J. Cell Sci. 36, 311–321 (1979)
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THE EVOLUTION OF EXCITABLE BEHAVIOUR IN *DICTYOSTELIUM*

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SUMMARY

Aggregation of *Dictyostelium discoideum* amoebae is effected by relayed cyclic AMP (cAMP) signals. The period of this wave propagation drops abruptly at the transition from aggregative to post-aggregative development. In this paper I demonstrate that the light-scattering response induced by a low concentration pulse of cAMP evolves from one lasting 5 min to one lasting 2 min. The definitive establishment of the 2-min response coincides with the beginning of post-aggregative gene expression. Amoebae at the aggregative stage are unable to respond to a second cAMP pulse delivered less than 4 min after the first, whereas at the post-aggregative stage they can respond to 2 pulses separated by 1 min or longer. Addition of cAMP phosphodiesterase to amoebae at the aggregative stage can in part mimic the change in excitable behaviour: the response is shortened and the amoebae can respond more frequently. However the shortened response is not post-aggregative in shape and the prolonged presence of cyclic nucleotides induces different responses at the aggregative and post-aggregative stage. Both these results suggest that the rate of destruction of the relayed signal is not solely responsible for the change in excitability.

INTRODUCTION

A few hours after starvation, the initially separate amoebae of *Dictyostelium discoideum* begin to aggregate by chemotaxis to cyclic AMP (cAMP), which is released in pulses from centres (Konijn, van de Meene, Bonner & Barkley, 1967; Konijn, Chang & Bonner, 1969; Gerisch, Hulser, Malchow & Wick, 1975). The cAMP binds to cell surface receptors (Malchow & Gerisch, 1973), and stimulates the cells to make a movement step and synthesize and release a pulse of cAMP (Shaffer, 1975; Roos, Nanjundiah, Malchow & Gerisch, 1975; Gerisch & Wick, 1975). This cAMP pulse stimulates the next region of cells, and in this way cells over a wide area are entrained to the centre. This signal propagation gives rise to either concentric or spiral wave patterns, which can be seen because the bands of moving and non-moving cells have different light-scattering properties (Alcantara & Monk, 1974). Concentric waves are thought to arise from a single cell (Durston, 1973), which spontaneously releases pulses of cAMP. Spiral waves are set up when some inhomogeneity in the aggregation field permits the establishment of a signal travelling around a loop of cells (Durston, 1973; Gross, Peacey & Trevan, 1976). Because cells are refractory for relay for a short time after excitation (Gerisch, 1971; Shaffer, 1962), the most stable loop size is such that each cell is stimulated just as it is no longer refractory for relay.

Therefore the period of these waves is a measure of the minimum time interval that can elapse between 2 successive excitations.

Cells developing in suspension exhibit similar excitable properties. Periodic oscillations in light scattering have been observed several hours after the onset of starvation (Gerisch & Hess, 1974). These stop after about 20–25 cycles (Wurster, 1976; Wurster & Schubiger, 1977). Externally applied pulses of cAMP given at various times during development also induce light-scattering changes (Gerisch & Hess, 1974). Shortly after the onset of starvation the response is a single rapid spike; later the response is biphasic and comprises a rapid spike and a longer response. During the biphasic response the added cAMP induces a rapid but transient rise in cyclic GMP (cGMP), which coincides with the light-scattering spike, followed by a transient rise in intracellular and then extracellular cAMP, with a similar timing to the longer light-scattering response (Wurster, Schubiger, Wick & Gerisch, 1977; Gerisch & Wick, 1975; Mato, Krens, van Haastert & Konijn, 1977).

It has recently been shown that, in cells aggregating on a surface, there is a gradual drop in signal period from about 6 min to 4 min, followed by an abrupt drop to 2 min after about 20 spiral waves have been emitted (Gross *et al.* 1977). Thereafter signal propagation continues with a 2-min period for several hours. The onset of the 2-min periodicity coincides approximately with the end of aggregative gene expression and the onset of post-aggregative gene expression (Gross *et al.* 1977; Town & Gross, 1978).

Since it is difficult to study the mechanism underlying the change in signalling behaviour in cells developing on a solid surface, I have looked for a related change in cells developing in suspension, by examining the responses of amoebae to pulses of cAMP applied at various times during development.

MATERIALS AND METHODS

Organisms and culture conditions

Strain HM2, a derivative of the strain V12/M2 obtained from Dr G. Gerisch, is temperature-sensitive for growth and resistant to cobalt and acriflavine (Trent & Kay, personal communication). Spore stocks were kept on silica gel and new working stock plates started every 2–3 months. Stock plates were kept at 7 °C: growth and development were at 22 °C. Amoebae were grown in suspensions of washed *Escherichia coli* B/r (10^{10}/ml) in KK_2 buffer (KH_2PO_4 2·25 g/l; K_2HPO_4 0·67 g/l; $MgSO_4.7H_2O$ 0·5 g/l; pH 6·1). The bacterial suspensions were heated at 80 °C for 10 min prior to use. Strain Ax2, originally obtained from Prof. J. Ashworth, was grown as described by Watts & Ashworth (1970).

For development, cells were washed 3–4 times in KK_2 buffer. They were then either resuspended at 5×10^7 cells/ml in KK_2 buffer and placed in a cuvette at once or suspended at 10^7 cells/ml in KK_2 and shaken in a conical flask on a rotary shaker at 120 rev/min for about 2 h prior to centrifugation and transfer to a cuvette at 5×10^7 cells/ml.

The convention $t_{0,1\ldots n}$ is used to refer to the time in hours after the initiation of development.

Optical methods

One to two millilitres of cells were placed in a cuvette with an optical path of 1 cm. Two No. 18 gauge needles were fixed opposite the wax slope across the bottom of the cuvette. Water-saturated air at a flow rate of about 28 ml/min (Meterate Flow Meter: Glass Precision Engineering Ltd) was passed through these needles, which were adjusted so that the bubbles

avoided the light path. The optical density at 405 nm was recorded using a Gilford spectrophotometer (Model 240) attached to a pen recorder. The cuvette temperature was kept at 21 ± 1 °C by a Churchill Chiller Thermo Circulator. Up to 4 cuvettes could be run in parallel. Pulses of cyclic nucleotides increased the volume of the suspension by 1 %.

Enzyme assays

Samples for enzyme assay were centrifuged; the pellets and supernatants were stored separately at −30 °C. Prior to assay the pellets were resuspended in 0·08 M tricine buffer, pH 7·5, containing 20 % (v/v) glycerol. The cell suspensions were sonicated for 2 bursts of 4 s, using the microprobe of a Virsonic Cell Disrupter (Model 16–850). cAMP phosphodiesterase was assayed as described by Henderson (1975), and glycogen phosphorylase as described by Town & Gross (1978), except that incubation was at 25°C.

Protein concentration was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

Cyclic nucleotide stability

Pulses of cyclic nucleotides, containing traces of ^{3}H-labelled cyclic nucleotides (The Radiochemical Centre, Amersham), were added to the cell suspensions. Samples were removed at different times and diluted with an equal volume of 10 % TCA, containing unlabelled markers (adenosine, 5'AMP, and cAMP). Cellulose thin-layer chromatograms (Eastman) were run in ethanol/1 M ammonium acetate (75:30), pH 7·5, to separate the nucleotides. The spots absorbing under ultraviolet were cut out and counted in Toluene + fluors: Triton: water (20:10:5).

Reagents

Reagents were of the purest grade commercially available. cAMP and cGMP were from Sigma.

Phosphodiesterase

The partially purified *Dictyostelium* cAMP phosphodiesterase preparation was a generous gift from Dr J. Sampson. The preparation contained 800 units of phosphodiesterase/ml. One unit of phosphodiesterase is the amount of phosphodiesterase required to hydrolyse 1 nmol of cAMP per min at 35 °C.

RESULTS

Evolution of the response to cyclic AMP pulses

Strain HM2, used in much of this work, frequently does not display spontaneous oscillations. This facilitates examination of the evolution of the responses to cAMP pulses. Typical light-scattering responses to 10^{-8} M cAMP during the development of HM2 are shown in Fig. 1. By about t_4 the response consists of a peak (peak I) lasting 25 ± 0·5 s (mean ± standard error of the mean), followed by a second peak (peak II) lasting 5·2 ± 0·1 min. At about t_6 a new profile is produced with a new peak intercalated between peaks I and II. Over the next 1·5 h peak II disappears and the amplitude of the intercalated peak (peak III) increases, until finally only peaks I and III remain. Peak III lasts 1·6 ± 0·1 min. Thereafter the response remains stable for at least 2 h. This evolution of response to cAMP was reproducibly observed in all 21 such experiments.

I shall refer to the response comprising peaks I and II as aggregative and to the later response (peaks I and III) as post-aggregative, since it will be shown below that these responses are associated with the phases of aggregative and post-aggregative development defined by Town & Gross (1978). At the earliest times in development tested ($t_{0.5}$) a single peak is induced; this pre-aggregative response gradually evolves into the aggregative response (Fig. 1).

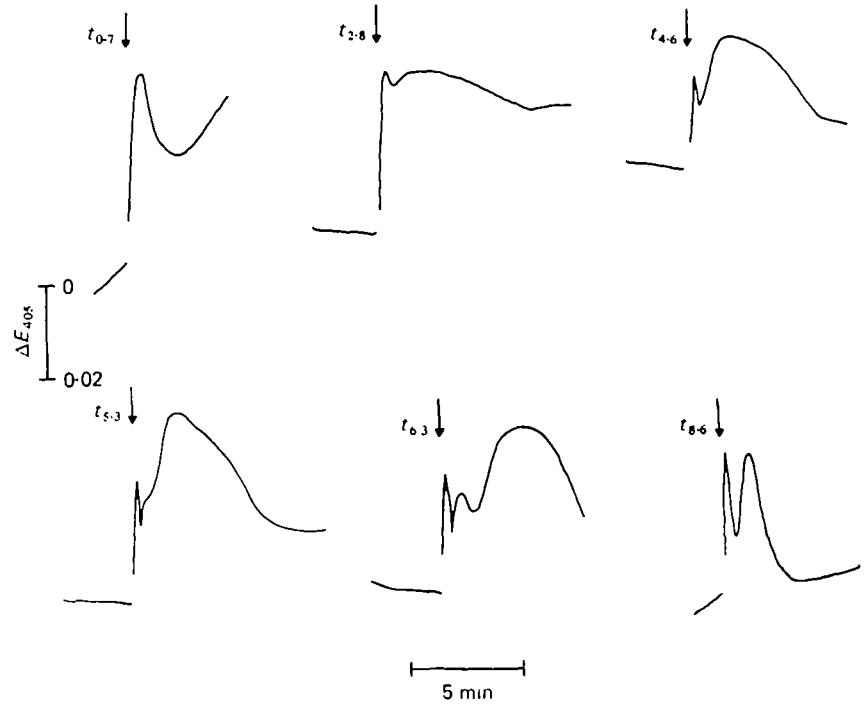

Fig. 1. Evolution of light-scattering responses to 10^{-8} M cAMP in HM2. Amoebae were prepared as described in Materials and methods and were subjected to several cAMP pulses throughout development. At the times indicated by an arrow, cAMP solution was added to the cell suspension and the optical density changes recorded. The responses were later traced. The figure is a composite of 2 different experiments, of which only some responses, typical of the various stages, are shown. Each type of response was obtained several times in most experiments. During the transition to the post-aggregative response 2 other responses were also occasionally observed. A long peak (8–14 min) sometimes followed peak III (7 out of 21 experiments). In 4 out of 21 experiments a further peak III was observed after the post-aggregative response.

Oscillations lasting from 1 to 7 cycles also arose in 13 out of 26 experiments. These began either after an aggregative response or during the transition to the full post-aggregative response. The period of the oscillations was $7{\cdot}4 \pm 0{\cdot}2$ min and the average peak width was similar to peak II of the aggregative response ($5{\cdot}3 \pm 0{\cdot}2$ min). Oscillations ended at about t_8.

Strain Ax2, which has been used in the previously published work on light scattering (Gerisch & Hess, 1974; Gerisch *et al.* 1977), shows a similar evolution of light-scattering profiles to HM2 (Fig. 2), except that peak II is shorter ($3{\cdot}5 \pm 0{\cdot}2$ min).

Gerisch & Hess (1974) demonstrated a change in response during the development of Ax2, by comparing the time delay between the first and second peaks. Using 5×10^{-6} M cAMP pulses, they observed a change from a 2-min delay in t_{2-4} cells to a 0·9-min

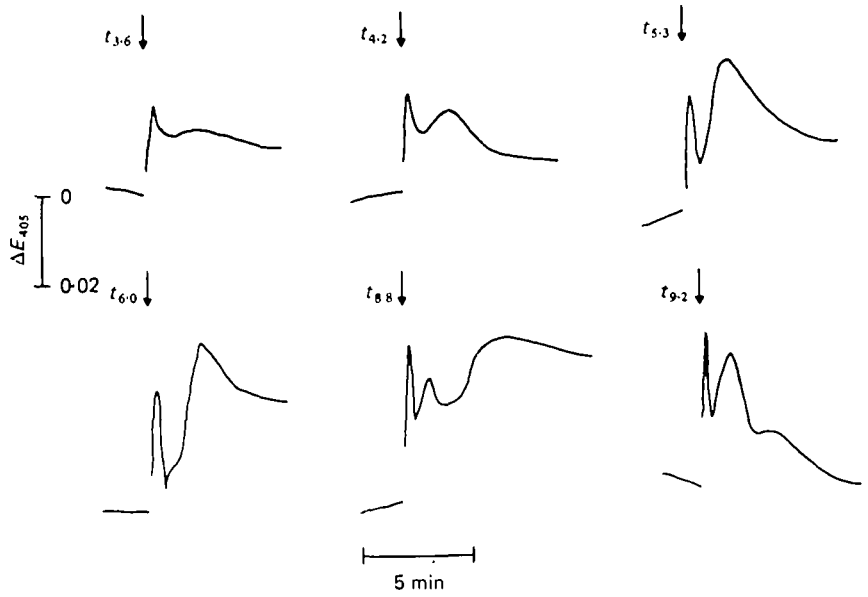

Fig. 2. Evolution of light-scattering responses to 10^{-8} M cAMP in Ax2. Details as for Fig. 1.

Table 1. *Effect of cAMP concentration on light-scattering response at the aggregative and post-aggregative stages*

Strain	Pulse concentration, M	Aggregative response: Peak delay, min	Aggregative response: Response duration, min	Post-aggregative response: Peak delay, min	Post-aggregative response: Response duration, min
Ax2	10^{-8}*	1·55 ± 0·05	4·0 ± 0·2	0·83 ± 0·04	1·9 ± 0·1
HM2	10^{-8}*	1·61 ± 0·05	5·6 ± 0·1	0·90 ± 0·03	2·0 ± 0·1
HM2	5×10^{-7}*	0·81 ± 0·07	5·7 ± 0·3	0·86 ± 0·06	2·2 ± 0·1
HM2	10^{-5}†	0·60	8·0	1·23	6·4
HM2	5×10^{-4}†	0·57	3·2	1·35	6·4

* Results are mean of 10–100 measurements ± S.E.M.
† Results are mean of 2 or 3 measurements.

delay in t_{10-15} cells. The second response was very weak in the t_{2-4} cells, especially with 10^{-8} M cAMP. In the present study, the peak-to-peak interval with Ax2 using 10^{-8} M pulses, was 1·55 min at the aggregative stage and 0·83 min at the post-aggregative stage (Table 1). Strain HM2 showed a similar change (Table 1). Gerisch & Hess (1974) showed that an increased cAMP concentration in the pulse caused a

greater peak to peak time in t_{2-4} cells; Table 1 shows that the opposite is true at the aggregative response stage.

The timing of the light-scattering responses in relation to the changes in gene expression

The transition to post-aggregative signalling in cells on a surface coincides with the cessation of accumulation of cAMP phosphodiesterase and the onset of glycogen phosphorylase accumulation (Town & Gross, 1978; Gross *et al.* 1977). In vigorously agitated suspensions of cells, accumulation of phosphodiesterase is prolonged and

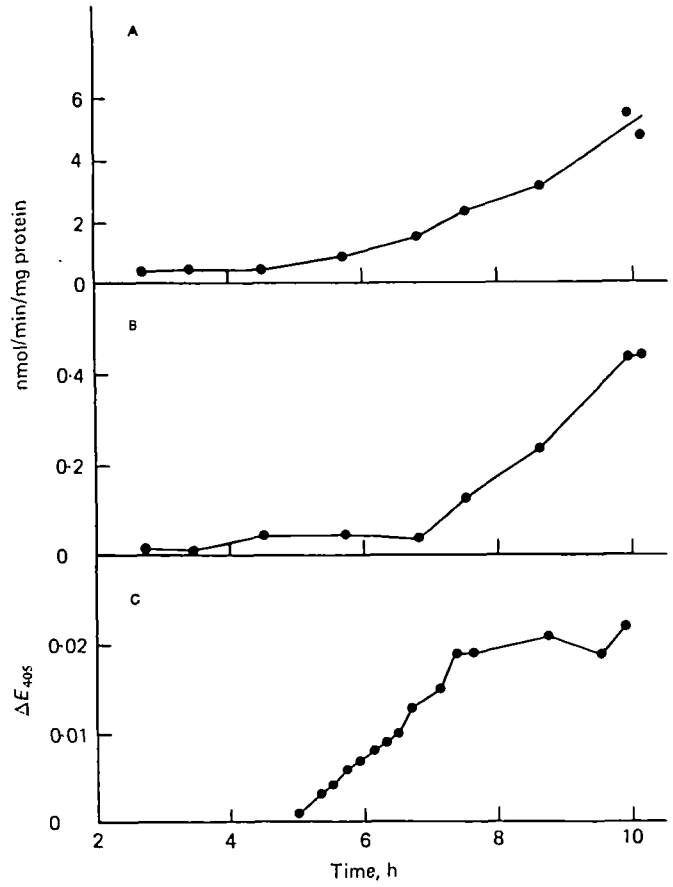

Fig. 3. Enzyme synthesis and the increase in peak III amplitude. HM2 amoebae were transferred to a cuvette at t_2 and given 10^{-8} M cAMP at various times. The amplitude of peak III in the recorded responses was measured. At the times indicated samples were removed and assayed for cell-associated cAMP phosphodiesterase and glycogen phosphorylase. A, phosphodiesterase; B, glycogen phosphorylase; C, peak III amplitude. A second experiment gave the same outcome.

accumulation of glycogen phosphorylase is reduced (Town & Gross, 1978). Fig. 3 shows that a similar pattern of accumulation was observed in bubbled suspensions. The results in Fig. 3 show that the gradual shift from peak II to peak III took place during the early phase of phosphodiesterase synthesis and that the amplitude of peak

III reached a maximum at the onset of glycogen phosphorylase accumulation. Thus the establishment of the definitive post-aggregative response in suspension and the transition to post-aggregative development on a surface occur at about the same time.

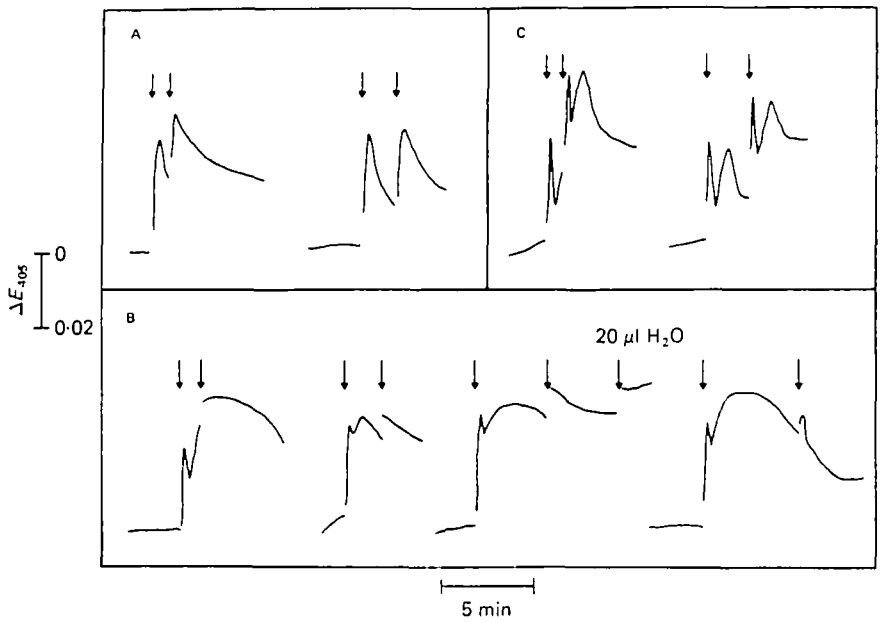

Fig. 4. Response to successive cAMP pulses. HM2 amoebae at the stages shown were subjected to successive 10^{-8} M cAMP pulses: A, pre-aggregative; B, aggregative; C, post-aggregative. (Details as for Fig. 1.) The discontinuity in the trace after the second pulse, even when no response was observed, is because the dilution of the suspension caused an optical density shift. An equal volume of water caused a similar optical density change (lower panel).

The response of cells to successive cAMP pulses

The signal period of spiral waves on a surface is a measure of the minimum possible time interval between successive excitations (Durston, 1973). Since this property changes at the signal transition on agar, I have examined how amoebae in suspension respond to two successive pulses of cAMP (Fig. 4). During both the pre-aggregative and the post-aggregative stages the cells could respond to both pulses at all time intervals tested (1 min or longer). However at the aggregative response stage the cells fail to respond when the second pulse is given less than 4 min after the first. Only after 6 min have elapsed between pulses is a full second response produced (Table 2). This behaviour was unchanged when the cAMP was added in a larger volume to facilitate rapid mixing as well as when successive pulses of 5×10^{-7} M cAMP were employed. In the presence of 5×10^{-7} M cAMP over 90% of the cell surface receptor sites will be occupied (Mato & Konijn, 1975).

The control of excitability by the rate of destruction of extracellular cyclic nucleotides

The difference in responsiveness is not directly related to the rate of destruction of the added cAMP; during the aggregative response only 1% of the added pulse

remains after 3 min, whereas during the pre-aggregative response about 15% of the pulse remains after 1 min at which time the amoebae are responsive to a further pulse (data not shown). However the extracellular cAMP concentration depends not only on the added cAMP pulse, but also on the induced cAMP release: during spontaneous

Table 2. *Response to successive pulses of* 10^{-8} M *cAMP at the aggregative response stage*

Response to second pulse	Interval between first and second pulse, min*					
	< 2	2–3	3–4	4–5	5–6	> 6
No response	8	6	13	2	0	0
Spike only	0	0	0	6	5	0
Full response	0	0	0	1	1	9

* Results are given as the number of experiments showing this response at the indicated time interval.

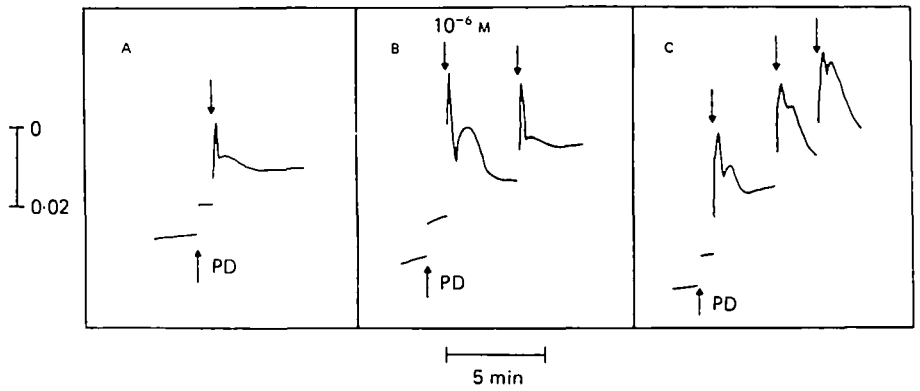

Fig. 5. Effect of cAMP phosphodiesterase on the aggregative response. Phosphodiesterase (25 u./ml) was added to suspensions of amoebae displaying the aggregative response at the times indicated by the arrows (PD). The cell suspensions were then given cAMP (at 10^{-8} M final concentration, except where stated otherwise). A, B and C are separate experiments. Details as for Fig. 1.

oscillations, the extracellular cAMP concentration reaches 10^{-6} M (Gerisch & Wick, 1975). Experiments in which extracellular cyclic nucleotides were either destroyed rapidly or kept at a relatively constant high concentration are reported in this section.

The addition of 25 u./ml of a partially purified cAMP phosphodiesterase to cell suspensions displaying the aggregative response altered the response to 10^{-8} M cAMP (Fig. 5A). Peak I was normal, but peak II had a lower amplitude and was shorter. Addition of 10^{-6} M cAMP in the presence of phosphodiesterase gave a similarly short response, but with a greater amplitude (Fig. 5B). If a 10^{-6} M pulse was followed 25 s later by the addition of cAMP phosphodiesterase a shortened response also resulted.

After phosphodiesterase addition the amoebae were responsive to a second pulse

given 2 min after the first (Fig. 5c). Although phosphodiesterase addition reduced the duration of the aggregative response, it is clear that the shape of the shortened response is not identical to the post-aggregative response (compare Figs. 1 and 5). The same quantity of phosphodiesterase did not alter the post-aggregative response. The normal increase in total phosphodiesterase from the aggregative response to the onset of the full post-aggregative response was in the range of 5–10 u./ml, while the minimum quantity of phosphodiesterase required to produce the effect was about 10 u./ml.

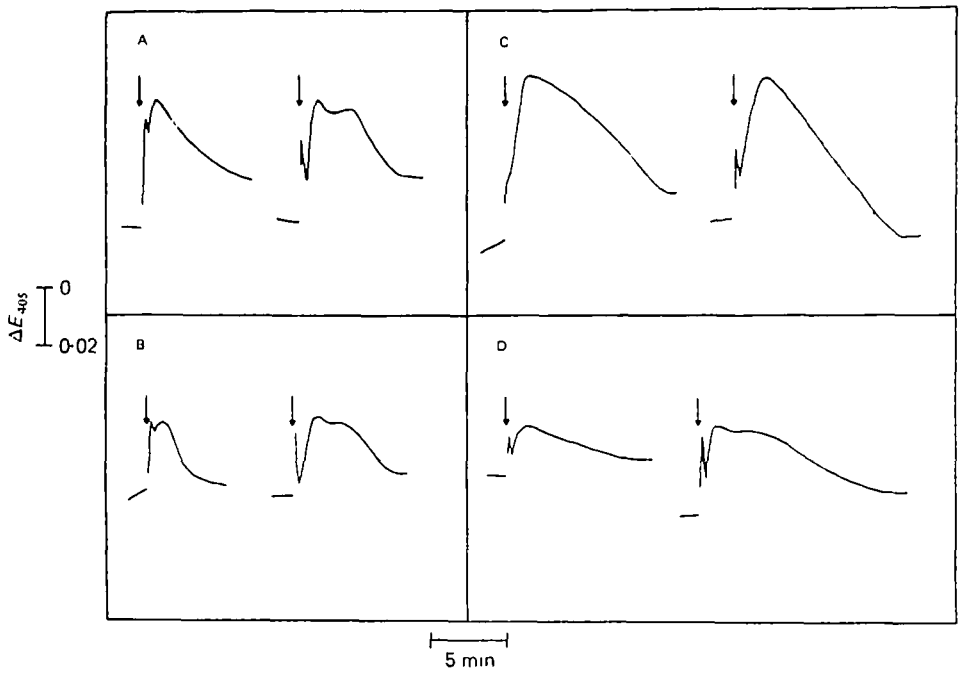

Fig. 6. Responses to high concentrations of cAMP and cGMP. Amoebae showing the aggregative or post-aggregative responses to 10^{-8} M cAMP were given: A, 10^{-5} M cAMP; B, 5×10^{-4} M cAMP; C, 10^{-5} M cGMP; D, 5×10^{-4} M cGMP. In each box, the first curve is aggregative, and the second, post-aggregative. Details as in Fig. 1.

When cAMP or cGMP is added to a concentration of 10^{-5} M it persists for several minutes at a high concentration (data not shown). I therefore compared the responses to sustained high levels of cyclic nucleotides at the aggregative and post-aggregative stages (Fig. 6 and Table 1). There were clear differences of shape between the aggregative and post-aggregative responses, indicating further that the difference in excitable behaviour is not just a function of the rate of destruction of the extracellular signal.

DISCUSSION

The period of spiral waves drops gradually from 6 to 4 min, then rather sharply to 2 min at about the time of the transition from aggregative to post-aggregative gene expression (Gross *et al.* 1977; Town & Gross, 1978). The observation that spon-

taneous oscillations in suspension cells have a similar shape to peak II of the aggregative response and that they stop by t_6 (see Results), the refractory behaviour of oscillating cells reported by Gerisch *et al.* (1977), and the fact that these oscillations cease at the time of the signal transition on agar (Peacey & Gross, personal communication) all indicate that such oscillations are restricted to the aggregative stage. Here I have presented evidence that there is a change in the shape of the light-scattering response of cells in suspension to single or successive pulses of cAMP, and that this change becomes definitively established at the transition to the post-aggregative stage. Aggregative and post-aggregative responses have been observed previously by Gerisch & Hess (1974), but the difference between them appears to have been overlooked probably because these workers used Ax2, which has a shorter aggregative response.

The experiments with added phosphodiesterase suggest that the duration of the released cAMP in the extracellular medium plays a significant role in controlling cellular response. Under conditions of rapid cyclic nucleotide hydrolysis the amoebae respond twice within 2 min at the aggregative stage, where they would normally be refractory to the second pulse. Malchow, Nanjundiah & Gerisch (1978) have recently shown that phosphodiesterase addition also affects the refractory properties of cells undergoing spontaneous oscillations. However my results indicate that internal processes also contribute to the change in response between the aggregative and post-aggregative stages. Thus amoebae at the two stages respond differently to high external concentrations of cyclic nucleotides, while addition of phosphodiesterase to aggregative stage cells does not generate a typical post-aggregative response.

I would like to thank Dr Julian Gross and my other colleagues for helpful discussion and advice.

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(*Received* 28 *June* 1978)