CHAPTER 3

cAMP Oscillations during Aggregation of *Dictyostelium*

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Abstract

For many years it has been known that developing cells of *Dictyostelium discoideum* show periodic surges as they aggregate. When it was discovered that the cells were responding chemotactically to cAMP gradients produced within the populations, experiments were carried out that demonstrated similar periodic changes in the concentration of extracellular cAMP. Moreover, homogenous populations of developed cells held in suspension could be shown to respond to cAMP by changes in cell shape. Such suspensions showed spontaneous oscillations in light scattering as well as cAMP levels as the result of entrainment of the cells. The molecular components necessary for the pulsatile release of cAMP were uncovered by analyzing the behavior of a large number of strains with defined mutations isolated from saturation mutagenic screens. Subsequent genetic and biochemical studies established the connections between a dozen proteins essential for spontaneous oscillations. Computer simulations of a molecular circuit based on these results showed that it is able to account for the temporal and quantitative aspects of the oscillatory system. The circuit also appears to be coupled to the construction and dismantling of the actin/myosin cortical layer that ensures that pseudopods are restricted to the anterior of cells during chemotaxis and that the cells do not back-track when the natural wave is behind them. Since the same molecular clock controls both signal production and signal response, these behaviors are always kept strictly in phase.

Introduction

Oscillations have been observed with a wide range of frequencies in different cell types. Neurons show periodicities in the millisecond range while circadian clocks show periodicities of about a day. Various mammalian cell types show periodicities in the range of a few minutes for the secretion of hormones.[1-3] The mechanisms underlying these oscillations depend on the time scale and the cell type, however, there are similarities to the molecular circuits that generate oscillations with similar periodicities. Many oscillations that occur every few minutes appear to be mediated in part by cAMP.[1,2,4]

cAMP is a ubiquitous second message in cells of bacteria, plants, yeast and animals. It is synthesized from ATP by adenylyl cyclase and hydrolyzed to 5'AMP by phosphodiesterases. In bacteria, cAMP activates the DNA binding protein CRP which regulates transcription of a large number of genes. In animal cells, cAMP activates the protein kinase, PKA, which phosphorylates a large number of proteins. In the social amoeba *Dictyostelium*, cAMP is not only a second message that activates PKA but also an extracellular chemoattractant that directs cellular

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motility.[5,6] The high resolution genetic and biochemical techniques that can be applied to *Dictyostelium* have uncovered the molecular circuits that can account for the production and cellular responses to pulses of cAMP.[7-10] During the aggregation stage of development cAMP is produced in pulses and released into the environment where it can diffuse to neighboring cells. When cAMP binds to the the high affinity surface receptor CAR1, it triggers several signal transduction pathways one of which stimulates the adenylyl cyclase ACA while another inhibits the internal phosphodiesterase RegA.[7] cAMP accumulates rapidly and is released to relay the chemoattractant signal. The activity of adenylyl cyclase peaks about 3 minutes after ligand binding to CAR1 and then decreases resulting in oscillations in cAMP production that show a 7 minute periodicity.

Dictyostelium is a eukaryotic microorganism that diverged from the evolutionary line leading to mammals shortly after the divergence of plants.[11,12] It grows in the soil by ingesting bacteria and divides by binary fission. In the laboratory it can be grown in simple defined media with a generation time of about 8 hours generating gram quantities of homogenous cells for biochemical analyses. The fact that these cells can grow and develop equally well as haploids or diploids facilitates microbial genetic techniques to rapidly isolate rare recessive mutations from populations of millions of cells. When cells are collected from nutrient environments, washed and deposited on buffer-saturated surfaces, they initiate a developmental program that results in the formation of aggregates of about 10^5 cells in 10 hours, migrating slugs by 14 hours, and finished fruiting bodies by 24 hours. The fruiting bodies have stalks that are about 2 mm high topped by a ball of spores (Fig. 1).

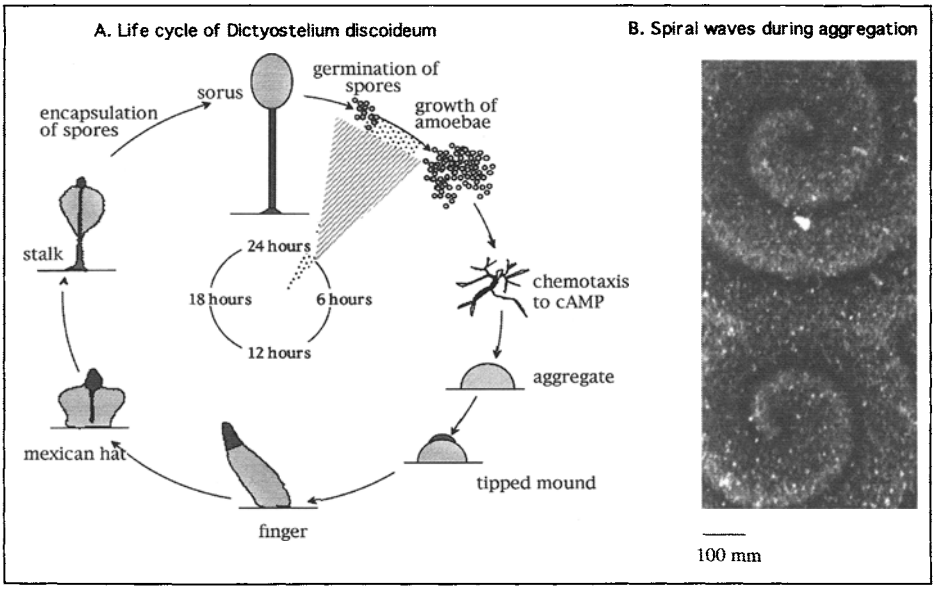

Figure 1. A) Schematic representation of the life cycle of *Dictyostelium discoideum*. Cells can grow for an indefinite period as long a there is a source of food. Upon starvation they initiate a developmental program that leads to the formation of fruiting bodies up to 2 mm high. B) Spiral waves in a chemoattractant field. About 6 hours after the initiation of development the cells become aggregation competent and signal each other with the chemoattractant molecule cAMP. Changes in cell shape in response to cAMP can be observed by dark field microscopy. There are approximately 10^6 cells in the field that are entrained by the pulses of cAMP emanating from the center and spreading as nondissipating spiral waves that mutually annihilate when they meet. Spirals rotate with a period of approximately 7 minutes.

During the first few hours following the initiation of development the cells express a dozen or so developmental genes that encode the components for extracellular signaling by cAMP. These include the receptor, CAR1, the adenylyl cyclase, ACA, the extracellular cAMP phosphodiesterase, PdsA, the Gα2 subunit of the trimeric G protein that mediates signaling from CAR1, and the MAP kinase ERK2.[13-15] Null mutations that knock out any of these genes result in strains that are unable to aggregate.[10] After about 4 hours the cells start to spontaneously release pulses of cAMP which rapidly entrain surrounding cells to release their own cAMP in phase. cAMP diffuses outward as nondissipating waves that become spirals when they encounter obstacles or nonresponding cells.[16] When cells are exposed to a wave of cAMP they not only relay the signal but also respond by modifying their cytoskeleton such that they round up and change their light scattering properties (Fig. 1). These spirals rotate with a period of about 7 minutes. A few hours after these waves first appear the cells start to move directionally towards high cAMP. They aggregate into streams that lead to mounds containing tens of thousands of cells. Pulses of cAMP continue to be released for a few hours but are then replaced by steady production of cAMP.

Proposed cAMP Oscillatory Circuit

Genes recovered from aggregateless strains that came out of saturation mutagenesis screens were cataloged as genes necessary for aggregation. As the list filled up it became apparent that simulations of the interactions of their products might account for the observed oscillations in cAMP accumulation. A successful model would generate spontaneous oscillations in cAMP with a periodicity of 7 minutes and a quantitative change in the range of 5 fold. It should also be able to account for the developmental stage at which spontaneous oscillations are observed. Critical variables could then be computationally explored.

We focused on those activities known to be controlled by cAMP or to interact with each other. It was clear that both ACA and ERK2 were activated when cAMP bound to CAR1.[10,17] ERK2 was found to inhibit the internal phosphodiesterase RegA leading to rapid accumulation of cAMP.[8,18] Most of the cAMP was secreted and slowly hydrolyzed on the outside by the secreted phosphodiesterase PdsA. The cAMP that was not secreted activated PKA which then indirectly led to the inactivation of both ACA and ERK2.[17] The connections in this circuit (Fig. 2) are all supported by biochemical studies in wild type cells as well as a wide range of mutant strains in which individual components were either missing or over-expressed. The kinetics of activation and inactivation of each component were calculated using a set of nonlinear interacting differential equations.[7,8] Parameters were set to account for the measured rate of activation of ACA, ERK2 and PKA. The remaining parameters were optimized to give rise to stable oscillations. A robust periodicity of 6 to 7 minutes was observed that did not change significantly even when individual parameters were varied 2 fold. The amplitude was affected but not the periodicity as has been observed in mutants with reduced ERK2 or RegA activities.[8,19] Only when several parameters were simultaneous changed was the periodicity affected.

Over 25 years ago Gunther Gerisch observed spontaneous oscillations in light scattering of populations of cells developing while suspended in buffer.[20,21] Since there were millions of cells in suspension that were synchronously changing their shape, it was clear that they had become entrained. When multiple cells are exposed to the external cAMP levels generated in the simulated circuit, they were found to rapidly become entrained such that they all produced cAMP at the same time.[7] Winfree[22] had predicted that cells with such oscillatory circuits will innately become synchronized if the cells interact in a common environment due to phase advancement and retardation. Gerisch found that cells in suspension had to develop for at least 3 hours before they would exhibit spontaneous oscillations and that the oscillations stopped after 7 hours of development. When the known changes in ACA and ERK2 during early development are incorporated into the circuit, the simulations captured this developmental pattern (Fig. 3). ACA activity increases at least 6 fold during the first 4 hours of development and does not produce sufficient cAMP to initiate the oscillations until 3 hours. The internal phosphodiesterase,

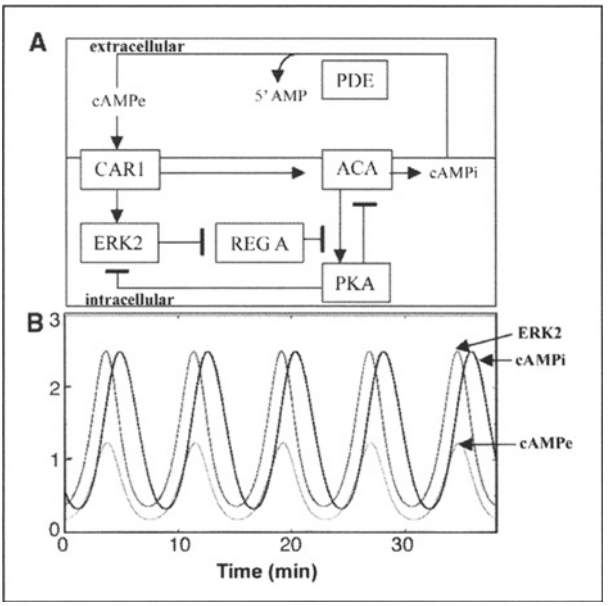

Figure 2. Proposed circuit and its output. A) cAMP accumulates as the result of activation of adenylyl cyclase (ACA) following ligand binding to the cAMP receptor CAR1 and the inhibition of the internal cAMP phosphodiesterase RegA by the MAP kinase ERK2 which is also activated following ligand binding to CAR1. Most of the cAMP is released into the extracellular space where it can stimulate adjacent cells before being broken down by the extracellular phosphodiesterase PDE. About 10% of the cAMP is retained in the cells where it can activate the cAMP dependent protein kinase PKA which indirectly inhibits both ERK2 and the stimulation of ACA activity. Phosphatases activate RegA which reduces the internal concentration of cAMP to reset the circuit. B) Using parameters that capture the kinetics at 4 hours of development, the circuit generates periodic changes in ERK2 activity and cAMP that closely mimic those observed in developing cells. (From Maeda et al, ref. 8.)

RegA, is subject to regulated turnover between 4 and 12 hours of development.[23] The circuit predicts that oscillations will stop when RegA activity decreases to 20% of peak activity.[8] Both the temporal and quantitative aspects of the measured oscillations were accurately predicted by the circuit giving considerable confidence that it includes the critical components and that the architecture is basically correct.

Intermediate steps that do not appear to be connected to the nodes were not included in the model because they just pass the signal along without modifying it. For instance, we know that ligand binding to CAR1 stimulates GTP exchange for GDP of the trimeric G protein containing $G\alpha 2$ and the release of $G\beta\gamma$ which carries the signal to activation of ACA in a process dependent on CRAC, a PH-domain protein that binds to phosphatidyl (3,4,5) phosphate [PIP3] on the inner face of the cell membrane. Furthermore, it is likely that ligand binding to CAR1 activates an upstream component of a MAP kinase cascade rather than directly activating ERK2. However, it was not necessary to include these intermediate steps into the circuit for successful simulations. All of the activation steps in the circuit were considered to be zero-order in the component activated while all inactivating steps were considered first order in the component affect. Such first order inactivation would be expected when the substrate is limiting. This structure to the differential equations is essential to give the time lags necesssary to generate cAMP pulses.

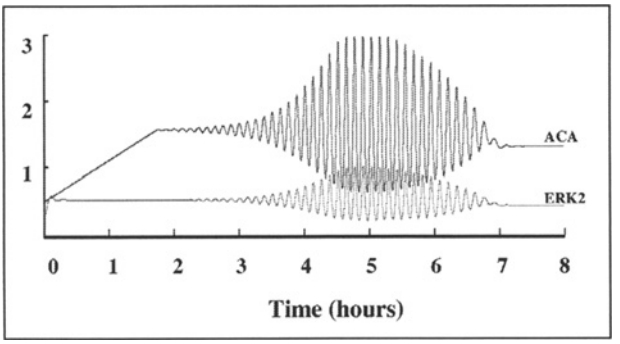

Figure 3. Developmental regulation. Spontaneous oscillations of ACA and activated ERK2 have been shown to occur only between 3 aned 7 hours of development (Gerisch et al, 1979; Maeda et al, 2004). ACA increases 6 fold during the first 4 hours of development while RegA activity decreases as the result of protein degradation. When these developmental parameters are entered into the circuit, spontaneous oscillations are limited to the developmental stage between 3 and 7 hours. (From Maeda et al, ref. 8.)

The fact that the model successfully captures the essential aspects of the observed spontaneous oscillations in cAMP and other components and can account for the transient occurance of oscillations during development suggests that most of the essential components are included and that the connections are physiologically relevant. The model has recently been used to successfully interpret the effects of the antipsychotic drugs, valproic acid and lithium, on early development of *Dictyostelium*.[24]

The model as presently implemented cannot account for the transient activation of adenylyl cyclase and ERK activity observed in cells exposed to constant saturating levels of cAMP. It is not written in kinetic terms and so cannot evaluate possible changes in the affinity of CAR1 for cAMP following activation as proposed in the model of Martiel and Goldbeter.[9] Moreover, adaptation may involve components not included in the present model such as a delayed pathway involving Gα9 or the activity of PI3 kinase that generates PIP3.[25,26] In any case, adaptation is not essential to account for the oscillations during natural waves.

Periodic Motility

Many eukaryotic cells move on a substratum by a crawling process that consists of a succession of events: cells extend an F-actin rich leading pseudopod, attach it to the substratum, and then retract the posterior in a process dependent on filamentous myosin in the cortex.[10,27] Lateral pseudopods are restricted by the cortical layer of actin/myosin that underlies the basolateral surfaces. *Dictyostelium* cells can translocate rapidly, moving about one cell length every minute, which permits high resolution analyses to be carried out using computer assisted motion analysis.[28-30] In response to periodic waves, aggregation competent cells move forward during the rising phase of the cAMP signal. At the peak of the cAMP signal, when the concentration is no longer increasing, the cells aburptly slow down. As the wave moves outward and decreases, the gradient is reversed. There is no net movement during the decreasing phase of the wave and the cells only form small pseudopods in random directions (Figs. 4, 5). As a result, the instantaneous rate of motility in natural waves shows a periodicity of 7 minutes coupled to the periodicity of cAMP release.

Somewhat surprisingly, loss of any of the components of the oscillatory circuit not only preclude the periodic production of cAMP pulses but also result in the inability of the cells to respond to natural waves produced by a surrounding population of wild type cells.[30-32] Cells lacking either RegA or ACA do not produce a leading pseudopod and dither around

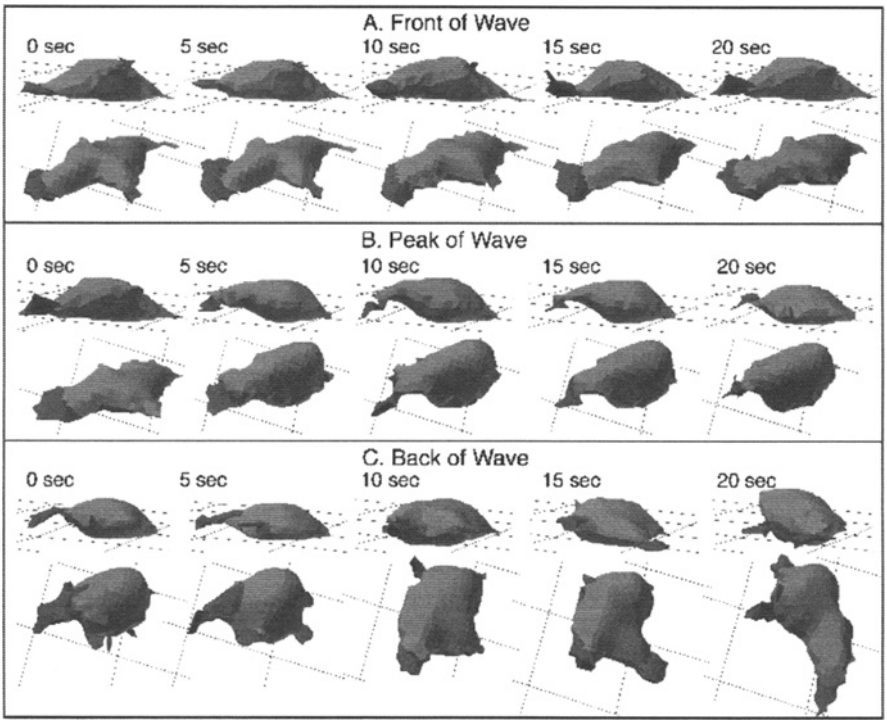

Figure 4. Responses to a wave of cAMP. Developing AX4 cells were given with a wave of cAMP that increased and decrease over a period of 7 minutes. As the cells migrated to the left, they were photographed through a confocal microscope and their shapes later reconstructed by 3D-DIAS.[32] The cell is shown at 15º and 60º from the surface as the cAMP started to increase, at the peak of cAMP at 3.5 minutes, and as the cAMP was decreasing. Nonparticulate pseudopods are darkened. (From Zhang et al,[32] with permission from the American Society for Microbiology.)

putting out small pseudopods in random directions. It is surprising that these mutant strains have almost exactly the same defects in chemotactic motility as each other since internal cAMP is high in one (*regA*$^-$) and low in the other (*acaA*$^-$). However, if the construction and dismantling of the cortical layer requires oscillations in internal cAMP, then the common phenotype could be explained.[31] Support for such a mechanism is based on the consequences to phosphorylation of myosin heavy chain II (MHCII). Unphosphorylated MHCII forms filaments in the cortex which dissociate when they are phosphorylated by myosin heavy chain kinases.[33] The myosin filaments associate with F-actin to form the rigid layer that precludes pseudopod formation along the sides and back of chemotaxing cells. The cortical layer is constructed during the rising phase of a natural wave and dismantled in the back of the wave. This periodic change in the cytoskeleton appears to be mediated by the oscillations in the internal concentration of cAMP.

Heid et al[34] were able to show that oscillations in phosphorylation of MHCII were critical to chemotactic movement. They studied *mhcA*$^-$ cells lacking endogenous myosin heavy chain that were transformed with constructs such that the cells express modified MHC II in which the threonine moities that are phosphorylated (1823, 1833 and 2029) are replaced with either alanine or aspartate. The 3XALA protein cannot be phosphorylated while the 3XASP protein mimics the phosphorylated form of MHC II. Cells expressing either the

3XALA or the 3XASP replacement of MHC II could not stream to form aggregates even when developed in mixed populations with wild type cells. F-actin normally increases in the cortex of wild type cells during the first phase of a temporal wave when the cAMP is increasing and then returns to the cytoplasm. However, in both 3XALA and 3XASP cells there is little or no increase in F-actin in the cortex.[34] It appears that MHC II must cycle between phosphorylated and unphosphorylated forms to bring F-actin to the cortex. Unphosphorylated myosin may bind F-actin in the cytoplasm and form a cortical layer by polymerizing with other myosin chains. When MHC II is phosphorylated it dissociates from the cortex and returns to the cytoplasm where it is dephosphorylated. In the 3XALA cells the myosin cannot be phosphorylated and remains in the cortex where it may interfere with F-actin localization. However, the fact that F-actin does not become membrane associated in the front of a wave in 3XASP cells where the MHC II does not form a cortex, argues against a simple physical barrier to actin assembly. Periodic recruitment of F-actin to the cortex appears to depend upon the dephosphorylation of MHC rather than just unphosphorylated MHC. Therefore, to generate a cortex that can limit the formation of lateral pseudopodia, it is necessary to have oscillation in MHC II phosphorylation and dephosphorylation.

One of the components of the oscillatory signaling circuit is PKA, which is activated when internal cAMP rises. PKA activity may lead to activation of other kinases or phosphatases that affect the phosphorylation state of MHC II. We determined the chemotactic properties of cells with constitutive PKA activity as the result of a null mutation in the regulatory subunit.[32] *pkaR*⁻ mutant cells were found to be defective in chemotaxis and failed to respond to natural waves when incubated in a predominantly wild type aggregation field. Moreover, when the mutant cells made up the great majority of the population, wild type cells failed to show any organized chemotactic response indicating that the *pkaR*⁻ cells also failed to generate pulses of cAMP.

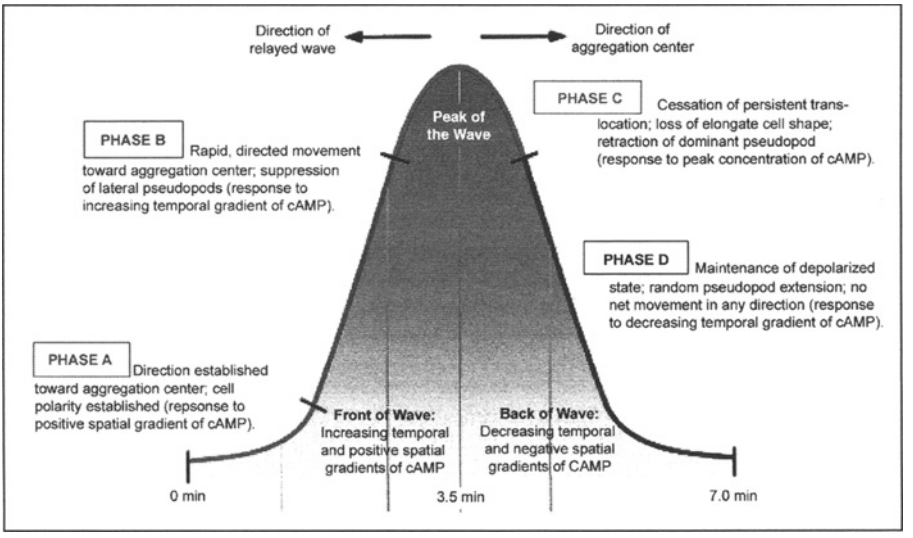

Figure 5. Phases of a natural wave. The direction of the gradient of cAMP is determined in the first minute when cAMP begins to increase. The cells orient their anterior pseudopod and move towards the high point for several minutes. When the concentration of cAMP no longer continues to increase, cells round up and cease moving. In the back of the wave, the cells extend multiple small pseudopods in random directions and show no net movement. The cortical layer is dismantled and the cells are ready to respond to the next wave. (From Zhang et al,[32] with permission from the American Society for Microbiology.)

In spatial gradients, constitutive PKA mutant cells put out twice as many lateral pseudopods as wild type cells and turn more frequently. Three dimensional computer asssisted reconstructions of cells in the front of a temporal wave showed that the dominant pseudopods were often off the substratum, waving in the buffer, accounting for the failure of the PKA mutant cells to show periodic changes in instantaneous velocity in phase with the changes in cAMP concentration.[32] Moreover, the cells are ovoid as are cells completely lacking MHC II as the result of deletion of the structural gene *mhcA*. Usually PKA reaches highest activity just after adenylyl cyclase reaches its highest activity near the peak of the wave.[7,35] In the *pkaR*⁻ mutant cells PKA activity is essentially constant. Maximum PKA activation may be necessary to dismantle polarity at the peak of the wave and allow the cells to round up.[32] The PKA mutants are always round.

Discussion

Oscillations in the time scale of minutes are often mediated by reversible enzymatic modifications of critical components. The circuit that underlies the periodic release of cAMP pulses in *Dictyostelium* relies heavily on phosphorylation and dephosphorylation although periodic activation of PKA by changes in the internal cAMP is a central step.[7,8] PKA appears to be responsible for inhibition of the activity of ERK2 as well as attenuating the activation of adenylyl cyclase. The effects on ACA may result from PKA activity acting on any of the steps leading from CAR1 to ACA although it is unlikely to affect the subunits of the trimeric G protein.[36] Alternatively, PKA could indirectly affect ACA itself to reduce its activity.

Oscillation in PKA activity may also be indirectly coupled to the construction and dismantling of the actin/myosin cortical layer that is essential for efficient chemotaxis. Coupling the response to the same oscillatory circuit that controls periodic release of cAMP ensures that the two processes are always in phase. If cAMP production and motility were out of phase, cells might back-track when the wave of cAMP had passed over them and the cAMP signal was being produced by cells further out from the aggregation center. PKA activity might directly or indirectly activate MHC kinases leading to the dismantling of the cortical layer in the back of the wave such that cells cannot translocate when the gradient is reversed but put out lateral pseudopods in all directions. MHCII is dephosphorylated in the cytoplasm and can then form filaments in the cortex when the next wave arrives. Recruitment of F-actin from the cytoplasm to the cortex appears to depend on the formation of new myosin filaments thereby insuring the coordinate construction of an actin/myosin layer that can direct pseudopods to the anterior.

While oscillatory signaling and responses have been far better characterized in *Dictyostelium* than other cells, there is compeling evidence that polymorphonuclear leukocytes respond in an almost identical manner to waves of chemoattractant.[37] These white blood cells move over long distances to sites of inflammation and infection. In a spatial gradient of the peptide fMLP, they move at the same rate as *Dictyostelium* cells and show excellent chemotactic directionality by suppressing lateral pseudopods. In a series of temporal waves in which the concentration of fMLP oscillated between 10^{-9}M and 10^{-7}M with a 7 minute periodicity the leukocytes showed oscillations in the instantaneous velocity similar to those of *Dictyostelium* cells exposed to temporal waves of cAMP. During the rising phase of each wave, they translocated more rapidly because of the suppression of lateral pseudopods. At the peak of each wave, motility decreased as the anterior psuedopod was retracted. And during the back of the wave, motility returned to basal levels as the result of extending many small lateral pseudopods.[37] These cells appear to have all the machinery to read a temporal gradient. While it is unlikely that leukocytes themselves generate waves of chemoattractant as *Dictyostelium* cells do, there is preliminary evidence for production of nondissipating periodic waves of chemoattractant in surrounding cells (David Soll, personal communication).

Oscillatory processes have an innate resiliance to perturbation as the result of their susceptability to phase advancement and phase retardation by signals emanating from other cells in the network. The advantage for *Dictyostelium* appears to be coordinated production of cAMP as well as integrated relay of the signal over long distances. This mechanism increases the

local concentration of cAMP by summing the output of multiple cells such that it is likely to be above threshold in populations over a wide range of cell densities.[38] Similar advantages may accrue from pulsatile production of hormones in metazoa.

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