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pH OSCILLATIONS IN CELL SUSPENSIONS OF *DICTYOSTELIUM DISCOIDEUM*: THEIR RELATION TO CYCLIC-AMP SIGNALS

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SUMMARY

Cells of *Dictyostelium discoideum* known to release cyclic AMP (cAMP) rhythmically in the form of pulses, change with the same period of about 8 min the pH of their medium. The pH is used here as an indicator to investigate the effect of externally added cAMP pulses on the oscillations. Both a temporary increase in amplitude and a permanent phase shift can be induced. The phase-response curve indicates that the period can be increased and decreased by rhythmic stimulation with cAMP pulses.

INTRODUCTION

Aggregating cells of *Dictyostelium discoideum* respond chemotactically to cAMP (Konijn, van de Meene, Bonner & Barkley, 1967; Konijn, 1972; Robertson, Drage & Cohen, 1972). They are also able to stimulate other cells by the periodic release of cAMP (Gerisch, Hülser, Malchow & Wick, 1975; Shaffer, 1975; Roos, Nanjundiah, Malchow & Gerisch, 1975). The alternation of release and response gives rise to spatio-temporal patterns of cellular activities in an aggregation territory, either in the form of concentric propagated waves or of rotating spirals (Shaffer, 1962; Gerisch, 1968; Alcantara & Monk, 1974; Durston, 1973). These patterns are based on the coupling of individual cAMP-generating cells *via* cell surface receptors: stimulation by a cAMP pulse induces, with a delay of several seconds, the release of an about 100-fold larger cAMP pulse which then stimulates cAMP release from other cells (Gerisch & Malchow, 1976).

The biochemical analysis of the cAMP-signal system in *D. discoideum* has been greatly facilitated by the possibility of studying synchronous periodic activities in cell suspensions (Gerisch & Hess, 1974). Initially light-scattering changes have been used to monitor oscillations of cellular activities. Subsequently the co-oscillation of intracellular and extracellular cAMP-concentrations and the oscillatory control of adenylate cyclase have been demonstrated (Gerisch & Wick, 1975; Roos, Scheidegger & Gerisch, 1977). These oscillations are accompanied by periodic changes of the redox state of cytochrome b (Gerisch & Hess, 1974). Here we firstly report on oscillations of the extracellular pH, and then apply the pH recordings to the computation of phase-response curves.

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METHODS

D. discoideum strain Ax-2 was grown in the medium of Watts & Ashworth (1970) containing 1·8 % maltose. Clones 206 and 214, both derived from clone 110, were used throughout. To induce differentiation, Ax-2 cells were harvested at 3–8 × 10^6 cells per ml, washed three times and resuspended in 17 mM Soerensen phosphate buffer, pH 6·0. Usually 1–2 h later the amoebae were washed twice in unbuffered salt solution containing 10 mM NaCl, 10 mM KCl and 3 mM $CaCl_2$ (Bonner, 1947), and resuspended at 2 × 10^7 cells per ml. Ten millilitres of the suspension were stirred at 23 °C and aerated through a Pasteur pipette with 25 ml oxygen per min. During the first hour the pH drifted from about 5·5 to 7·2; thereafter it increased more slowly up to 7·7. All measurements were done between pH 7·2 and 7·7, using a Metrohm EA 125 or Ingold LOT M3405 glass electrode together with a Metrohm E 510 pH-meter. Light scattering was measured as described previously (Gerisch & Hess, 1974). For calibration purposes, constant influxes of 1 or 2 mM HCl were produced using an Ingold dosimeter driven through a gear by a synchro-motor.

RESULTS

Sustained oscillations of the extracellular pH

About 4 h after their removal from nutrient medium and suspension in an unbuffered salt solution, cells started a periodic change in the extracellular pH (Fig. 1). The period varied between 6 and 9 min, the amplitude was about Δ pH = 0·01. Buffering due to cell surface proteins and CO_2 production during respiration was unavoidable. The recorded pH changes can be considered as net changes resulting from processes which periodically increase the extracellular H^+ concentration, and from non-periodic processes acting in the opposite direction. The decline of the extracellular H^+ concentration after an excursion from its steady state can be demonstrated by the addition of HCl (Fig. 2). The decline can be explained by removal of CO_2 from the cell suspension through the oxygen stream, possibly complemented by ion exchange between the extracellular and intracellular compartments.

To quantitate the buffering capacity and the rate of H^+ changes, the pH changes during and after a constant influx of HCl were recorded. The initial rate of H^+ increase was a linear function of the rate of inflow (Fig. 2C) and was therefore used to calculate the buffering capacity. During continuous inflow of HCl the pH reached plateaus which depended on the rate of inflow, indicating its equilibration with the rate of H^+ decrease (Fig. 2A). After cessation of the HCl influx the initial rate of H^+ decrease proved to be a linear function of the H^+ excursion, within the limits of experimental error (Fig. 2B). This plot was used to calculate the rate of H^+ decay during spontaneous pH-oscillations. This is shown in Fig. 1 for 2 cases differing in the shape of the pH oscillations. The maxima and minima of the rates of H^+ increase coincided with the inflexion points of the pH curves (Fig. 1). Integration of the rate curves yielded a mean of 10·7 μmol H^+ per pulse per l. for Fig. 1A, and 3·7 μmol H^+ for Fig. 1B. In both cases the suspension contained 2 × 10^7 cells per ml. The mean numbers of protons generated per cell per pulse were therefore 3·3 and 1·1 × 10^8, respectively.

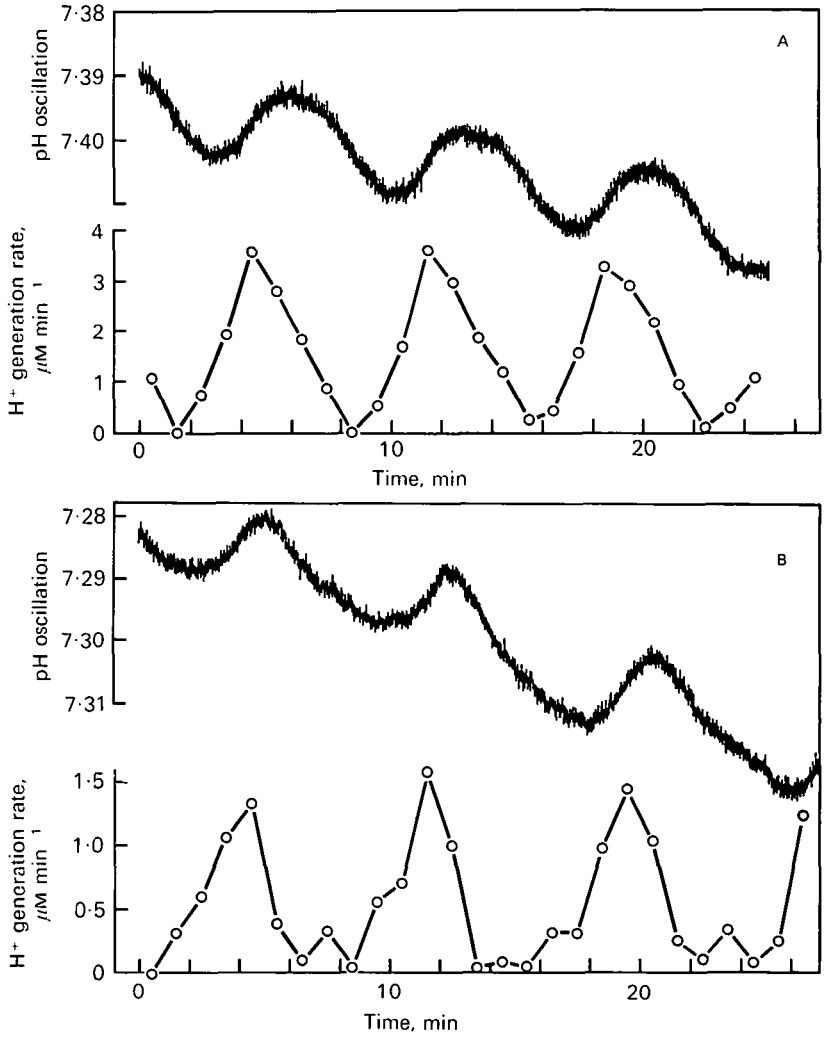

Fig. 1. Two examples of spontaneous oscillations (A, B) of extracellular pH (top curves), and of the rates of H^+ generation (○). The maxima and minima of the rate curves coincide with the inflexion points of the pH curves. The rates of H^+ generation were calculated from Fig. 2B, and the minima were set to zero. Buffering in the cell suspension was measured by test pulses of HCl.

Relationship of pH oscillations to cAMP pulses

Peaks of decreased light scattering are known to mark the phase in which cyclic-AMP pulses are formed by the activation of adenylate cyclase (Roos *et al.* 1977). In order to correlate the pH oscillations with cAMP production, light scattering was measured at the same time as pH. Before the onset of spontaneous oscillations, application of cAMP pulses elicited changes similar to those generated spontaneously (Fig. 3). After the cessation of pulsatile cAMP application, the changes in both light scattering and pH continued in the form of autonomous oscillations.

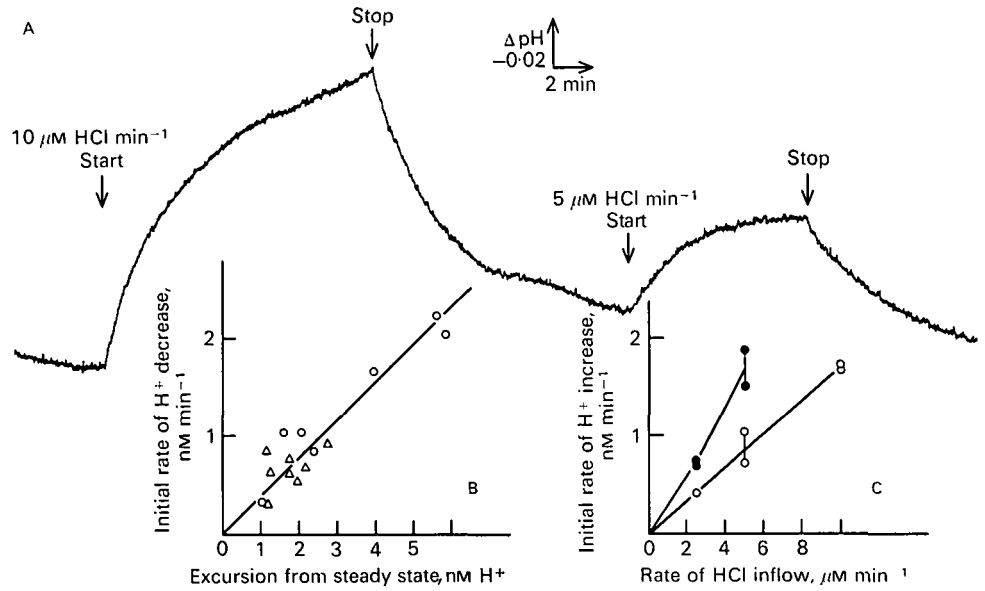

Fig. 2. pH changes during and after continuous inflow of HCl. A, excursions of the pH in response to 2 different rates of HCl inflow. B, the initial rate of H^+ decrease after cessation of inflow, as a function of the excursion from the basal H^+ concentration (data from 2 experiments: △, ○). C, rate of H^+ increase at the beginning of HCl inflow in 2 experiments (●, ○). Varying slopes indicate differences of the buffering capacity.

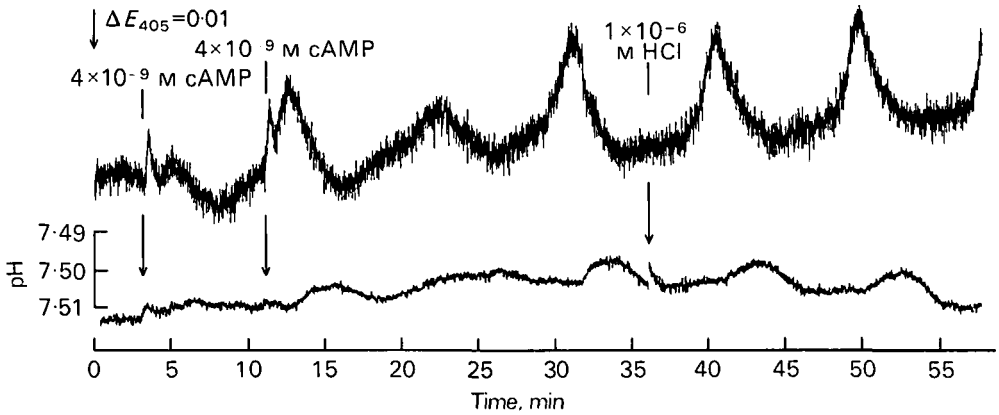

Fig. 3. Beginning of autonomous oscillations (right) after a series of cAMP pulses (left). Top, light-scattering changes; bottom, induced and spontaneous pH changes. 1 μM HCl did not cause a response in light scattering.

In all experiments light scattering and pH oscillated with the same period and with approximately the same phase relationship (Table 1), suggesting that both variables are coupled to the same oscillating system which also controls the activity of adenylate cyclase. In all cases the peaks of the extracellular H^+ concentration were preceded by the light-scattering peaks. With the exception of one out of 7 experiments, this

applied also to the inflexion points of the pH curve (Table 1). This, together with the results shown in Fig. 1, indicates that the highest rate of H^+ increase was reached after the light-scattering peak. According to Table 1 the mean phase difference between the light-scattering peak and the pH minimum was 0·31 periods (= 112°). Between the light-scattering peak and the maximal rate of H^+ increase the mean difference was 0·12 periods (= 43°).

Table 1. *Temporal relation of the pH oscillations to the light-scattering peaks*

Experiment no.	Interval between light-scattering peaks and pH minimum, min	Interval between light-scattering peaks and steepest pH decrease, min	Mean period, min
1	2·6	0·6	8·5
2	1·9	−0·2	8·2
3	2·2	0·9	7·7
4	2·6	1·3	8·1
5	2·7	1·4	8·0
6	2·5	1·3	9·1
7	2·7	1·3	7·0
Mean	**2·5**	**0·9**	**8·1**

Averages from 2 to 5 periods per experiment. According to Fig. 1 the steepest pH decrease coincides with the highest rate of proton increase in the extracellular medium. Light-scattering peaks were found to precede the peak concentrations of intracellular cAMP on the average by 0·5 min, and those of extracellular cAMP by 1 min (Gerisch & Wick, 1975).

The oscillations of light scattering and the periodic activities of aggregation centres show a temperature dependence corresponding to an activation energy of about 16 kcal mol^{-1} (Nanjundiah, Hara & Konijn, 1976; Wurster 1976; Gross, Peacey & Trevan, 1976). The pH oscillations showed the same temperature dependence as those of light scattering: from 21 to 30 °C the frequency of both oscillations increased by a factor of 1·6.

Using light scattering as an indicator, cAMP pulses have been found to shift the phase of the oscillations (Gerisch & Hess, 1974). The other oscillating variables, cAMP formation and pH, showed a similar phase shift (Fig. 4), indicating that they remained coupled to the light-scattering changes.

HCl pulses did not induce phase shifts, nor did they elicit light-scattering changes (Fig. 3), which excludes that any one of these responses is caused by the observed changes of the extracellular proton concentration.

The phase–response curve

Depending on the phase of the oscillations at which a cAMP pulse was applied, either phase advances, delays, or no shifts were obtained (Fig. 5). In Fig. 6 the difference between the old and the new phase is plotted as a function of the phase at which the cAMP pulse was given. This phase-response curve has a shape such as is characteristic of a signal that strongly interacts with the oscillator (Type O resetting in

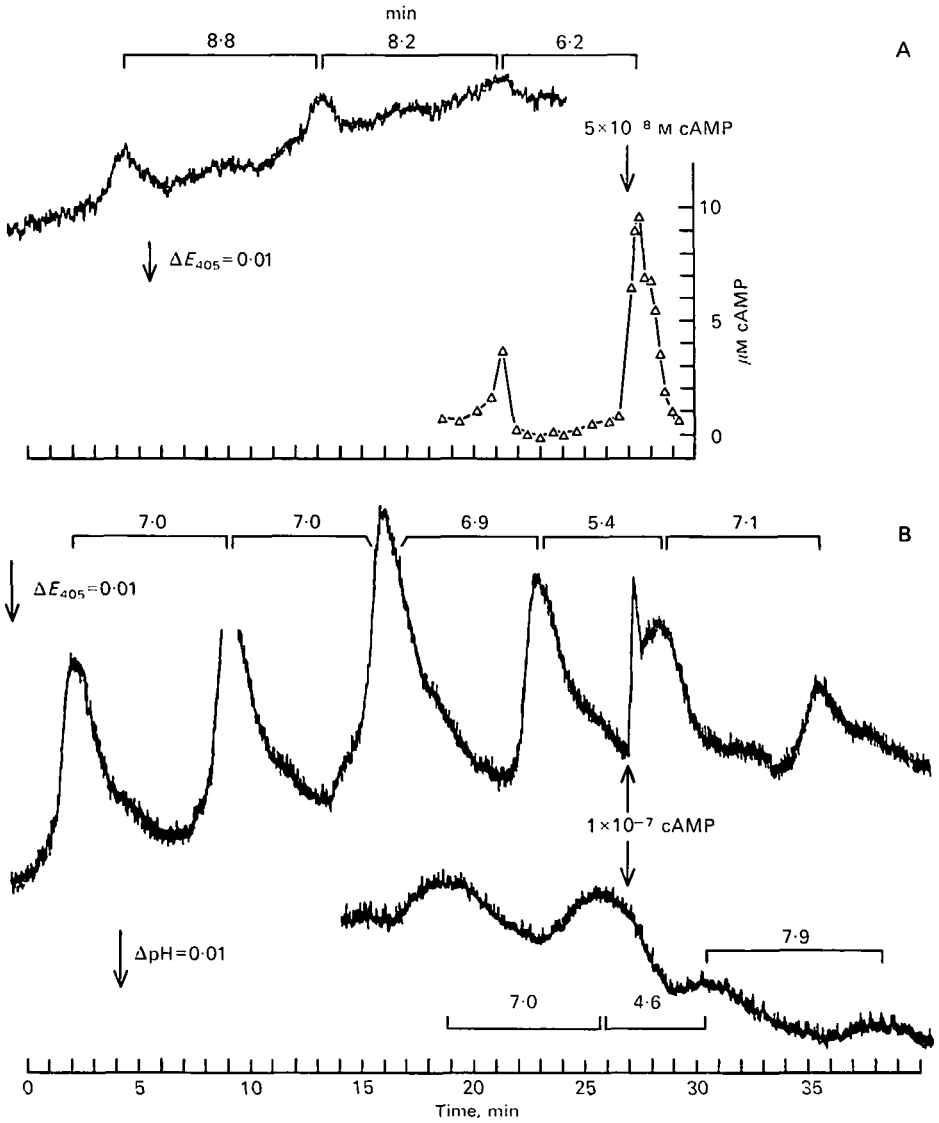

Fig. 4. Phase advances induced by cAMP pulses. A, recording of light scattering (top) and measurement of the total cAMP in the cell suspension (△). The last spontaneous light-scattering peak was small and, accordingly, the cAMP increase was low. The applied cAMP pulse amplified the cAMP amplitude. (Light scattering could not be recorded at the end of the experiment because the cell suspension was used for cAMP samples.) cAMP was determined by the Gilman assay as described (Gerisch & Wick, 1975) and plotted as concentration per l. of densely packed cells. The cell concentration was 5×10^7 cells per ml. B, light scattering (top) and concomitant phase shift in pH oscillations (bottom).

the terminology of Winfree (1977)). During and immediately after the light-scattering peaks no phase shifts were observed. This time coincides with the phase of autonomous formation of cAMP pulses, indicating unresponsiveness of the oscillating system during and after adenylate cyclase activation.

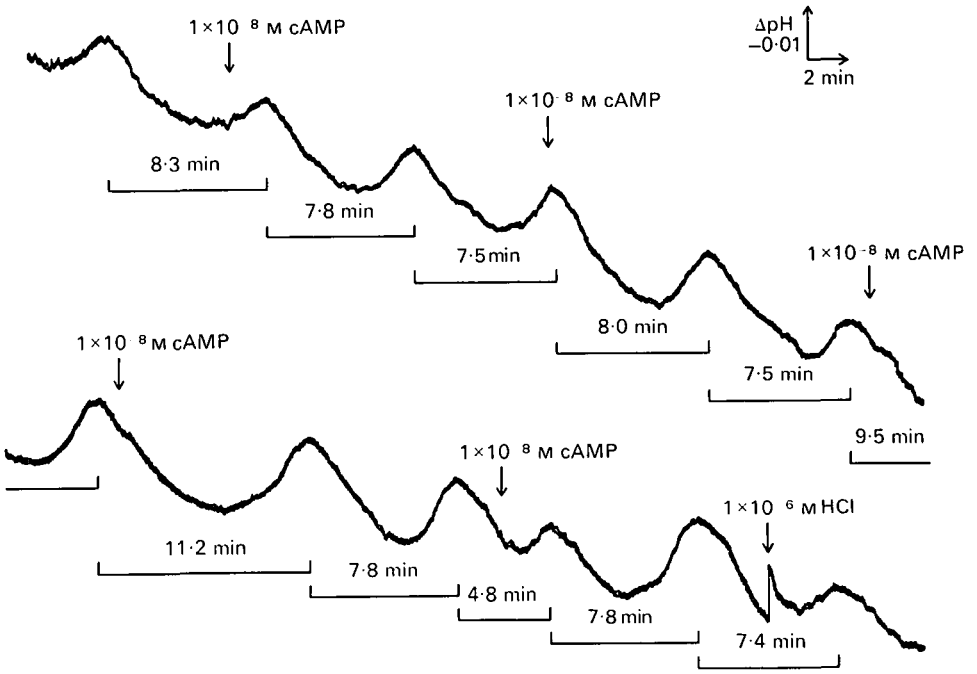

Fig. 5. Phase advances and delays induced in pH oscillations by cAMP pulses.

Phase–response curves provide information about the range of entrainment of the endogenous oscillations by stimuli of various periods. According to Fig. 6 harmonic entrainment might be possible within the limits of $T_f(1-0{\cdot}4) < T_s < T_f(1+0{\cdot}4)$. ($T_f$ free-running period of the *D. discoideum* oscillator; T_s period of extraneous cAMP pulses). The mean of T_f was 7·8 min in these experiments, so that the limits for entrainment correspond to $4{\cdot}8 < T_s < 10{\cdot}9$ min. These limits for entrainment are applicable only to cAMP pulses of the shape and size used.

For various reasons the actual range of stable entrainment might be smaller than indicated by the above limits. Some peculiarities and uncertainties of the responses which have implication for the interpretation of these data must be discussed here. Sometimes a cAMP pulse given at about 0·5 of the period caused a shoulder on the pH curve. This can be seen twice in Fig. 5. Although a small pulse of cAMP was possibly formed in these cases, only the next pulse was evaluated. Accordingly, the phase shift was plotted as a delay. This observation shows that the response to cAMP pulses is not of the all-or-none type.

In one of the experiments shown in Fig. 6, cAMP-phosphodiesterase was added in order to lower the background level of cAMP. In this case the region of phase advance was extended to the left. This suggests that the shape of the phase-response

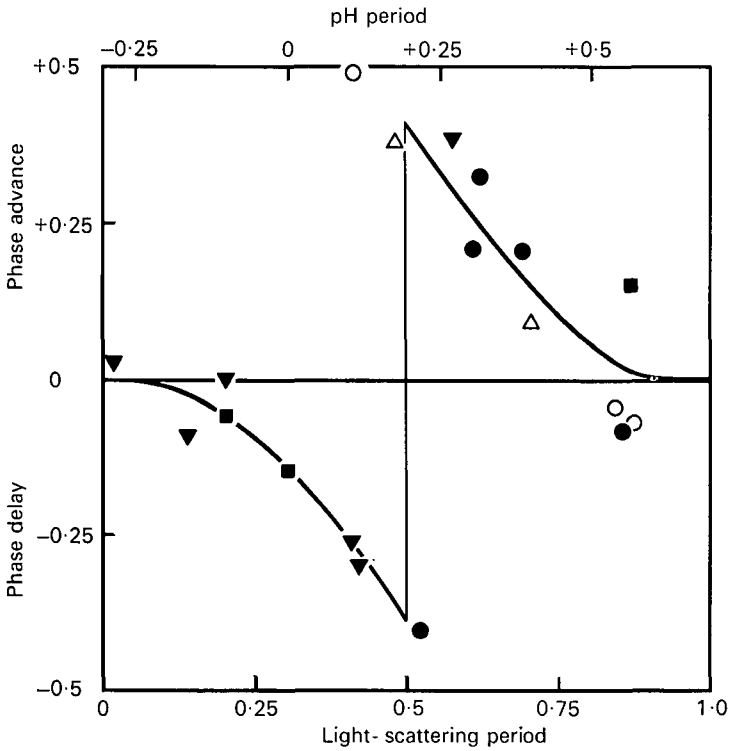

Fig. 6. Phase-response curve compiled from 5 experiments (different symbols). Phase advances and delays are plotted as a function of the phase of pH oscillations at which perturbing cAMP pulses were given. The pulse size was either 5 nM (open symbols) or 10 nM cAMP (closed symbols). The mean period was 7·8 min, and all periods were normalized to 1. In the pH period at the top, the zero point is the phase of the pH minimum. The light-scattering scale on bottom is calculated from the pH oscillations using the data of Table 1. Phases 0 and 1 correspond to the peaks of decreased light scattering shown in Figs. 3 and 4. In one experiment (○) partially purified cAMP-phosphodiesterase of *Dictyostelium purpureum* was added. Activity, 14 nmol cAMP per ml per min, measured at 35 °C. The phase shift was manifest already during the period in which the perturbing cAMP pulse was given (Fig. 5). Therefore the shift was evaluated immediately in order to reduce errors caused by frequency changes (Fig. 7C). Only if the pH minimum occurred at less than 0·25 cycles after the perturbing pulse was the following period evaluated. By checking also the subsequent periods it was shown that the data plotted here provide reliable estimates of permanent phase shifts.

curve is affected by the background of cAMP against which the cells have to detect an extraneous pulse.

Sometimes an extraneous cAMP pulse increased the amplitude of the oscillations. This effect was evident when the pulse was given just before or at the beginning of an autonomous pulse (Fig. 7). It indicates that the system does not always oscillate with its maximal amplitude. This is also evident from the pulse-size measurements shown in Fig. 4A. The increase in pH amplitude was always limited to one period. In the subsequent periods the amplitudes were sometimes lower than before the extraneous pulse (Fig. 7A), or they even became undetectable (Fig. 7B).

Signal pulses which affect only the state variables of the oscillating system should not permanently change the frequency of free-running oscillations (Pavlidis, 1973). A pulse of cAMP, however, sometimes increased the frequency abruptly, and no return to the original level was observed (Fig. 7 c). This implies the possibility that cAMP, in addition to its effect on the state variables, may also change parameter values of the oscillating system.

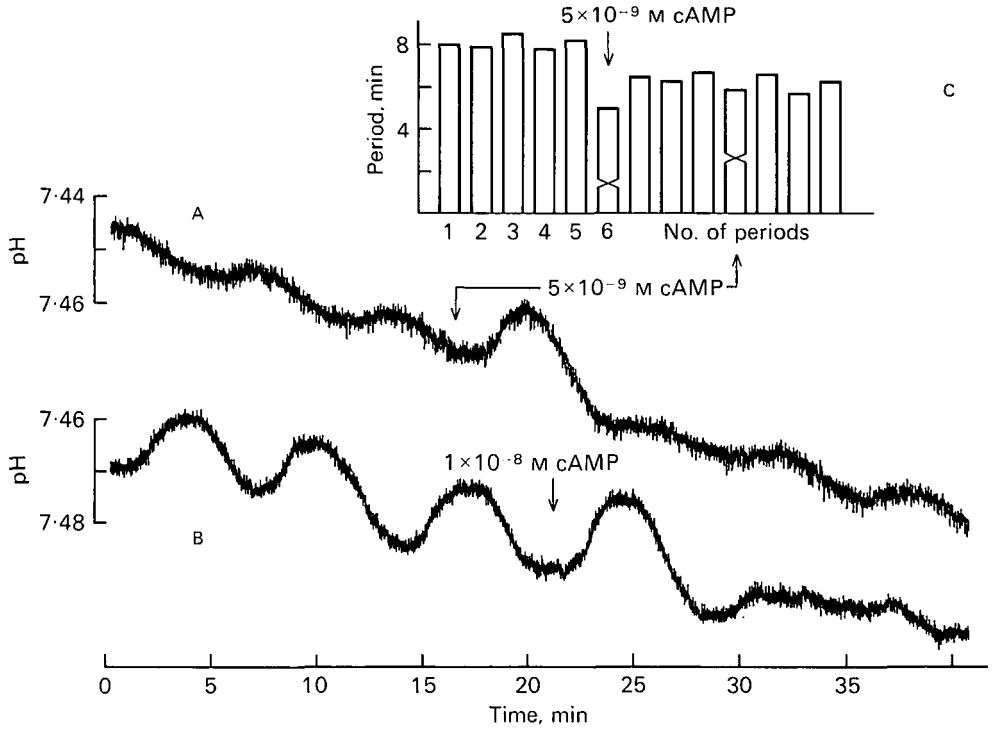

Fig. 7. Modification of the amplitude and frequency of pH oscillations by a cAMP pulse. A, temporary increase in amplitude after a cAMP pulse given in step with the autonomous oscillations. B, cessation of the oscillation after a cAMP pulse, as has been repeatedly observed. C, increase of the frequency in response to a cAMP pulse in the same experiment as shown in A. The constriction of the bar no. 6 indicates the time of cAMP application, which was at the beginning of a pH period. The second cAMP pulse (bar no. 10) did not cause a further increase of the frequency.

DISCUSSION

The periodic pH changes reported here can be used as convenient indicators to detect oscillations and to monitor their phase in a cell suspension. The pH changes also provide an indication of biochemical reactions which accompany periodic cAMP synthesis. Both the highest rate of extracellular proton increase and of cAMP release were found at 1 min after the light-scattering peak (Table 1). If hydrolysis of the released cAMP were the basis of the pH oscillations, the H^+ increase should be in a stoichiometric relationship to the amount of cAMP produced. Previous estimates of the pulse size yielded a value of 1×10^7 cAMP molecules per cell (Gerisch & Malchow, 1976). The data derived from Fig. 1 yield a value of 1 to 3×10^8 protons per pulse per

cell. The cell concentration and the composition of the medium were not identical under both conditions. Nevertheless, the large divergence of the number renders it unlikely that most of the protons result from phosphodiesterase action, although this will undoubtedly contribute. Another oscillating source of protons is a periodic change of the CO_2 output from the cells (Gerisch *et al.* 1977). The situation may be complicated by the superposition of multiple reactions contributing to the pH changes and reaching their peaks at different phases of a cycle. This is suggested by the shape of the oscillations shown in Fig. 1B, and also by a biphasic pH-change observed after cAMP stimulation in the absence of autonomous oscillations (Nanjundiah & Malchow, 1976; Malchow *et al.* 1977).

In the present paper emphasis has been focused on perturbations of the oscillating system by cAMP pulses, using pH as an indicator both of phase shifts and of modifications of the amplitude. The phase-response curve of Fig. 6 shows an interval of about 0·35 cycles (= 2·7 min) during which no effect on the oscillating system is obtained. This interval coincides with the time during which the intracellular cAMP concentration increases and the cAMP is released as a signal (Gerisch & Wick, 1975; and Table 1). The time after signalling during which no cAMP-pulse can be elicited by a cAMP-stimulus, has been referred to as the refractory phase for signal relay (Gerisch, 1968). Under our conditions this is the interval of about 0·30 cycles (= 2·3 min) during which a phase delay is induced. This estimate of the refractory phase is based on the assumption that the formation of every cAMP pulse is detected by an accompanying pH change; and it applies to perturbing cAMP-stimuli of 5–10 nM, a size which is likely to be reached near the surface of responding cells under 'normal' conditions of cell aggregation. The refractory phase is most probably not absolute. This means the phase-response curve may change with the size of the stimuli. Even with the stimuli used here shoulders on the pH curves indicate small responses during the phase of relative refractoriness (Fig. 5).

The maximal phase advance was nearly 0·5 cycles in the experiments shown in Fig. 6. A slight change in either the parameter values of the oscillating system or in the size of the stimuli might shift the phase advance to $\geqslant$ 0·5 cycles. Then it might be possible that cAMP signals entrain the system with half of its free-running period. Such a change may be related to the abrupt increase in frequency of propagated signals by multiples of 2, which have been observed during development (Durston, 1974; Gross *et al.* 1977).

As shown by the phase–response curve, *D. discoideum* cells can be entrained also at frequencies lower than their free-running oscillations. In a cell layer elements with relatively low frequency would tend to decrease the frequency of other oscillators in their vicinity, thus inhibiting centre formation. Elements with a high frequency would tend to initiate propagated waves, thus functioning as aggregation centres. An analogous behaviour has been analysed in pacemaker neurons of abdominal ganglia in *Aplysia* (Perkel *et al.* 1964; Winfree, 1977).

The increase of the amplitude in response to a cAMP pulse, as shown in Fig. 7, indicates that pulse formation during spontaneous oscillations depends upon a subtle control system, rather than being an all-or-none response. This effect indicates that

not only frequencies but also amplitudes are variables to be taken into account when the coupling of oscillators is studied as a basis of pattern formation in cell aggregation of *D. discoideum*.

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