca^{2+} \) pathways, respectively. \( m_2 \) is the maximum \( IP_3 \)K hydrolysis rate, \( ap \) is the basal level of \( IP_3 \) hydrolysis, \( reg \) represents normalized activity of \( RegA \), \( j_{AMP} \) and \( j_{AMP} \) are the normalized flows of \( cAMP \) released by an individual \( Dictyostelium \) cell and surrounding cells, respectively. \( \psi_i \) is the cytoplasmic membrane potential. \( \psi_i = \frac{V_m}{R} \), where \( V_m \) is the cytoplasmatic transmembrane potential. \( aca = (1 - aca) \cdot ACA_{ac} \) is a normalized function of \( ACA \) activity, \( D_{ACA} \) is the max ACA hydrolysis rate, \( RegA \), is the max RegA hydrolysis rate, \( k_{RegA} \) is \( cAMP \) affinity to \( RegA \), and \( k_{ER} \) is modulation of \( RegA \) activity by \( Ca^{2+} \) release from ER. \( d_1 \) and
Derivation of the Ca\(^{2+}\) module equations

The first two parts of eqn (2) represent the Ca\(^{2+}\) concentration alterations in a given volume due to the flows through membranes and channels. The Nernst–Planck current density equation,\(^{69,70}\) has been employed in order to make the transition from Ca\(^{2+}\) ion flows to Ca\(^{2+}\) currents:

\[
J = \frac{I}{z \cdot F},
\]

where I is a current generated by a flow, J is the Faraday’s constant, \(z\) is the Ca\(^{2+}\) ion charge. Eqn (3) allows the representation of the intracellular and ER Ca\(^{2+}\) concentration alterations as a sum of Ca\(^{2+}\) currents:

\[
\frac{d([\text{Ca}^{2+}])}{dt} = \sum_{i} J_{i}
\]

Below we derive the terms for the currents through the PMCA, SERCA, and IP\(_{3}\)Rs.

The PMCA binds one ion per cycle and extrudes Ca\(^{2+}\) from the extracellular space (using the ATP hydrolysis energy), when it is bound to calmodulin with four Ca\(^{2+}\) ions. The term for PMCA activation when \([\text{Ca}^{2+}]\) = const (extracellular Ca\(^{2+}\) concentration is approximately 1 mM) is given by the following equations:

\[
\begin{align*}
\dot{I}_{\text{PM}}^{\text{PM}}([\text{Ca}^{2+}]) & = N_{\text{PM}}^{\text{PM}} \cdot \dot{I}_{\text{PM}}^{\text{PM}} \cdot \dot{p}_{\text{PM}}^{\text{PM}}([\text{Ca}^{2+}]) \cdot \dot{p}_{\text{PM}}^{\text{PM'}}([\text{Ca}^{2+}]), \\
\dot{p}_{\text{PM}}^{\text{PM}}([\text{Ca}^{2+}]) & = \frac{\text{CaM}([\text{Ca}^{2+}])}{k_{m} + \text{CaM}([\text{Ca}^{2+}])}, \\
\dot{p}_{\text{PM}}^{\text{PM'}}([\text{Ca}^{2+}]) & = \frac{k_{out}^{\text{out}}}{k_{out}^{\text{out}} + \text{CaM}([\text{Ca}^{2+}])},
\end{align*}
\]

where \(N_{\text{PM}}^{\text{PM}}\) is the number of PMCA in one \(\mu\m\) of cytoplasmic membrane, \(\dot{I}_{\text{PM}}^{\text{PM}}\) is the time-averaged current per single activated PMCA channel, \(\dot{p}_{\text{PM}}^{\text{PM}}([\text{Ca}^{2+}])\) is the probability of one Ca\(^{2+}\) ion binding PMCA, and \(\dot{p}_{\text{PM}}^{\text{PM'}}([\text{Ca}^{2+}])\) is the probability of Ca\(^{2+}\) ions being released by PMCA on the outer side of the cytoplasmic membrane. Calmodulin activation by Ca\(^{2+}\) is modeled as \(\text{CaM}(\text{Ca}^{2+}) = \frac{[\text{Ca}^{2+}]}{(k_{m} + [\text{Ca}^{2+}])}\), because PMCA requires activated calmodulin to be active itself. \(k_{m}\) is the calmodulin affinity constant for Ca\(^{2+}\), and \(p_{1} = \frac{[\text{CaM}]}{k_{m}}\) is the calmodulin affinity constant to PMCA divided by total calmodulin concentration.

Various cells have different types of Ca\(^{2+}\) channels on the cytoplasmic membrane, the conductivity of which can depend on transmembrane potential, ligands, etc. SACs are one such example, since the activation of these channels is driven by membrane tensions, transmembrane potential, and possibly other factors. Currents through Ca\(^{2+}\) channels located on cytoplasmic membrane are modelled as:

\[
\begin{align*}
\dot{I}_{\text{PM}}^{\text{PM}} & = (N_{\text{PM}}^{\text{PM}}(S) + N_{\text{PM}}^{\text{PM}}(G_0)) \cdot \dot{I}_{\text{PM}}^{\text{PM}}, \\
\dot{p}_{\text{PM}} & = -g_{\text{PM}} \cdot \frac{R \cdot T}{F} \left(\psi_i - 0.5 \cdot \ln\left(\frac{[\text{Ca}^{2+}]_{\text{in}}}{[\text{Ca}^{2+}]_{\text{out}}}\right)\right),
\end{align*}
\]

In the case of the ER, Ca\(^{2+}\) concentration can vary over a wide range on both sides of the ER membrane; this situation is essentially different from the description of Ca\(^{2+}\) currents through the cytoplasmic membrane, because the extracellular concentration does not vary nearly as much. Thus, in the ER case, equations similar to eqn (6) can no longer be used. The current through SERCA, \(i_{\text{ER}}\), is the time-averaged current per single channel, \(\dot{I}_{\text{ER}}(\text{Ca}^{2+})\) is the probability of two Ca\(^{2+}\) ions binding to ATPase, and \(\dot{p}_{\text{ER}}^{\text{2+}}(\text{Ca}^{2+})\) is the probability of Ca\(^{2+}\) ion release by SERCAs. If the two Ca\(^{2+}\) ions have a consecutive binding mechanism, then Ca\(^{2+}\) binding and release probabilities are given as:

\[
\begin{align*}
\dot{p}_{\text{ER}}^{\text{2+}}(\text{Ca}^{2+}) & = \frac{(\text{Ca}^{2+})^2}{k_2 + k \cdot \text{Ca}^{2+} + (\text{Ca}^{2+})^2}, \\
\dot{p}_{\text{ER}}^{\text{2+}}(\text{Ca}^{2+}) & = \frac{k_1^2 \cdot \text{Ca}^{2+}}{k_1^2 + k_1 \cdot \text{Ca}^{2+} + (\text{Ca}^{2+})^2}.
\end{align*}
\]

In the case of the ER, Ca\(^{2+}\) concentration can vary over a wide range on both sides of the ER membrane; this situation is essentially different from the description of Ca\(^{2+}\) currents through the cytoplasmic membrane, because the extracellular concentration does not vary nearly as much. Thus, in the ER case, equations similar to eqn (6) can no longer be used. The passive currents through the ER membrane, \(\dot{I}_{\text{ER}}\), were presented differently from currents through the cytoplasmic membrane. General equations.\(^{71}\) for currents that depend on both internal and external ion concentrations are:

\[
\begin{align*}
\dot{p}_{\text{ER}} & = N_{\text{ER}}^{\text{Ca}} \cdot \dot{p}_{\text{ER}}^{\text{Ca}} \cdot \dot{p}_{\text{ER}}^{\text{2+}} \frac{R \cdot T}{F} \left(\psi_i - 0.5 \cdot \ln\left(\frac{[\text{Ca}^{2+}]_{\text{in}}}{[\text{Ca}^{2+}]_{\text{out}}}\right)\right) - [\text{Ca}^{2+}] \exp(-n \cdot z \cdot (\psi_0 - \psi_{\text{ER}})),
\end{align*}
\]
Given that \( \kappa_{ER} \approx 10^{-4} \text{M} > 10^{-6} \text{M} \approx \kappa_i \) and \( e^k > e^{-k} \) (for \( k > 0 \)) we can approximate the current through single IP3Rs as:

\[
\dot{i}_{IP3} \approx z F \nu \exp(n \cdot z \cdot (\psi_0 - \psi_{ER})) [Ca_{ER}^{2+}].
\]

Structural and physiological studies lead to the following equation for Ca\(^{2+}\) and IP3-dependent probability of IP3Rs being open:

\[
\rho_{IP3}^{Ca^{2+}} = p_{st} + \frac{\dot{i}_{IP3}}{n \cdot z \cdot (\psi_0 - \psi_{ER})} \cdot \frac{k_{ip} \cdot (\frac{Ca_{3+}}{k_{ip} + Ca_{3+}}) \cdot (1 - p_{st})}{(k_{ip} + Ca_{3+})} \cdot \frac{ip3^4}{(k_{ins} + ip3)^4} + \frac{ip3^4}{(k_{ins} + ip3)^4},
\]

where \( p_{st} \) is the Ca\(^{2+}\) and IP3-independent basal level of probability for the channel to be open, \( k_{k} = \frac{Ca_{3+}}{k_{ip} + Ca_{3+}} \) and \( k_{close} \) is the IP3 channel closing rate, \( k_{open} \) is the IP3 channel opening rate and \( k_{eq} \) is the equilibrium dissociation constant in the reaction where Ca\(^{2+}\) interacts with the IP3R channel. \( k_{ins} \) is the IP3 binding affinity constant. This equation explicitly incorporates all four IP3 and twelve Ca\(^{2+}\) binding sites in determining the conductance of the IP3R channel.

**Derivation of the IP3 module equation**

The protein activities that regulate the IP3 concentration (third line in eqn (2)) are given according the following considerations. Fig. 1 schematically illustrates the IP3 formation and degradation pathways. PIP2 hydrolysis by PLC is the source of IP3, and further phosphorylation by IP3K decreases the concentration of IP3 in the cytoplasm. PLC in Dictyostelium is reported to be regulated by both Ca\(^{2+}\) and G-protein subunits. PLC has four Ca\(^{2+}\) binding sites formed by EF hands. PLC activity in our model is represented by Ca\(^{2+}\)-dependent and Ca\(^{2+}\)-independent terms reflecting the Ca\(^{2+}\)-dependence and extracellular factor-dependence via the G-protein pathway:

\[
\text{PLC}(u_i, \text{extracellular cAMP}) = \text{PLC}_{Ca^{2+}} + \text{PLC}_G
\]

\[
= N_{PLC_{Ca^{2+}}} \cdot \frac{(Ca_{3+})^4}{(k_{Ca^{2+}} + Ca_{3+})^4} + N_{PLC_G} \cdot \frac{\text{camp}}{k_G + \text{camp}},
\]

where \( k_{Ca^{2+}} \) is the Ca\(^{2+}\) affinity constant for PLC, \( k_{PLC_{Ca^{2+}}} \) and \( k_{PLC_G} \) are the coefficients that define the contributions to the PLC activation by the Ca\(^{2+}\) and G-protein pathways, respectively, \( \text{camp} \) is the extracellular cAMP concentration, \( k_G \) is the equilibrium dissociation constant for the extracellular cAMP binding to cAR1.

In our model, IP3 is further phosphorylated into IP4 by IP3K. Although IP3 can also be dephosphorylated into IP2, we find that incorporation of the IP3 dephosphorylation term into the model does not lead to any significant effects, and we have, therefore, only considered the phosphorylation reaction of IP3 into IP4. The stationary velocity of this phosphorylation reaction is given by:

\[
\text{IP3}K(\text{ip3}) = \mu_{IP3,K} \cdot \frac{ip3}{k_{ip} + ip3},
\]

where \( k_{ip} \) is the Michaelis constant and \( \mu_{IP3,K} \) is the maximal rate of IP3 conversion into IP4.

**Derivation of the G protein-coupled module equations**

The module that includes the intra- and extracellular cAMP alterations (fourth and fifth lines in eqn (2)) is represented mathematically as follows. Extracellular cAMP binds to cAR1 which in turn regulates the activities of ERK2 and ACA via G-proteins. Since the activation of ERK2 appears to depend on cAR1 activation, so that both oscillate in the same phase, cAR1 was not included into the model as an independent variable. On the other hand, ACA has been reported to depend on intracellular Ca\(^{2+}\). As mentioned earlier, given the lack of detailed biochemical characterization of ACA dependence on Ca\(^{2+}\), various ACA activation mechanisms were tested computationally in the model, via the CaM protein, in analogy with the mammalian adenylyl cyclases. Since the interplay of signals via the cAR1/G-protein pathways and Ca\(^{2+}\) signals can have a highly non-linear nature, a comprehensive dependence of ACA on these two factors has been developed in our model.

ACA in this model is assumed to be indirectly activated by Ca\(^{2+}\)-CaM pairs and by two types of G-proteins: G2 \( \beta y \) subunits via CRAC protein and Grz2 subunits. The cAMP production rate by an individual ACA complex is given by:

\[
\mu_i = \frac{k_{ACA} \cdot ACA \cdot ATP}{K_{ATP} + ATP}
\]

where \( K_{ATP} \) is the equilibrium dissociation constant for the ACA-ATP interaction, and \( i \cdot ACA_i = ACA_0 \), where \( ACA_0 \) is the total concentration of ACA.

The total cAMP production rate is given by the sum of the individual production rates of ACA-CaM or ACA-G-protein subunits as defined by eqn (13). Thus,

\[
\mu_{cAMP} = \sum_i \mu_i
\]

Where the probability of ACA to be activated by Ca\(^{2+}\)-CaM and with G-proteins is given by the following probabilities:

\[
ACA_{const} = ACA_0 \cdot p_{CaM}^{C1} \cdot p_{G0_2}^{C2} \cdot p_{G2}^{C3},
\]

where \( p_{CaM}^{C1}, p_{G0_2}^{C2}, \) and \( p_{G2}^{C3} \) are the probabilities for ACA regulation by CaM bound to a number of Ca\(^{2+}\) ions, and with the two types of G-protein pathways, respectively.

The probabilities for CaM-ACA interactions are given by:

\[
p_{CaM}^{C1=0} = \frac{CaM}{K_{CaM-ACA} + CaM},
\]

\[
p_{CaM}^{C1=1} = \frac{K_{CaM-ACA} + CaM}{K_{CaM-ACA} + CaM},
\]

where \( p_{CaM}^{C1=0} \) and \( p_{CaM}^{C1=1} \) are the probabilities of ACA to be in a complex with and without CaM, respectively. \( C1 = 1 \) if...
where $p_{G1}^2$ and $p_{G1}^0$ are the probabilities of ACA to be in a complex with and without $G_1$, respectively. $C_2 = 1$ when $i = 2, 4, 6, 8$, $C_2 = 0$ if $i = 1, 3, 5, 8$, $K_{G_1,ACA}$ is the equilibrium dissociation constant for G$_1$-ACA interactions. Similarly, for the second type of G-protein subunit we have:

$$
p_{G2}^{C3=1} = \frac{G_2}{K_{G2} + G_2},
$$

$$
p_{G2}^{C3=0} = \frac{K_{G2}}{K_{G2} + G_2},
$$

where $p_{G2}^i$ and $p_{G2}^0$ are the probabilities of ACA to be in a complex with and without $G_2$, respectively. $C_3 = 1$ if $i = 2, 4, 6, 8$, $C_3 = 0$ when $i = 1, 3, 5, 8$, $K_{G_2,ACA}$ is the equilibrium dissociation constant for G$_2$-ACA interactions.

ACA is activated by CaM with $j = 0$, 1, or 2 bound Ca$^{2+}$ ions. Fig. 3 shows the normalized ACA dependence on Ca$^{2+}$ and G-protein concentrations for these 3 cases. A more detailed description of Ca$^{2+}$-CaM-dependent regulation can be found in ref. 47, 48 and 78.

Additional complexity in the dynamics of ACA activity modulation comes from the PKA phosphorylation of the cAR1 receptor. Since cAR1 activation by extracellular cAMP has been shown to activate ACA almost linearly, the cAR1 receptor has not been included as an independent state variable in the model. However, the effects of cAR1 phosphorylation by cAMP-dependent PKA and the associated effects on the level of ACA have been modelled according to eqn (26), as discussed in the section on phosphorylation dynamics below.

Intracellular cAMP is produced by ACA and hydrolysed by intracellular phosphodiesterase RegA. The activity of RegA is given by:

$$
\text{RegA}(\text{Ca}^{2+}, \text{ERK}2) = \text{RegA}_0 \cdot \left( \frac{k_{\text{ERK2}}}{k_{\text{ERK2}} + \text{erk}2} + \text{regA}_H \right),
$$

where $\text{RegA}_0$ is the total RegA concentration, $k_{\text{ERK2}}$ is the equilibrium dissociation constant for RegA interaction with ERK2, regA$_H$ is the stationary RegA activity in the absence of any stimulation.

The release of cAMP from the intracellular space into the extracellular compartment is modelled as the sum of intracellular cAMP and Ca$^{2+}$ terms:

$$
J_{\text{cAMP}} = \frac{V_i \cdot d}{S_i} \left( k_{\text{out}} \cdot \frac{(\text{camp})^2}{(k_{\text{AMP}} + \text{camp})^2} - K_{\text{Ca}^{2+}} \cdot \frac{\text{Ca}^{2+}}{K_{\text{Ca}^{2+}} + \text{Ca}^{2+}} \right),
$$

where $K_{\text{Ca}^{2+}}$ and $K_{\text{out}}$ are the relative strengths of the cAMP and Ca$^{2+}$-dependent terms of the cAMP release, and $k_{\text{Ca}^{2+}}$ and $k_{\text{Ca}^{2+}}$ describe the sensitivity of cAMP release to intracellular cAMP and Ca$^{2+}$ concentrations, respectively. The activation of PKA by intracellular cAMP is given by:

$$
\text{PKA} = \text{PKA}_0 \cdot \left( \frac{\text{camp}}{k_{\text{PKA}} + \text{camp}} \right)^4.
$$

where $\text{PKA}_0$ is the total PKA concentration, and $k_{\text{PKA}}$ is the equilibrium dissociation constant for cAMP-PKA interactions.

**Relationship between parameters in original and normalised model equations**

The relationship between the parameters in the normalized system of differential equations with the original description for the flows in the cellular volume is thus given by:

$$
a_1 = \frac{S_i \cdot N_{\text{PM}} \cdot \delta_{\text{PM}} \cdot R \cdot T}{z \cdot F \cdot \left( V_i - V_{\text{ER}} \right) \cdot d} = \frac{\dot{j}_\text{PM}}{d},
$$

$$
a_2 = \frac{S_i \cdot \tau_1}{z \cdot F \cdot \left( V_i - V_{\text{ER}} \right) \cdot d} = \frac{\dot{j}_\text{PM}}{d},
$$

$$
a_3 = \frac{S_{\text{ER}} \cdot N_{\text{ER}} \cdot \nu \cdot \exp(a \cdot z \cdot (\psi_0 - \psi_{\text{ER}}))}{(V_i - V_{\text{ER}})} = \frac{\dot{j}_{\text{ER}}}{d},
$$

$$
a_4 = \frac{S_{\text{ER}} \cdot N_{\text{ER}}^4 \cdot \dot{j}_{\text{ER}}}{z \cdot F \cdot \left( V_i - V_{\text{ER}} \right) \cdot d} = \frac{\dot{j}_{\text{ER}}}{d},
$$

$$
m_0 = \frac{r_0 \cdot S_i \cdot N_{\text{PLC}} \cdot \left[ \text{PIP}_2 \right]}{(V_i - V_{\text{ER}}) \cdot d} = \frac{\mu_{\text{PLC}}}{d},
$$

$$
m_1 = \frac{r_1 \cdot S_i \cdot N_{\text{PLC}} \cdot \left[ \text{PIP}_2 \right]}{(V_i - V_{\text{ER}}) \cdot d} = \frac{\mu_{\text{PLC}}}{d},
$$

$$
m_2 = \frac{\mu_{\text{PKA}}}{d},
$$

$$
ap = \frac{r \cdot S_i \cdot N_{\text{PLC}} \cdot \left[ \text{PIP}_2 \right]}{N_{\text{A}} \cdot (V_i - V_{\text{ER}}) \cdot d} = \frac{\mu_{\text{PKA}}}{d},
$$

$$
V_i - V_{\text{ER}} \approx 4.4.
$$

The final system of differential equations, which were solved numerically to generate all the results presented in the paper is thus given by:

$$
\frac{d(\text{Ca}^{2+})}{dt} = \frac{\dot{j}_\text{PM}}{d} \cdot \left( \psi_0 - 0.5 \cdot \ln \left( \frac{\text{Ca}^{2+}}{\text{Ca}^{2+}} \right) \right) - \frac{\dot{j}_\text{PM}}{k_d + \text{Ca}^{2+}} \cdot \frac{\text{CaM}(\text{Ca}^{2+})}{K_{\text{CaM}} + \text{CaM}(\text{Ca}^{2+})}
$$

$$
+ \frac{\dot{j}_{\text{ER}}}{d} \cdot \frac{\text{Ca}^{2+}}{\text{Ca}^{2+} - a_4 \cdot \dot{j}_{\text{ER}} \cdot \text{ER}}.
$$

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\[
\frac{d(\text{cat}^{2+}_{\text{ER}})}{dt} = \frac{V_i - V_{\text{ER}}} {V_{\text{ER}}} \left( -j_{\text{ER}} - j_{\text{ER}} \cdot \text{cat}^{2+} + a_d \cdot j_{\text{ER}} \cdot j_{\text{ER}}^2 \right),
\]
\[
\frac{d(\text{ip3})}{dt} = \mu_{\text{PLC}_{\text{Ca}^{2+}}} \cdot \frac{(\text{cat}^{2+})^4} {k_{\text{Ca}^{2+}} + \text{cat}^{2+}} + \mu_{\text{PLC}_{\text{G}}} \cdot \text{camp}_{p} + ap - \mu_{\text{IP}_{\text{K}}} \cdot \text{ip3} \cdot \frac{\text{ip3}} {k_{\text{IP}_{\text{K}}} + \text{ip3}}. \tag{23}
\]
\[
\frac{d(\text{camp})}{dt} = D_{\text{ACA}} \cdot \text{aca}^* - \text{RegA} \cdot \frac{\text{camp}_i} {k_{\text{RegA}} + \text{camp}_i} \cdot \frac{k_{\text{ER}}}{k_{\text{ER}} + \text{cat}^{2+}_{\text{ER}}}
\]
\[-k_{\text{out}} \cdot \frac{(\text{camp})^2} {k_{\text{AMP}} + (\text{camp})^2} - k_{\text{out}} \cdot \frac{\text{cat}^{2+}} {k_{\text{Ca}^{2+}} + \text{cat}^{2+}_{\text{Ca}^{2+}}},
\]
\[
\frac{d(\text{camp})_{\text{out}}}{dt} = K_{\text{AMP}} \cdot \frac{\text{camp}^2}{(k_{\text{AMP}} + \text{camp})^2} \cdot (\text{camp}_i - \text{camp}_f)
\]
\[+K_{\text{out}} \cdot \frac{\text{cat}^{2+}} {k_{\text{Ca}^{2+}} + \text{cat}^{2+}_{\text{Ca}^{2+}}} - \text{PDE}_{\text{Ca}^{2+}} \cdot \frac{\text{camp}_p} {k_{\text{PDE}} + \text{camp}_p},
\]
\[
\frac{d(\text{erk})}{dt} = d_{1} \cdot \text{pk}_{\text{eri}} \cdot (1 - \text{erk}) - \text{erk}
\]
\[
\frac{d(\text{aca})}{dt} = d_{2} \cdot \text{pk}_{\text{eri}} \cdot (1 - \text{aca}) - \text{aca}
\]

where $j_{\text{PM}}$ represents the sum of currents into the membrane, $j_{\text{PM}}$ is the max $\text{Ca}^{2+}$ current flow through the PMCA, $j_{\text{ER}}$ is the max $\text{Ca}^{2+}$ current flow through the IP$_3$Rs, $j_{\text{ER}}$ is the max

### Table 2 Model parameters

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Value</th>
<th>Meaning</th>
</tr>
</thead>
<tbody>
<tr>
<td>$j_{\text{PM}}$</td>
<td>0.05 $\mu$M sec$^{-1}$</td>
<td>The sum of currents into the membrane</td>
</tr>
<tr>
<td>$j_{\text{PM}}$</td>
<td>12 $\mu$M sec$^{-1}$</td>
<td>Max $\text{Ca}^{2+}$ flow through PMCA</td>
</tr>
<tr>
<td>$j_{\text{ER}}$</td>
<td>0.1 $\mu$M sec$^{-1}$</td>
<td>Max current flow through IP$_3$R</td>
</tr>
<tr>
<td>$j_{\text{IP}_3}$</td>
<td>0.3 $\mu$M sec$^{-1}$</td>
<td>Max current flow through IP$_3$ “proportion” of IP$_3$Rs</td>
</tr>
<tr>
<td>$j_{\text{ER}}$</td>
<td>20000 $\mu$M sec$^{-1}$</td>
<td>Max $\text{Ca}^{2+}$ flow through SERCA</td>
</tr>
<tr>
<td>$k_{d}$</td>
<td>0.4 $\mu$M</td>
<td>PMCA $\text{Ca}^{2+}$ affinity</td>
</tr>
<tr>
<td>$V_{\text{in}}/V_{i}$</td>
<td>0.185</td>
<td>ER to cell volumes ratio</td>
</tr>
<tr>
<td>$k_{\text{K}}$</td>
<td>0.00025</td>
<td>Ratio of IP$_3$R opening to closing rates</td>
</tr>
<tr>
<td>$k_{\text{in}}$</td>
<td>0.1 $\mu$M</td>
<td>IP$_3$ binding affinity to IP$_3$</td>
</tr>
<tr>
<td>$k_{\text{ca}}$</td>
<td>0.12 $\mu$M</td>
<td>$\text{Ca}^{2+}$ binding affinity to IP$_3$R</td>
</tr>
<tr>
<td>$k_{\text{m}}$</td>
<td>1 $\mu$M</td>
<td>$\text{Ca}^{2+}$ affinity to CaM</td>
</tr>
<tr>
<td>$k_{\text{CaM}}$</td>
<td>0.003 $\mu$M</td>
<td>CaM affinity to PMCA</td>
</tr>
<tr>
<td>$k_{\text{d}}$</td>
<td>0.4 $\mu$M</td>
<td>$\text{Ca}^{2+}$ binding affinity to PLC</td>
</tr>
<tr>
<td>$k_{\beta}$</td>
<td>0.4 $\mu$M</td>
<td>Sensitivity of PLC to extracellular cAMP-cAR1 interactions</td>
</tr>
<tr>
<td>$\mu_{\text{PLC}_{\text{Ca}^{2+}}}$</td>
<td>0.7 $\mu$M s$^{-1}$</td>
<td>Max $\text{Ca}^{2+}$-dependent PLC hydrolysis rate</td>
</tr>
<tr>
<td>$\mu_{\text{PLC}_{\text{G}}}$</td>
<td>0.1 $\mu$M s$^{-1}$</td>
<td>Max G-protein pathway PLC hydrolysis rate</td>
</tr>
<tr>
<td>$\mu_{\text{PLC}_{\text{M}}}$</td>
<td>0.01 $\mu$M s$^{-1}$</td>
<td>Basal level of PLC hydrolysis rates</td>
</tr>
<tr>
<td>$k_{\text{ip}}$</td>
<td>1 $\mu$M</td>
<td>IP$_3$ binding affinity to IP$_3$K</td>
</tr>
<tr>
<td>$\mu_{\text{PKA}}$</td>
<td>0.5 $\mu$M s$^{-1}$</td>
<td>Max IP$_3$K hydrolysis rate</td>
</tr>
<tr>
<td>$k_{\text{PKA}}$</td>
<td>0.1 $\mu$M</td>
<td>Equilibrium dissociation constant for cAMP-PKA interactions</td>
</tr>
<tr>
<td>$\phi_{i}$</td>
<td>$-70$ $\text{mV}$</td>
<td>Resting membrane potential of cytoplasm</td>
</tr>
<tr>
<td>$\phi_{\text{ER}}$</td>
<td>$-70$ $\text{mV}$</td>
<td>Resting membrane potential of ER</td>
</tr>
<tr>
<td>$D_{\text{ACA}}$</td>
<td>3.6 $\mu$M s$^{-1}$</td>
<td>Max ACA hydrolysis rate</td>
</tr>
<tr>
<td>$\text{RegA}_{\text{CaM}}$</td>
<td>138 $\mu$M s$^{-1}$</td>
<td>Max RegA hydrolysis rate</td>
</tr>
<tr>
<td>$K_{\text{AMP}}$</td>
<td>4000 $\mu$M sec$^{-1}$</td>
<td>Relative strength of the cAMP-dependent cAMP release</td>
</tr>
<tr>
<td>$K_{\text{CaM}}$</td>
<td>3000 $\mu$M sec$^{-1}$</td>
<td>Relative strength of the $\text{Ca}^{2+}$-dependent cAMP release</td>
</tr>
<tr>
<td>$K_{\text{AMP}}$</td>
<td>0.5 $\mu$M</td>
<td>Sensitivity of cAMP release to intracellular $\text{Ca}^{2+}$ concentration</td>
</tr>
<tr>
<td>$k_{\text{Ca}^{2+}}$</td>
<td>0.3 $\mu$M</td>
<td>Sensitivity of cAMP release to intracellular $\text{Ca}^{2+}$ concentration</td>
</tr>
<tr>
<td>$k_{\text{ER}}$</td>
<td>0.1 $\mu$M</td>
<td>cAMP affinity to RegA</td>
</tr>
<tr>
<td>$PDE_{\text{Ca}^{2+}}$</td>
<td>1580 $\mu$M sec$^{-1}$</td>
<td>Modulation of RegA activity by $\text{Ca}^{2+}$ release from ER</td>
</tr>
<tr>
<td>$k_{\text{PDE}}$</td>
<td>0.2 $\mu$M</td>
<td>Max extracellular PDE hydrolysis rate</td>
</tr>
<tr>
<td>$c_{1}$</td>
<td>0.75</td>
<td>cAMP affinity to extracellular PDE</td>
</tr>
<tr>
<td>$c_{2}$</td>
<td>0.625</td>
<td>Defined by eqn (29)</td>
</tr>
<tr>
<td>$d_{1}$</td>
<td>0.5 $\mu$M s$^{-1}$</td>
<td>ERK2 dephosphorylation rate</td>
</tr>
<tr>
<td>$d_{2}$</td>
<td>0.5 $\mu$M s$^{-1}$</td>
<td>ERK2 dephosphorylation rate</td>
</tr>
<tr>
<td>$K_{\text{ACA}}$</td>
<td>0.3, 100, 100, 100, 100, 100</td>
<td>ACA activities in complex with variable combinations of CaM and/or G-protein subunits bound</td>
</tr>
<tr>
<td>$k_{\text{ATP}}$</td>
<td>1 $\mu$M</td>
<td>Equilibrium dissociation constant for ACA-ATP interaction</td>
</tr>
<tr>
<td>$\text{ATP}$</td>
<td>$4 \times 10^{3}$ $\mu$M</td>
<td>ATP concentration</td>
</tr>
<tr>
<td>$K_{\text{G-ACA}}$</td>
<td>0.1 $\mu$M</td>
<td>Equilibrium dissociation constant for CaM-ACA interaction</td>
</tr>
<tr>
<td>$K_{\text{CaM-ACA}}$</td>
<td>0.5 $\mu$M</td>
<td>Equilibrium dissociation constant for G-ACA interaction</td>
</tr>
</tbody>
</table>
current flow through the SERCAs, $\mu_{PLC_{Ca^2+}}$ and $\mu_{PLC_{Ca^2+}}$ are the max PLC hydrolysis rates induced by the $Ca^{2+}$ and G-protein pathways, respectively, $\mu_{IP_{K}}$ is the max IP$_{K}$ hydrolysis rate, $\mu_{PLC_{Cat}}$ is a basal level of PIP$_2$ hydrolysis, $aca^* = (1 - aca)$, $ACA_{rz}$ is a normalized function of ACA activity, $D_{ACA}$ is the max ACA hydrolysis rate, $RegA_0$ is the max RegA hydrolysis rate, and $K_{CAMP}$ and $K_{Ca^{2+}}$ are the relative strengths of the cAMP- and Ca$^{2+}$-dependent cAMP release, respectively. $k_{RegA}$ is cAMP affinity to RegA. $k_{ERK}$ is the modulation of RegA activity by $Ca^{2+}$ release from ER, and $k_{CAMP}$ and $k_{Ca^{2+}}$ are the sensitivities of cAMP release to intracellular cAMP and Ca$^{2+}$ concentrations, respectively. $d_1$ and $d_2$ are the normalized phosphatase activities that dephosphorylate ERK2 and ACA, respectively. $c_1$ and $c_2$ are the complex ratios of the phosphatase and the protein kinase–donor protein complexes (ERK2 and ACA, respectively), multiplied by the phosphorylation/dephosphorylation constants as defined in eqn (29) below. All parameter values used in the above equations are given in Table 2.

The G protein-coupled receptor model analysed in this study is taken from, and may be viewed as a “minimal” model for the system of differential equations for eqn (24) is given by:

$$\frac{dM}{dt} = -k_1 \cdot M \cdot DP + k_{-1} \cdot MDP + k_4 \cdot MAF,$$

$$\frac{dMA}{dt} = -k_3 \cdot MA \cdot F + k_{-3} \cdot MAF + k_2 \cdot MDP,$$

$$\frac{dMDP}{dt} = k_1 \cdot M \cdot DP - (k_{-1} + k_2) \cdot MDP,$$

$$\frac{dMAF}{dt} = k_3 \cdot MA \cdot F - (k_{-3} + k_4) \cdot MAF.$$

We will next consider the pseudo steady-state approximation when the concentrations of the MDP and MAF are constant. In this case, the two last lines of eqn (25) give:

$$MDP = \frac{k_1}{k_{-1} + k_2} \cdot M \cdot DP,$$

$$MAF = \frac{k_3}{k_{-3} + k_4} \cdot MA \cdot F.$$

By substituting eqn (26) into the second equation from eqn (25), one can obtain:

$$\frac{dMA}{dt} = -\frac{k_3 \cdot k_4}{k_{-3} + k_4} \cdot MA \cdot F + \frac{k_1 \cdot k_2}{k_{-1} + k_2} \cdot M \cdot DP.$$

Since the sum of the phosphorylated M and unphosphorylated MA molecules is a constant number $M_0$: $M + MA = M_0$, eqn (24) can be represented as:

$$\frac{dMA}{dt} = -\frac{k_3 \cdot k_4}{k_{-3} + k_4} \cdot MA \cdot F + \frac{k_1 \cdot k_2}{k_{-1} + k_2} \cdot (M_0 - MA) \cdot DP.$$

or in the non-dimensional representation as:

$$\frac{dma}{d\tau} = d \cdot \left(\frac{c}{\tau} \cdot (1 - ma) - ma\right).$$

where $ma = \frac{MA}{M_0}$, $\tau = \frac{k_1 + k_2}{k_{-1} + k_2} \cdot F_0 \cdot t$, $d = \frac{DP}{DP_0}$, $c = \frac{k_3 \cdot k_4 \cdot (k_{-1} + k_2)}{k_1 + k_2 \cdot (k_{-3} + k_4) \cdot DP_0}$, $x = \frac{p}{c} \cdot F_0$, and $DP_0$ are the total concentrations of the protein phosphatase and the protein kinase–donor protein complex, respectively.

Eqn (28) and (29) have been used in our model to describe the modulation of the ACA and ERK2 activities when phosphorylated by PKA.22

**Procedure for comparing the robustness of the proposed model with the G protein-coupled receptor model**

The G protein-coupled receptor model analysed in this study is taken from, and may be viewed as a “minimal” model for...
generating stable cAMP oscillations. The set of nonlinear differential equations describing the dynamics of this model are given by:

\[
\begin{align*}
\frac{d[ACA]}{dt} &= k_1[CAR1] - k_2[ACA][PKA] \\
\frac{d[PKA]}{dt} &= k_3[cAMPf] - k_4[PKA] \\
\frac{d[ERK2]}{dt} &= k_5[CAR1] - k_6[PKA][ERK2] \\
\frac{d[RegA]}{dt} &= k_7 - k_8[ERK2][RegA] \\
\frac{d[cAMP]}{dt} &= k_9[ACA] - k_{10}[RegA][cAMPi] \\
\frac{d[cAMPe]}{dt} &= k_{11}[ACA] - k_{12}[cAMPe] \\
\frac{d[CAR1]}{dt} &= k_{13}[cAMPe] - k_{14}[CAR1]
\end{align*}
\]

where ACA is adenylyl cyclase, PKA is the protein kinase. ERK2 is the mitogen-activated protein kinase, cAMPi and cAMPe are the internal and external cAMP concentrations, respectively, RegA is the internal cAMP phosphodiesterase and CAR1 is the ligand-bound G protein-coupled receptor. Initial concentrations and nominal values for the parameters in the above model are as given in ref. 22.

To ensure a consistent procedure for checking the robustness of both models, the Monte-Carlo simulation technique is used to simultaneously vary four parameters which determine the dynamics of the extracellular cAMP positive feedback loop in both the minimal and the proposed model. The four parameters which are varied in the minimal model are \(k_9, k_{10}, k_{11}\) and \(k_{12}\), while in the proposed model they are \(D_{ACA}, RegA, K_{cAMP}\) and \(PDE_6\).

For each model, the uncertain parameters are sampled uniformly from the following:

\[
\text{param} = \text{param}_i(1 + p_6 \delta_i)
\]

where \(\text{param}_i\) is the nominal value of the parameter \(i, p_6\) is the maximum level of perturbation, \(\delta_i\) is a uniformly distributed random number between \(-1\) and \(+1\).

Although some of the nominal parameter values in each model were derived from (inherently noisy) biological data, others were tuned to values that generated the required oscillatory behaviour. Thus, we have very little a priori information on the likely distributions of the parameters as a result of environmental variations and modelling uncertainty. In such cases, the uniform distribution is the standard choice for the type of statistical robustness analysis performed in this paper.22 The simulations for both models were performed using standard numerical routines in MATLAB.

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References
