J. Cell Sci. **20**, 21–27 (1976)
Printed in Great Britain

THE DEVELOPMENT OF THE RELAYING COMPETENCE IN *DICTYOSTELIUM DISCOIDEUM*

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SUMMARY

The fraction, X_2, of a population of *D. discoideum* cells competent to relay an aggregative signal has been measured as a function of time, t, spent in interphase. $X_2(t)$ is less than 0·1 up to 5 h, increases slowly to 0·4 by 8 h 20 min, and saturates at 1 by 10 h.

The hypothesis that cellular interactions might enhance $X_2(t)$ was tested by mixing populations of cells set into interphase at different times. No interactions were found. External stimulation did not affect $X_2(t)$.

INTRODUCTION

In this paper we report the results of measuring $X_2(t)$, the proportion of cells in a synchronized population of interphase amoebae capable of relaying a pulsed signal when stimulated by a pulse of cAMP released from a microelectrode. Such a signal can initiate and control aggregation, replicating a normal centre (Robertson, Drage & Cohen, 1972). Artificial stimulation is necessary as $X_2(t)$ approaches unity while $X_3(t)$ is still zero. That is, almost all cells are capable of relaying a signal before there are any autonomous signals to relay. In order to make accurate measurements of $X_2(t)$ interphase cells must be well synchronized. This is accomplished by centrifugation of vegetative cells from growth plates, freeing them of the food bacteria. It must be done while all cells are still in the vegetative state (see Bonner, 1963, 1967, for a review). (See the accompanying paper for more details (Gingle, 1975).)

METHODS

The experimental methods are as published previously except where differences are noted below (Robertson & Drage, 1975; Cohen, Drage & Robertson, 1975). For symbols used see p. 27.

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Culturing

Growth cultures of *D. discoideum* (NC-4) were prepared in the following manner. Suspensions of *Aerobacter aerogenes* were inoculated with *D. discoideum* spores and then plated on to nutrient agar growth plates. The cultures were then incubated at 22 °C for 30 h. At this time approximately 10^9 cells were harvested per plate. Amoebae were washed from plates in cold phosphate buffer and separated from the food bacteria by differential centrifugation. The amoebae were resuspended in buffer and diluted to the desired concentration using a haemocytometer. Suspensions (0·05 ml) were then plated on to 2 × 2 cm buffered agar blocks for filming. Times into interphase and cell density (cells/cm^2) were recorded for each field of cells prepared.

Filming

The agar 'filming' blocks were placed in clear plastic boxes with optically flat windows and provisions for a microelectrode. A water-soaked square of filter paper was also placed in the filming box to maintain proper humidity conditions. The temperature was maintained at 22 °C while filming.

Fields of *D. discoideum* were filmed with Nikon microscopes equipped with Bolex H-16 movie cameras and Nikon CFMA camera drives. Rates of 2, 4, or 7·5 frames per min were used depending on the needs of the experiment. All experiments were filmed with Kodak 4x-R, 16-mm film. Typically, filming was preceded by positioning a microelectrode on to the *D. discoideum* field on the agar block surface.

The microelectrode, filming chamber, and associated electronics have been described (see fig. 1 of Cohen *et al.* 1975). The microelectrodes consisted of glass micropipettes containing buffered solutions of 10^{-4} or 5×10^{-5} M cAMP. Electrical contact was made via a chlorided silver wire. The electronics consisted of a Grass stimulator Model no. S88 driven by a timing pulse generator and coupled to the microelectrode via a Grass isolation unit Model no. S1V5. Typically, the microelectrodes were pulsed with 2 s, 10 μA, negative pulses every 5′. A positive bias was applied to prevent cAMP leakage between pulses. Therefore the microelectrodes pulsed cAMP into the field of amoebae at intervals of 5′. In early experiments the pulsing was begun within an hour after starvation. However, as data accumulated pulsing was typically begun approximately 100′ before the expected time for field-wide relaying. The data obtained in both cases were identical.

Measurement

All measurements were taken from the time-lapse films, the running records of the experiments. The initiation times for field-wide relaying, that is the time at which the microelectrode first initiated a propagated wave, were measured. Cell densities were measured by counting the number of cells and dividing by the field area. All errors in the time measurements were within ± 20′ real time. While the filming interval was typically 15 s, the errors in wave recognition were between 10′ and 20′ because the pattern of cell movement waves was only recognized with the film in motion at 24 frames/s. Thus, an observation error of 3 s would correspond to an 18′ real time error. Errors in cell density were typically ± 2 %.

Theory of measurement

When the microelectrode begins pulsing cAMP into the field the amoebae, having been centrifuged free of bacteria, are developing the competences for aggregation. When a cell becomes relaying competent it responds to a super-threshold concentration of cAMP by moving towards the source and secreting a pulsed signal, probably also of cAMP, to be received by neighbouring cells. However, as the concentration profile of the signal decreases with respect to distance (Cohen *et al.* 1975), the relaying signal has a finite range beyond which relaying cannot be stimulated (Cohen & Robertson, 1971). The decreasing concentration profiles are due to the hemispherical diffusion of the cAMP as well as to the presence of an enzyme, probably phosphodiesterase (PDE), which is secreted and degrades the signal (Chang,

1968; Robertson & Grutsch, 1974). The signal range R is a function of cell density and of time after centrifugation because enzyme activity is also a function of both and because at sufficiently high densities many cells will contribute to the signal.

Because there is a finite relaying signal range, there is a critical density N^* of relaying cells below which long-range signal propagation cannot occur (Cohen & Robertson, 1971). Therefore, field-wide signalling cannot occur until the critical density of relaying-competent cells is reached during interphase. When this occurs the microelectrode excites field-wide signal propagation, often in the form of circular waves which can easily be seen in the time-lapse films. At the onset time, t^*, the fraction of relaying-competent cells in a field of total cell density N is $X_2(t^*) = N^*/N$. Repeating the experiment with fields of different cell densities, each with a characteristic t^*, generates an $X_2(t)$ curve. Proper interpretation of the results is contingent upon knowing critical density, which is an increasing function of N and t, partly because of enzyme secretion (Cohen & Robertson, 1972; Robertson & Cohen, 1974). Critical density has been measured as a function of N and t. While we use the data here, the measurements are reported separately. The technique is described in the accompanying paper (Gingle, 1975).

RESULTS

The relaying competence

The $X_2(t)$ data were first plotted assuming a constant critical density of $2{\cdot}5 \times 10^4$ cells/cm^2, the limiting value of critical density. That is, for a field of cell density N,

$$X_2(t^*) = \frac{2{\cdot}5 \times 10^4/\text{cm}^2}{N},$$

where t^* is the initial time for field wide relaying. The data are plotted as the broken line in Fig. 1. The curve represents data from 21 experiments. As critical density is

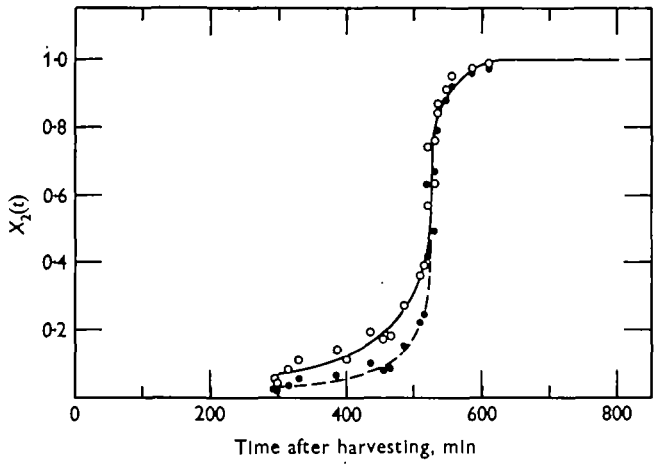

Fig. 1.

not a constant, but rather an increasing function of cell density and developmental time, the $X_2(t)$ data were also corrected for critical density variation, and the corrected data are plotted as the solid line in Fig. 1. This is the true fraction of cells which are relaying competent, as a function of time.

Possible interactive effects

In addition to the above experiments which yielded values of $X_2(t)$, we performed mixed population experiments to test for interactive effects on the development of relaying. In these experiments, early populations of cells were harvested and plated a time t_D before late populations. The remainder of the experiment, positioning of the microelectrode, filming, etc., was as before.

The results of these experiments, the t^*'s obtained for mixed fields, indicate that the early and late populations develop independently and that interactions between cells do not play any significant role in relaying development. The data, from 3 such experiments, are shown in Table 1 along with predicted results to be discussed in the next section of this paper.

Table 1. *Effect of interactions on* $X_2(t)$

Experimental data				Expected results		
N(Early), cells/cm^2	N(Late), cells/cm^2	t^*, min	t_D, min	t^*_{EL} (Early advances Late), min	t^*_{LE} (Late advances Early), min	t^*_{Ind} (Independent development), min
$2{\cdot}42 \times 10^5$	$2{\cdot}42 \times 10^5$	468	150	380	530	455
$3{\cdot}00 \times 10^5$	$3{\cdot}00 \times 10^5$	442	150	355	505	435
$3{\cdot}32 \times 10^5$	$3{\cdot}32 \times 10^5$	417	150	345	495	425

Table 2. *Effect of applied signal on* $X_2(t)$

Microelectrode concn. of cAMP, M	Cells/cm^2	t^*, min	t^* (expected from 10^{-4} M cAMP data), min
5×10^{-4}	$1{\cdot}02 \times 10^5$	513	513
5×10^{-4}	$5{\cdot}08 \times 10^4$	529	525
10^{-3}	$3{\cdot}00 \times 10^4$	536	536
10^{-3}	$2{\cdot}61 \times 10^4$	586	585
10^{-3}	$2{\cdot}50 \times 10^5$	436	455
10^{-2}	$3{\cdot}67 \times 10^5$	384	390
10^{-2}	$2{\cdot}57 \times 10^4$	608	600

Effect of applied signal on $X_2(t)$

Finally, we varied microelectrode cAMP concentration between 10^{-2} and 10^{-4} M to see whether there was any effect on $X_2(t)$ development as observed from the onset times for relaying. We also varied the starting time of microelectrode pulsing from several hundred minutes to 30 min before t^*. Neither manipulation had a detectable effect as seen in Table 2.

DISCUSSION

Shape of the function

The $X_2(t)$ plot shows a rapid transition of populations of amoebae to the relaying state shortly after 500 min. The fraction of relaying-competent amoebae increases from 0·1 to 0·9 in a 200-min period. This corresponds well with the cell division cycle, which is about 210 min under our growth conditions. That is, the harvested amoebae have a distribution of phases in their cell division cycle which introduces a broadening into the $X_2(t)$ curve. This mitotic phase distribution is probably responsible for at least

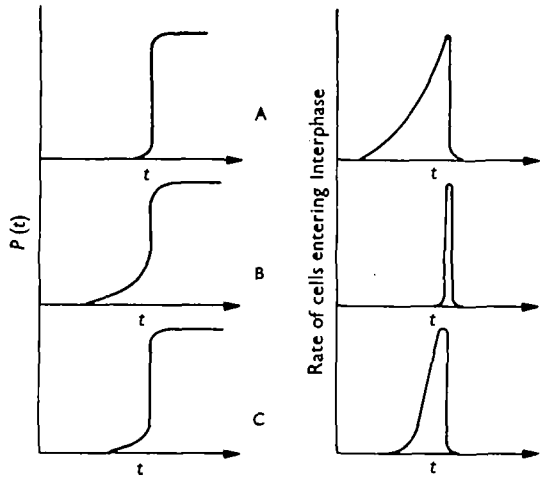

Fig. 2.

some of the broadness and skewness of the $X_2(t)$ curve. $X_2(t)$ can be related to the mitotic phase distribution by the integral

$$X_2(t) = \int_0^\infty dt' P(t-t') R(t'), \tag{1}$$

where $R(t')$ is the rate of amoebae entering interphase at t' and $P(t-t')$ is the probability for becoming relaying competent by t when entering interphase at t'. Possible effects of the mitotic phase distribution are represented schematically in Fig. 2. The 2 extremes and an intermediate case are shown in parts A, B, and C respectively. Fig. 2A represents a broad and skewed mitotic phase distribution which almost completely accounts for the broadness in the $X_2(t)$ data. The corresponding $P(t)$ curve is very sharp. Fig. 2B represents the opposite extreme in which the mitotic phase distribution is sharp with the corresponding $P(t)$ curve similar to the $X_2(t)$ data. Experiments using 'heat shocks' to better synchronize cell mitotic phases should sharpen the $X_2(t)$ data and thereby determine the actual phase distribution in amoebae after normal harvesting.

Cell interactions

The possible role of cell-cell interactions in relaying development was studied with mixed population experiments. In these experiments, there were 2 extreme possibilities: first, the early population, N_E, completely advances the late population, N_L, yielding a t^*_{EL} characteristic of a population (N_E+N_L) of early cells; second, the late population completely retards the early population yielding a t^*_{LE} characteristic of a population (N_F+N_L) of late cells. If no interactions occur, the t^*_{lin} will be characteristic of a linear superposition of the early and late populations developing independently. The three cases are, in summary:

(1) Early advances Late: $(N_E+N_L)X_2(t^*_{EL}) = N^*$
(2) Late retards Early: $(N_E+N_L)X_2(t^*_{LC}-t_D) = N^*$
(3) No interactions: $N_E X_2(t^*_{lin})+N_L X_2(t^*_{lin}-t_D) = N^*$,

where t_D is the delay time between early and late harvesting and N^*'s were determined by the technique described above. Therefore the onset time t^* is

$$t^*_{EL} < t^* < t^*_{LE},$$

and is equal to t^*_{lin} in the case of no interactions, as was found in the experiments (Table 1). In these experiments we chose $N_E = N_L$ so that t^*_{lin} would be approximately midway between t^*_{EL} and t^*_{LE}.

Effects of stimulation

When the $X_2(t)$ results are interpreted, a natural question is whether or not the artificial stimulus, the microelectrode, had any effects on the natural time course of relaying development. Any possible effects could, of course, depend on pulse concentration or on the total number of pulses. Neither variation in cAMP pipette concentration over 2 orders of magnitude, nor variation in total number of pulses before onset, had any detectable affects on the X_2 time development (see Table 2). Therefore the results obtained are indeed the fraction of relaying-competent cells as a function of developmental time.

Significance of these results

After the removal of food the amoebae undergo a series of differentiations leading to chemotactic sensitivity, relaying, and finally autonomy. The extent to which each of these competences is independent of the other is of major importance to an understanding of the signalling system. A detailed and quantitative understanding of the wild-type characteristics such as $X_2(t)$ is necessary to characterize and quantify the individual competences. It is noteworthy, for example, that $X_2(t)$ saturates before autonomy begins, just as $X_1(t)$ saturates before relaying can occur (Robertson *et al.* 1972). Thus it is possible to obtain a population of cells which has attained one competence, but has not yet begun to manifest the next. Therefore, the cell structure, membrane, extracellular medium, etc., can be monitored or asayed before and after each competence develops. Variations observed in each case can then be correlated

with the differentiations leading to a particular competence. This feature should help work on the biochemistry of differentiation.

This work was supported by NSF grant No. GB-30784 and NIH grant No. HD-04722 to the University of Chicago.

The authors are grateful for suggestions and criticism from Morrel H. Cohen.

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(*Received* 13 *June* 1975)

SYMBOLS USED IN TEXT

N	Total amoeba density.
N^*	Critical amoeba density for relaying.
N_E	Amoeba density in early population.
N_L	Amoeba density in late population.
$P(t-t')$	Probability of an amoeba becoming relaying competent at time t, if entering interphase at time t'.
$R(t')$	Rate of amoebae entering interphase at time t'.
t^*	Onset time for long-range signalling.
t_D	Delay time between harvesting late and early amoebae populations.
t^*_{EL}	Onset time if early amoebae advance late amoebae.
t^*_{LE}	Onset time if late amoebae retard early amoebae.
t^*_{lln}	Onset time for no interactions between amoebae.
X_1	Proportion of chemotactically competent amoebae.
X_2	Proportion of relaying-competent amoebae.
X_3	Proportion of autonomously competent amoebae.