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OSCILLATIONS OF CYCLIC NUCLEOTIDE CONCENTRATIONS IN RELATION TO THE EXCITABILITY OF *DICTYOSTELIUM* CELLS

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SUMMARY

Aggregating cells of *Dictyostelium discoideum* are able to release cyclic AMP periodically. The oscillations of cAMP generation are associated with changes in adenylate cyclase activity. Cyclic AMP receptors on the cell surface are functionally coupled to the oscillating system as evidenced by phase shifts that are induced by small pulses of extracellular cAMP. An important element of the oscillating system is the signal processing from surface receptors to the adenylate cyclase. This pathway exhibits adaptation resulting in the suppression of responses to constant, elevated concentrations of cAMP. The signal input for adenylate cyclase activation is, therefore, a change in the extracellular cAMP concentration with time.

Oscillations in the absence of detectable changes of intra- or extracellular cAMP concentrations suggest the possibility that there is a metabolic network in *D. discoideum* cells that undergoes oscillations without coupling to adenylate cyclase. Cyclic GMP concentrations oscillate with a slight phase difference in advance of that of cAMP, suggesting that the two nucleotide cyclases might not be activated by the same mechanism. Elevation of extracellular calcium exerts an inhibitory effect on the accumulation of cAMP and on the second of the two cGMP peaks.

INTRODUCTION

Cyclic AMP is the chemotactic agent of aggregating cells of *Dictyostelium discoideum*. Time-lapse films made by Arthur Arndt (1937) revealed, more than 40 years ago, that the streaming of cells towards aggregation centres is often periodic, and that excitation can spread in waves from a centre towards the periphery of an aggregation territory. As emphasized by Shaffer (1962), this propagation of excitation waves is due to a relay system in which the cells act as amplifiers of the signals that originate in the centre. Wave patterns are formed in layers of preaggregation cells by self-organization (i.e. by interaction of randomly mixed cells) and often assume spiral shapes (Gerisch, 1965). The speed of propagation and period of the oscillations in a layer of densely packed cells is shown in Fig. 1. From the speed of propagation a minimal delay of about 15 s between signal input and output has been calculated (Cohen & Robertson, 1971).

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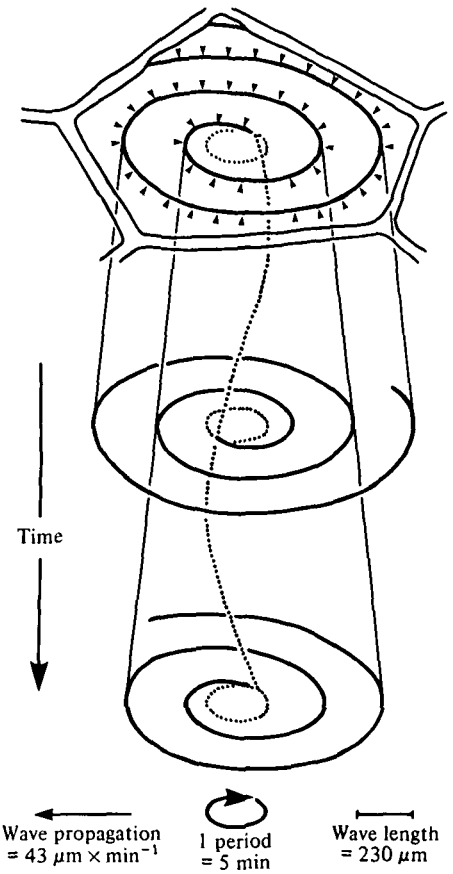

Fig. 1. A spiral wave of chemotactic activity. Top: cells are attracted towards the centre in an aggregation territory of *D. discoideum*. As it is often observed, the line connecting all cells that at a given moment are chemotactically stimulated has the shape of a spiral. From top to bottom the circulation of the origin of the spiral around a central area (dotted) and the propagation of the wave over the aggregation territory is shown. The data on bottom apply to aggregation in dense cell layers. (From Gerisch (1978).)

The waves visualize the pattern in space and time of cellular activities which are under oscillatory control. The spiral lines, which connect cells of identical phase, become visible in the aggregation territories because the cells change shape and location during their chemotactic response (Gerisch, Kuczka & Heunert, 1963; Alcantara & Monk, 1974). The evolution of spatio-temporal order indicates that the activities of the individual cells are coupled to each other. The following experiments were attempted to show that cAMP is a coupling factor and that the waves reflect the spatio-temporal pattern of adenylate cyclase activities.

For the biochemical analysis of the oscillating system it is important to minimize phase differences between cells (i.e. to eliminate spatial heterogeneity but to preserve temporal pattern). This was performed by studying spontaneous and cAMP-induced activities in stirred cell suspensions (Gerisch & Hess, 1974). Fig. 2 illustrates the developmental stage where autonomous oscillations of adenylate cyclase have been observed. For a period before and also after this stage, the cells proved to be excitab

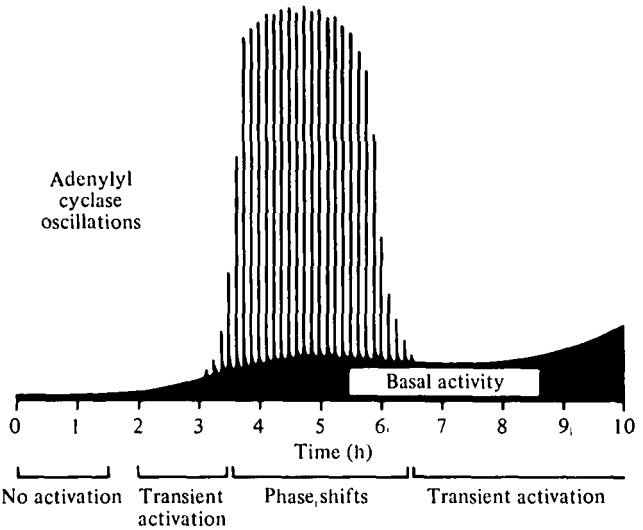

Fig. 2. Diagram showing the increase of basal adenylate cyclase activity (Klein, 1976) and sustained oscillations of activity (Roos *et al.* 1977*b*) during development of *D. discoideum* strain Ax-2. 'Transient activation' refers to the induction of a cAMP pulse by cAMP application to intact cells. At 0 time cells are washed free of nutrients, resuspended on 0·017 M phosphate buffer, pH 6·0 and allowed to develop into aggregation competent ones. (From Gerisch *et al.* 1977*b*).

in the sense that they are able to release cAMP in response to small pulses of cAMP. It is assumed that the changes in cells stimulated by an extraneous cAMP pulse resemble those occurring during a period of limit cycle oscillations (Goldbeter & Segel, 1977). The sequence of events following stimulation can thus be considered as the analogue of a cycle of sustained oscillations.

There are two responses to cAMP stimuli that can be easily registered in cell suspensions: light-scattering changes (Gerisch & Hess, 1974) and transient increases in the extracellular proton concentration (Malchow *et al.* 1978*a*, *b*). Either one of these responses has been routinely used by us as an indicator for the responsiveness of the cells.

BIOCHEMISTRY OF EXCITATION

Cyclic AMP binds to receptors on the cell surface (Malchow & Gerisch, 1974). Within the first few seconds after cAMP application two changes are observed: an increased influx of extracellular ^{45}Ca (Wick, Malchow & Gerisch, 1978) and an increase of the cellular cGMP concentration (Mato *et al.* 1977; Wurster *et al.* 1977). The behaviour of cAMP during the early phase of a response has proven to be more variable. In some of our experiments, no or only small increases in cellular cAMP occurred during this early phase; in others an appreciable increase was observed.

Fig. 3A shows that the early changes may be followed within 1 min (or 2 min at 12 °C) by a second cGMP peak and a peak of cAMP. Whereas the first cGMP peak is correlated with chemotactic stimulation (Mato & Konijn, 1977; Wurster *et al.* 1977; Wurster, Bozzaro & Gerisch, 1978), the second cGMP peak could be involved in signal propagation. This is definitely the case for the cAMP peak.

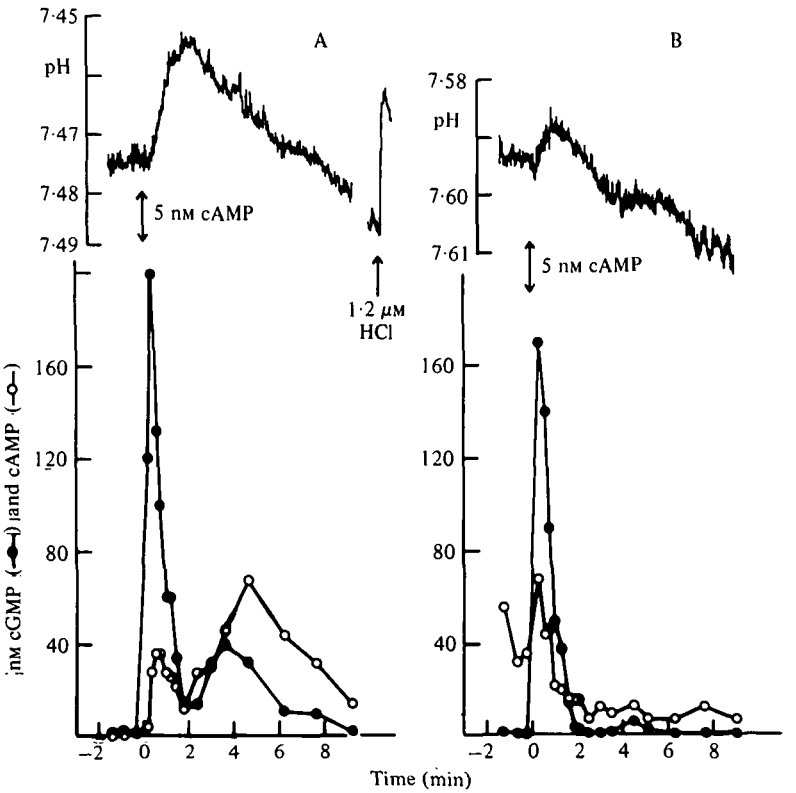

Fig. 3. pH-changes in unbuffered salt solution (top) and changes in cyclic nucleotides (bottom) before (A) and 30 min after the addition of 100μM-Ca^{2+} (B). Calcium addition prior to cAMP stimulation reduced the decrease of pH (Malchow *et al.* 1978*b*) and suppressed the second cAMP and cGMP peaks. HCl was added for calibration of the pH-changes. The cyclic nucleotide concentrations are averaged over the total volume of a suspension of 2×10^7 cells per ml. The temperature was reduced to 12 °C in order to improve temporal resolution of the responses. For methods see legend to Fig. 1 in Gerisch *et al.* (1977*b*).

Stimulation of *D. discoideum* cells resulted in the activation of adenylate as well as guanylate cyclase (Roos & Gerisch, 1976; Mato & Malchow, 1978) (Fig. 4). Therefore, the rise of the cyclic nucleotide concentrations can be explained by activation of the enzymes that synthesize these compounds. The cAMP synthesized in response to cAMP stimuli was released into the extracellular space where it was rapidly hydrolysed by extracellular and cell surface phosphodiesterases (Gerisch *et al.* 1977*a*). The release was specific for cAMP and probably other cyclic nucleotides, as was shown by the absence of an increased rate of 5′-AMP extrusion from stimulated cells (Fig. 5).

A knowledge of the temporal relationship between fluctuating stimulant concentrations and responses is essential for the mathematical analysis of oscillations in *D. discoideum* (Sperb, 1979). Analysis of this relationship is complicated by the short lifetime of cAMP in the extracellular space. By using high concentrations of stimulant or, alternatively, by using a slowly hydrolysed analogue of cAMP it is possible to keep the stimulant concentration for a reasonable time at a virtually constant high level. In all cases the responses were transient even under these conditions, indicating that they were not time-independent functions of stimulant concentrations. The responses rather

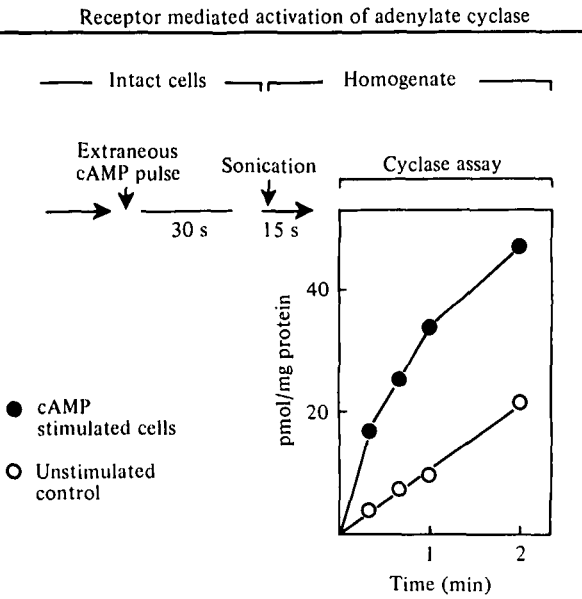

Fig. 4. Protocol used for the demonstration of adenylate cyclase activation in response to cAMP pulses. Similar protocols have been used for guanylate cyclase activation and for the measurement of activity changes of adenylate cyclase under conditions of sustained oscillations. (In the latter case cAMP application to intact cells was omitted.) Adenylate cyclase activation has not been observed in cell homogenates. When, however, intact cells were stimulated by a cAMP pulse and then rapidly sonicated, short-lived activation of adenylate cyclase was detected in homogenates. Open circles show basal adenylate cyclase activity. Ordinate: amount of cAMP synthesized in the homogenate. Methods as described by Roos & Gerisch (1976).

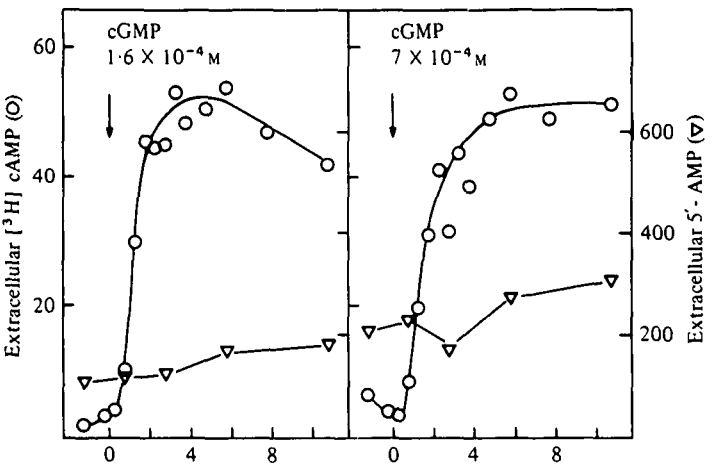

Fig. 5. Specific stimulation of cAMP release. High concentrations of cGMP stimulate cAMP release similar to small pulses of cAMP. The advantage of high cGMP concentrations is that cAMP-phosphodiesterase is competitively inhibited. Concentrations higher than $1{\cdot}6 \times 10^{-4}$ M cGMP were required for sufficient phosphodiesterase inhibition. Under these conditions the integral release of cAMP can be directly measured. Since, by the same token, hydrolysis of cGMP is negligible, the data show that cAMP is released in response to a rise of the stimulant, but does not continue for more than 4 min in the presence of high stimulant concentrations. Specificity is indicated by the absence of an increased 5'-AMP release from stimulated cells. The cells have been prelabelled with ^{3}H-adenine. The data on the ordinates are cpm of extracellular ^{3}H-cAMP (○, left) or ^{3}H-5'-AMP (▽, right) per 10^{+4} cpm of total intracellular radioactivity. (From Roos, (1977).)

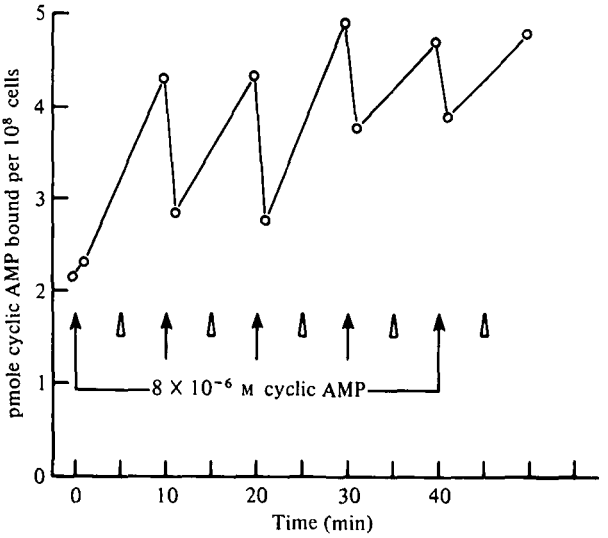

Fig. 6. Experimental attempts to estimate changes of cAMP binding ability in strongly cAMP-stimulated cells. Prior to the experiment the cells were entrained in 0·017 M phosphate buffer, pH 6·0, at 23 °C by pulses of 5 nM cAMP given every 5 min. Entrainment signals of 5 nM size (open arrows) were alternated during the experiment with stronger stimuli of 8×10^{-6} M cAMP. For the measurement of cAMP binding, samples of the cell suspension were centrifuged for 10s in a Beckman Microfuge and washed twice with ice-cold buffer. Binding was measured at 4–6 °C using ^{3}H-cAMP as described by Malchow & Gerisch (1974). On Scatchard plots the binding data behave as if affinity is decreased in cAMP-stimulated cells.

depend on changes of stimulant concentrations with time. This has been demonstrated for the light-scattering response (Gerisch *et al.* 1975*a*), for adenylate cyclase activation (Gerisch *et al.* 1977*b*) and, its consequences, the increase of the intracellular cAMP concentration (Rossier *et al.* 1978) and the rate of cAMP release into the medium (Gerisch *et al.* 1977*b*). The exact relationship of the latter response to fluctuating extracellular cAMP concentrations has been recently examined by Devreotes, Derstine & Steck (1979) and Devreotes & Steck (1979). These authors elegantly demonstrated, by the use of a perfusion technique, that the duration of cAMP release can be extended to more than 30 min provided that the cells were stimulated by continuously increasing cAMP concentrations. The efflux of cAMP from the cells stopped soon after the rise of the stimulant concentration was discontinued.

The mechanism of the adaptation demonstrated by these experiments remains to be clarified. The alternatives are changes at the receptor level on one hand and alterations of subsequent steps of signal processing on the other. 'Desensitization' (a decrease of either the number of overt receptors or their affinity) refers to a change of the first category. A decrease in the number of available cAMP binding sites on the surface of cells exposed to 10^{-5} to 10^{-3} M cAMP has been demonstrated by Klein & Juliani (1977). This effect progressed slowly as compared to the adaptation which is relevant for oscillations. The latter should be manifested within 1 min after cAMP addition, and it should require not more than 1–2 μM cAMP (i.e. extracellular concentrations which are actually reached during spontaneous oscillations (Gerisch & Wick, 1975)).

The cAMP released from stimulated cells competes for the radioactive cAMP that is added for the testing of cAMP-binding to surface receptors. This complicates th

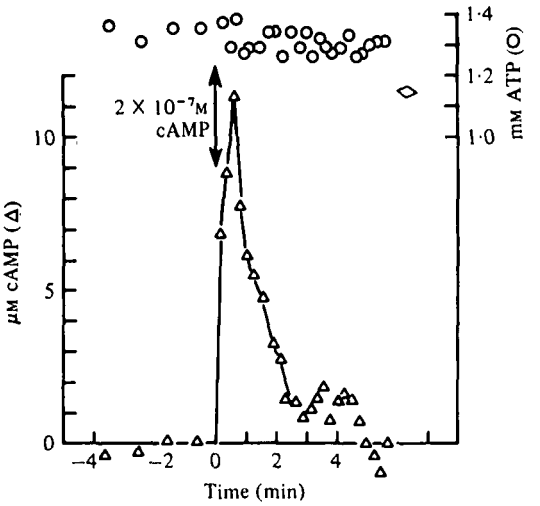

Fig. 7. cAMP and ATP in suspended cells stimulated by cAMP. Molarities have been calculated per volume of densely packed cell sediment, although the measurements include extracellular cAMP. Note that the variation of cAMP concentrations is in the range of 2–3 orders of magnitude below the ATP concentration. (The first point after 0 time includes cAMP added for stimulation.) Thus only a small fraction of the cells' ATP seems to be used up by activated adenylate cyclase. Conditions: about 3×10^7 cells of strain Ax-2 per ml in Soerensen phosphate 0·017 M, at 4–5 hours after the end of growth, pH 6·0, at 23 °C, supplied with oxygen.

measurement of affinity changes. Using a protocol which attempted to minimize this artifact (Fig. 6) we found after cAMP stimulation a transient decline in the affinity constant of the cAMP-receptor complexes by a factor of 2. This value refers to a range of cAMP concentrations between 10 and 120 nM, and thus mainly concerns the low affinity branch of the biphasic binding kinetics as they have been measured by Green & Newell (1975).

The presumed change in affinity of the cAMP receptors appears not to be extensive enough to explain the complete cessation of cAMP-synthesis and release in adapted cells. A subsequent step in signal processing seems therefore also to be affected. The relevant change in adapted cells is certainly not depletion of the substrate of adenylate cyclase, since the total ATP content is not detectably reduced after stimulation (Fig.7).

MODULATION OF THE RESPONSE BY CALCIUM

Calcium had a clear-cut effect on the responses when it was added shortly before stimulation (Fig. 3). The experiment shown in Fig. 3 has been performed in a salt solution before and after the addition of 0·1 mM $CaCl_2$. When calcium was added shortly before cAMP was applied – and only then – the second cGMP and cAMP peaks were suppressed (Fig. 3B). It is not clear whether calcium acts directly on nucleotide cyclases. Guanylate cyclase of *D. discoideum* was not inhibited by 1 mM-$CaCl_2$ (Mato, Roos & Wurster, 1978) and adenylate cyclase was only half-inhibited by 0·1 M $CaCl_2$ (Klein, 1976). Furthermore, the first sharp increase of cGMP was virtually unchanged in cells previously supplemented with calcium.

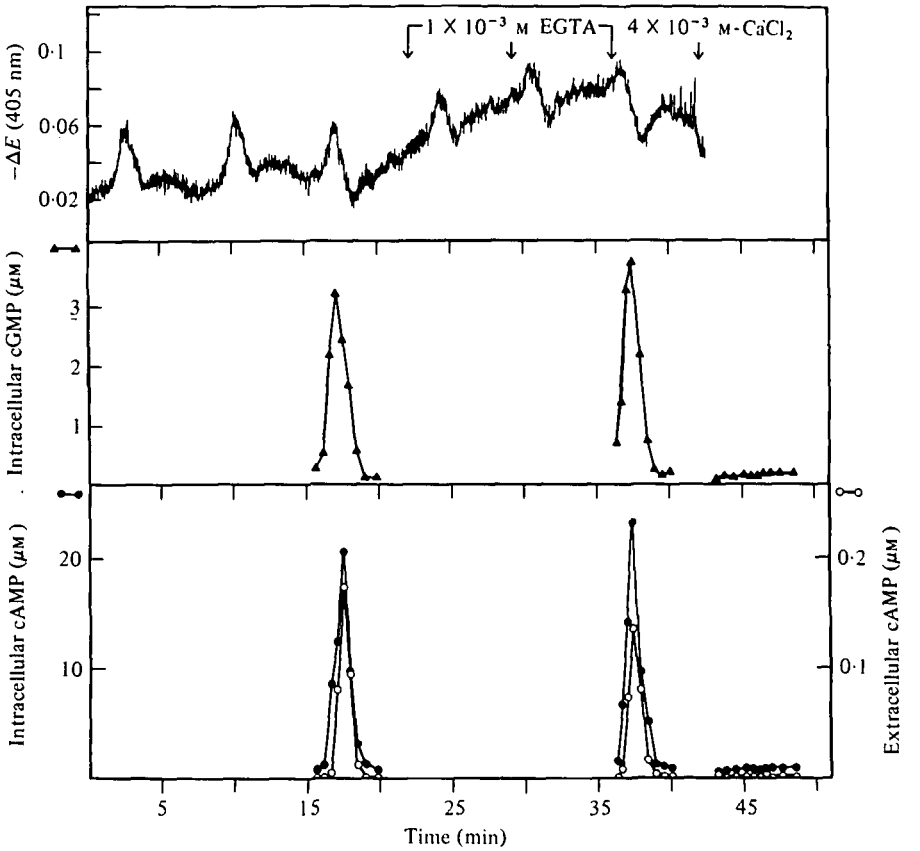

Fig. 8. Sustained oscillations showing spikes of decreased light-scattering (top), intracellular cGMP (middle) and intra- and extracellular cAMP (bottom). By EGTA the amplitude of light scattering was reduced, but the cyclic nucleotide pulses were virtually unchanged. After the addition of calcium the oscillations stopped. (Recording of light scattering was not possible at the end of the experiment because the volume left in the optical cuvette was reduced by the removal of samples.) The strain was Ax-2. Cyclic nucleotides were determined by radio-immuno-assay. Other methods as described by Gerish & Wick (1975). (From Wick, 1978.)

PERIODIC CELLULAR ACTIVITIES ARE LINKED TO ADENYLATE CYCLASE OSCILLATIONS

The investigation of synchronized periodic activities in agitated cell suspensions began with the discovery of rhythmically generated spikes of decreased light scattering (Gerisch & Hess, 1974). These spikes were produced in cell suspensions at the same developmental stage as propagated waves in cell layers. Measurement of intra- and extracellular cAMP concentrations revealed that the spontaneous light-scattering changes were related to the periodic generation of chemotactic stimuli (Gerisch & Wick, 1975). At the beginning of a light-scattering spike cAMP was accumulated within the cells before it was released, after a delay of about 0·5 min, into the extracellular medium where it was rapidly hydrolysed. Simultaneously with cAMP accumulation the number of vesicles increased within the cells, and declined thereafter (Maeda & Gerisch, 1977). This can be interpreted as indicating that cAMP is released by exocytosis, but as yet no evidence is available which shows that the vesicles which change in number contain cAMP.

The cAMP oscillations are associated with periodic changes in adenylate cyclase activity (Roos, Scheidegger & Gerisch, 1977). However, it is not only the cAMP concentration which oscillates, but also that of cGMP (Wurster *et al.* 1977). Moreover, the cGMP peaks slightly precede those of cAMP (Fig. 8). This raises the question of whether guanylate cyclase is more directly linked to the oscillating system than adenylate cyclase, which might be secondarily activated by some event accompanying the cGMP increase.

Fig. 8 shows an experiment in which elimination of extracellular calcium by EGTA reduced the amplitude of light scattering, as it has been previously shown for EDTA (Gerisch *et al.* 1975*b*). Nevertheless, the peaks of intracellular cGMP and cAMP were of essentially unchanged amplitude. The transport of cAMP into the extracellular space was also unaffected. After, however, calcium had been returned, the cyclic nucleotide concentrations remained at the basal levels, similar to the suppression of the second cGMP and cAMP peaks in excitable cells stimulated by cAMP (Fig. 3B).

The blockage of oscillations by a sudden increase in the extracellular calcium concentration can be hypothetically explained by two assumptions. (1) Cytoplasmic calcium mediates a reaction which inhibits guanylate and adenylate cyclases. This does not necessarily mean a direct action on the respective enzymes. (2) The cytoplasmic calcium concentration is determined by the rate of calcium influx and the activity of calcium pumps that remove calcium from the cytoplasmic space. If the pumps adapt their activity only slowly in response to increasing calcium concentrations, the cytoplasmic calcium concentration would transiently rise to an elevated level, and would decline after adaptation of the pumps.

SIGNAL INPUT FROM CELL SURFACE RECEPTORS TO THE OSCILLATOR

Small pulses of cAMP shift the phase of the oscillations. The direction and extent of the shifts depend on the phase in which the pulses are applied (Malchow *et al.* 1978*a*). Only during their own cAMP production are the cells insensitive to extraneous pulses (Fig. 9). These results show that at all phases of an oscillatory cycle (apart from the signalling phase) the receptors can be activated by extracellular cAMP.

It is nevertheless likely but not definitely established that the affinity of the receptors for cAMP changes during the oscillations. Oscillatory changes of cAMP binding have been reported by Klein, Brachet & Darmon (1977) and by King & Frazier (1977). Because affinity changes can be simulated by the periodic release of cAMP, which competes with the labelled cAMP added, the latter authors have studied oscillations in cells whose cAMP production was blocked by dithiothreitol. The relationship of the oscillations observed under these conditions to those discussed in this paper is uncertain. The oscillations observed by King and Frazier were of shorter period (2 min) and apparently ran with almost undamped amplitude in the absence of cAMP. They also appeared to be associated with periodic modification of surface sites by epicellular protein kinase. The periodically phosphorylated sites were suggested to be cAMP receptors. In contrast, ^{32}P incorporation, from extracellular ATP into intact cells, was extremely low, if not absent, under our conditions and remained so after stimulation with cAMP (Rahmsdorf, Malchow & Gerisch, 1978). Cell surface phosphorylation is therefore unlikely to be a candidate for receptor modification during oscillations as they have been studied in our laboratory.

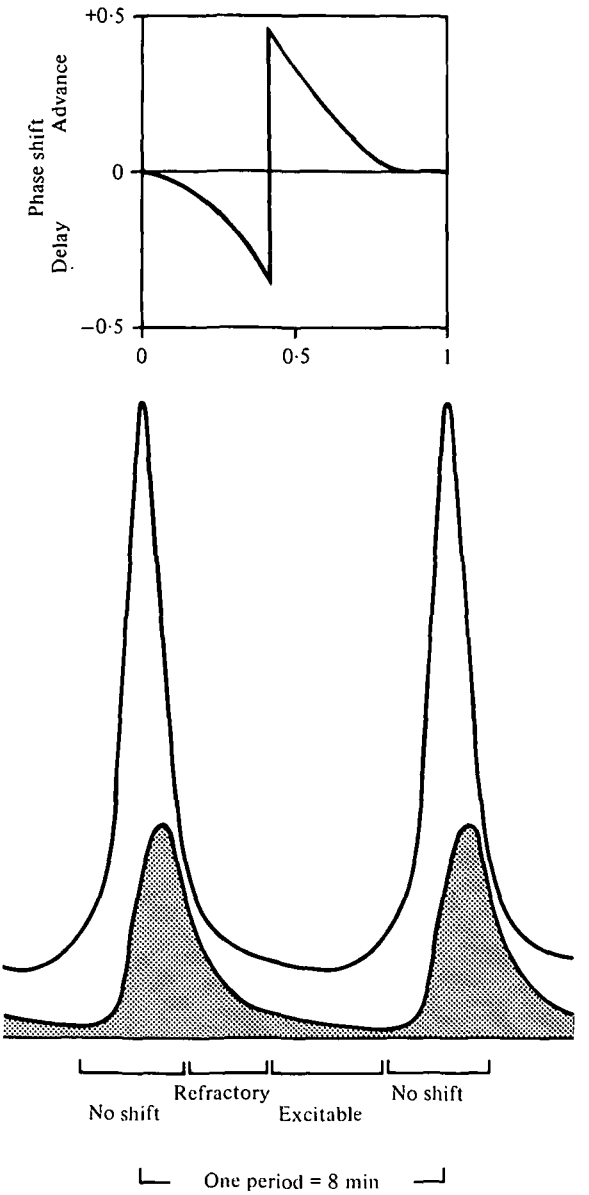

Fig. 9. A period of free-running cAMP oscillations (bottom) and phase shifts induced in different phases of a cycle of normalized length (top). The shifts were induced by pulses of 5–10 nM amplitude. On the bottom the phases in which no advanced cAMP pulse could be induced by the small disturbing pulses applied are labelled 'refractory'. Accordingly, 'excitable' refers to phases in which an advanced pulse of cAMP synthesis was obtained. The open area shows the shape of intracellular cAMP oscillations, the filled area that of extracellular oscillations. The decline of the extracellular cAMP concentration is due to phosphodiesterase action. (From Gerisch *et al.* 1977*b*.)

IS ADENYLATE CYCLASE ACTIVATION INDISPENSABLE FOR OSCILLATIONS TO OCCUR?

Models for oscillations in *D. discoideum* have been designed in which adenylate cyclase plays a central role (Goldbeter, 1975; Cohen, 1977). Such models are supported, in principle, by the finding that pulses of cAMP cause phase-shifts, indicating that cAMP receptors are coupled to the oscillating pathway, probably through guanylate or adenylate cyclase activation. The importance of extracellular cAMP pulses for the maintainance of sustained oscillations is also supported by experiments in which continuous fluxes of cAMP were applied in order to establish steady, elevated extracellular cAMP concentrations. These fluxes irreversibly abolished light-scattering oscillations, probably because the cells could no longer detect and produce their own cAMP pulses (Gerisch & Hess, 1974). It is possible, however, that cAMP only couples the oscillations of the individual cells to each other and thus acts as a synchronizer in cell suspensions, without being essential for oscillations at the single cell level.

As discussed above, periodic light-scattering changes are clearly correlated with oscillations of intra- and extracellular cAMP concentrations. This is only true, however, for oscillations in which light scattering decreases in the form of spikes. After some time this type of oscillation is often converted into a sinusoidal pattern in which the spike-like depressions are replaced by smoothly curved increases of light scattering (Fig. 10). Sometimes small peaks of cAMP concentrations were still observed in the phases in which the spikes were to be expected, but these peaks became progressively smaller from one period to the next (Fig. 11). In other cases neither intra- nor extracellular changes of cAMP concentrations could be detected during sinusoidal oscillations (Fig. 12). Similar results have been obtained by determining adenylate cyclase activities which either slightly increased, in the appropriate phase, or remained at basal levels without any obvious oscillations. These data leave the following possibilities open. (1) Rudimentary cAMP oscillations are present during sinusoidal oscillations which the cells can detect and use for the synchronization of their activities. Our assays are, however, less precise than the recognition system of the cells. This possibility is improbable because sinusoidal oscillations proved to be fairly resistant against disturbing cAMP pulses, suggesting that during these oscillations the cells are relatively insensitive to cAMP. (2) Sinusoidal oscillations occur in the absence of any cAMP changes. If this were true, the question would be whether there is a synchronizing agent other than cAMP. Sinusoidal oscillations are, however, slightly damped. Thus it is not impossible that they continue, after initial synchronisation, without any coupling between the cells.

FUNCTIONAL CONNEXION OF THE OSCILLATING SYSTEM TO MEMBRANE DIFFERENTIATION

In one particular strain of *D. discoideum* (Nc-4, including its axenically growing derivative Ax-2), the periodically generated cAMP pulses are important for cell differentiation from the growth phase to the aggregation competent stage. Small pulses of cAMP stimulated the differentiation, whereas a continuous influx of cAMP of the same average rate inhibited (Gerisch *et al.* 1975*c*). The inhibitory effect of

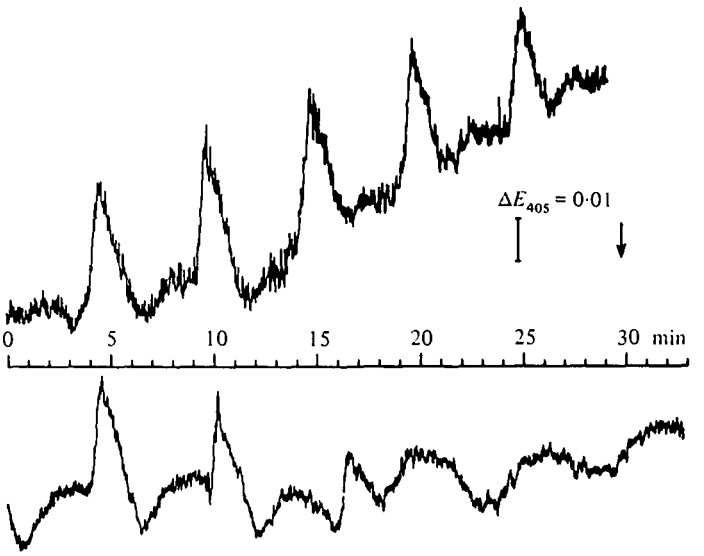

Fig. 10. Transition from a spike pattern, as it is shown on top, to sinusoidal oscillations. Note that the phase in which spikes of decreased light scattering are formed corresponds, during sinusoidal oscillations, to maxima of light scattering. (Decreased light scattering is plotted upwards). (From Gerisch *et al.* 1977*b*).

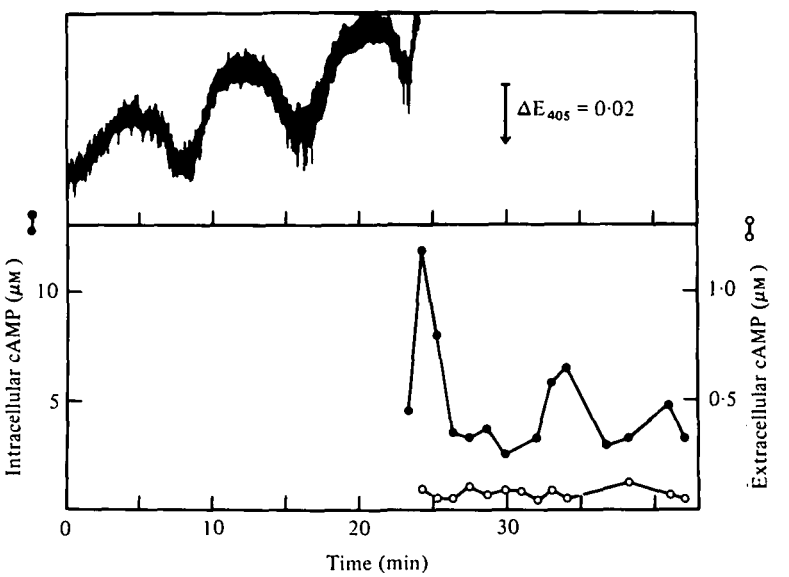

Fig. 11. Damped cAMP oscillations associated with sinusoidal oscillations of light scattering. In this example sharp intracellular cAMP peaks were found at the beginning of the measurements. No concomitant increase of the extracellular cAMP concentration was detected. The strain was Ax-2. Methods as described by Gerisch and Wick (1975). (From Wick, 1978).

steady concentrations was particularly strong in the case of a slowly hydrolysable cAMP analogue, adenosine-3′,5′-cyclic phosphorothioate (Rossier *et al.* 1978). The effect of cAMP pulses was particularly clear-cut in the case of certain non-aggregating mutants (Darmon *et al.*, 1975). The importance of oscillations for cell differentiation is underlined by results obtained with a phosphodiesterase-negative mutant

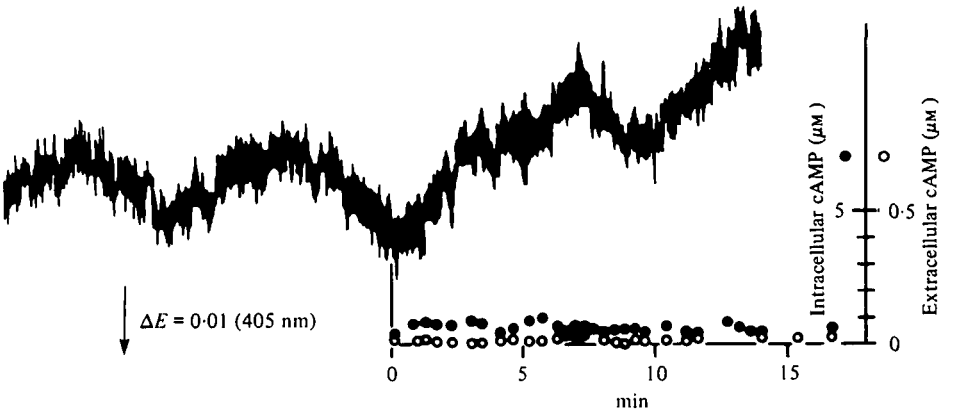

Fig. 12. Sinusoidal oscillations of light scattering in the absence of detectable oscillations of intra- or extracellular cAMP. The strain was Ax-2. Methods as described by Gerisch & Wick, (1975).

(Darmon, Barra & Brachet, 1978). In this mutant no oscillations were observed and aggregation competence was not acquired. However, the mutant exhibited oscillations and development proceeded when purified phosphodiesterase was added to the medium.

The reason for the inefficiency of steady cAMP concentrations is apparently the adaptation of the response system. Fluctuation of cAMP production – not necessarily periodically – is, therefore, a prerequisite for optimal stimulation of development. The intracellular factors which connect the control of development to the activation of receptors at the cell surface are unknown. Calcium, cGMP and cAMP are certainly candidates. Soluble cAMP or cGMP binding proteins specific for either cAMP or cGMP are present in the cells and could mediate effects of intracellular cyclic nucleotides on development. *D. discoideum* has been reported to possess cAMP-dependent developmentally regulated protein kinases (Sampson, 1977). The protein kinases tested in our laboratory were neither activated by cAMP nor cGMP and did not clearly co-fractionate with the binding proteins for these cyclic nucleotides (Rahmsdorf & Gerisch, 1978).

The stimulation of cell development by periodic cAMP pulses implies that the control of membrane proteins occurs through the activation of membrane receptors. The membrane components controlled by cAMP pulses include the constituents of the cAMP signal system: adenylate cyclase, cAMP-phosphodiesterase and cell surface cAMP receptors (Roos, Malchow & Gerisch, 1977*a*). In addition, the expression of a specific glycoprotein on the cell surface is enhanced by cAMP pulses (Gerisch *et al.* 1975*c*). This glycoprotein has been suggested to be involved in a specific type of cell adhesion which is characteristic of cells in the aggregation stage (Müller & Gerisch, 1978).

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