The influence of pH on the choice between sexual and asexual development in *Dictyostelium mucoroides*

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The cellular slime mould *Dictyostelium mucoroides* exhibits clear dimorphism in development which, depending upon environmental conditions, leads to (1) macrocyst formation, a sexual process or (2) sorocarp formation, an asexual process. The present work was undertaken to determine the role of protons in the determination of the developmental mode. Intracellular pH (pH_i) was measured at the single-cell level microfluorometrically by use of the pH-sensitive fluorescent dye carboxyfluorescein. The proton-pump inhibitor diethylstilboestrol, the protonophore 2,4-dinitrophenol and a weak acid, propionate, were found to convert the developmental mode from macrocyst to sorocarp formation under conditions otherwise favouring macrocyst formation. All of these drugs lowered pH_i . Although no significant difference in pH_i was detected between dissociated cells shaken in buffers of pH 5.0 and pH 7.0, a marked difference in pH_i arose between the two if 5 mM- NH_4Cl was included to simulate the expected microenvironment in the cell mass during development. Comparison of the pH_i of cells developing toward sorocarps with that of cells developing toward macrocysts revealed a few cells with extremely high pH_i (> 7.30) in the latter population. The results obtained suggested that pH_i , or something related to pH_i , influences the developmental choice.

Introduction

Vegetative cells of cellular slime moulds divide and increase in number as solitary amoebae while feeding on bacteria. Upon exhaustion of the bacterial food supply, starving cells gather together, forming aggregation streams. Some strains of *Dictyostelium mucoroides* exhibit clear dimorphism in development depending upon various physical and chemical factors: sorocarp formation is an asexual process whereas macrocyst formation occurs during the sexual process (Raper, 1951; Blascovics & Raper, 1957; Filosa & Dengler, 1972; Erdos *et al.*, 1973; MacInnes & Francis, 1974). During sorocarp development a tip is formed on top of the cell mass, and this is followed by the migration of a slug-shaped mass. The slug is eventually transformed into a sorocarp consisting of a mass of spores and a supporting stalk. At the early stage of macrocyst formation, large aggregates form first, but tip-like structures never develop. The aggregate is subdivided into smaller masses

(precysts). In each precyst there arises a cytophagic cell (zygotic giant cell) that is formed by the fusion of two cells (Amagai, 1989). The cytophagic cell increases in size, engulfing in turn all the other cells in the precyst. The enlarged cytophagic cell finally becomes surrounded by a thick wall to form a macrocyst. Under certain conditions, the mature macrocyst germinates to release many amoebae which initiate a new life-cycle (Filosa & Dengler, 1972; Erdos *et al.*, 1973; Nickerson & Raper, 1973; Abe & Maeda, 1986).

The developmental choice toward sorocarp or macrocyst formation in *D. mucoroides* 7 (Dm7) is determined by exogenous factors such as light, water and volatile substance(s) (Weinkauff & Filosa, 1965; Filosa, 1979). For instance, Dm7 cells form sorocarps in the light and macrocysts in the dark, whereas the mutant MF1, derived from Dm7, forms macrocysts even in the light (Filosa, 1979). Recently, ethylene was found to be an intrinsic macrocyst-inducing factor (Amagai, 1984, 1987), while cAMP, a chemoattractant at the aggregation stage, was found to inhibit macrocyst formation and induce sorocarp formation (Amagai & Filosa, 1984; Amagai, 1987). Amagai (1989) also found that ethylene specifically induces formation of zygotic giant cells through cell fusion.

Abbreviations: CFDB, carboxyfluorescein-dibutyrate; DES, diethylstilboestrol; DNP, 2,4-dinitrophenol; pH_i , intracellular pH; pH_o , extracellular pH.

In monolayer cultures of *Dictyostelium discoideum*, Gross *et al.* (1983) have demonstrated that extracellular pH (pH_o), exogenously added weak acids, bases, or proton-pump inhibitors affect the spore-to-stalk ratio, thus suggesting the intracellular pH (pH_i)-dependent choice of differentiation pathway. Recently, Van Duijn & Vogelzang (1989) have reported that the membrane potential of *D. discoideum* is mainly generated by an electrogenic proton pump and that decreasing pH_o or addition of diethylstilboestrol (DES) directly depolarizes the membrane.

pH_o is also known to be involved in determination of the developmental mode in *D. mucoroides*; higher pH_o (7.0–8.0) favours macrocyst formation, while lower pH_o (4.0–5.0) leads to sorocarp formation (Amagai & Filosa, 1984). The present work was undertaken to examine the role of protons in the choice between the sexual and asexual processes in *D. mucoroides*. The results show that pH_i , or something related to pH_i , could influence the mechanism that determines the developmental mode.

Methods

Organisms and culture. A wild-type *Dictyostelium mucoroides* (Dm7), a spontaneous mutant, MF1, derived from Dm7 (Filosa, 1979), and a mutant, BK1, derived from MF1 were used. MF1 cells, which form macrocysts even in the light, were used for the experiments with DES and 2,4-dinitrophenol (DNP) and for estimation of the relationship between pH_o and pH_i . BK1 is a strain that readily forms sorocarps.

Vegetative cells were grown with *Escherichia coli* B/r by shaking culture at 22 °C. They were harvested during the exponential growth phase and washed four times in Bonner's salt solution (Bonner, 1947). Dm7 and MF1 cells were put either on 1 ml 2% (w/v) agar (Difco) containing 20 mM-MES buffer (pH 7.0) in a plastic dish (3.5 cm diameter), or on 2 ml 2% (w/v) plain agar in a glass dish (3.5 cm diameter), at a density of 1×10^6 cells cm^{-2} . After the amoebae had settled on the substratum for 30 min, excess water was removed and the surface was allowed to dry. The cells were incubated at 22 °C for 2 h, and overnight at 4 °C to synchronize the developmental stage. Development was analysed by shifting the cells back to 22 °C. All developmental times refer to the time in hours after the shift to 22 °C. In the case of BK1 cells, the washed cells were put at a density of 5×10^6 cells cm^{-2} on cellulose membranes in 3.5 cm plastic dishes, which were then filled with 20 mM-MES buffer (pH 6.0). The plastic dishes were sealed with vinyl tape and incubated in the dark.

Application of DES, DNP and propionate. The proton-pump inhibitor DES and the protonophore DNP were applied to MF1 cells at various developmental stages after the temperature was shifted from 4 °C to 22 °C. Portions (1 ml) of the agar on which cells had been plated were transferred to the same volume of agar containing the drugs at concentrations twofold higher than those to be tested. The cells were allowed to develop at 22 °C in the light. Potassium propionate, a weak acid, was added to BK1 cells developing on cellulose membranes. This was followed by incubation at 22 °C in the dark. The final developmental forms were observed under a dissecting microscope after 30 h incubation.

Measurement of pH_i . pH_i was measured by a modification of the method of Inouye (1988) using a fluorescent pH indicator, carboxyfluorescein. Cells at various developmental stages were washed once by centrifugation and resuspended in 20 mM-potassium phosphate (pH 7.0) or 20 mM-MES buffer (pH 5.0 or pH 7.0). The cell suspension was put on the centre of a coverglass in the perfusion chamber to allow the cells to settle. A few minutes later, the buffer was removed and immediately carboxyfluorescein-dibutyrate (CFDB) solution (approx. 100 μM in 20 mM-potassium phosphate buffer, pH 6.0) was added. After about 3 min of CFDB treatment, the chamber was perfused with the buffer for 3 min, followed by measurement of pH_i . Perfusion was continued during the measurement.

The pH_i of many individual cells was calculated, based on a standard curve derived using 50 mM-potassium propionate and 25 mM- $(\text{NH}_4)_2\text{SO}_4$ (Inouye, 1988).

Chemicals. CFDB was kindly provided by Dr K. Inouye (Kyoto University, Japan). DES and DNP were purchased from Wako Chem.

Results

Effects of DES, DNP and potassium propionate on the developmental mode

Several drugs that might lower pH_i were tested to determine if they would affect the developmental pathways of *D. mucoroides*. Under the conditions favouring macrocyst formation (pH 7.0, in the light), DES was applied to aggregating MF1 cells (2 h after the temperature surrounding the culture plates was increased from 4 °C to 22 °C), followed by incubation for 30 h at 22 °C. In the presence of 5 μM -DES, masses of macrocysts were formed (Fig. 1b), though they were smaller than control macrocysts formed without DES (Fig. 1a). Both macrocysts and sorocarps were formed in the presence of 10 μM -DES (Fig. 1c). A higher concentration (15 μM) of DES was found to direct the developmental mode completely from macrocyst to sorocarp formation (Fig. 1d). Above 20 μM -DES, many cells failed to aggregate and cellular differentiation never occurred. To determine the effective period for DES, 15 μM -DES was applied to MF1 cells at various developmental stages (Fig. 2). The sorocarp-inducing activity was most conspicuous at the early aggregation stage (t_2 : 2 h after the temperature-shift of culture plates from 4 °C to 22 °C), while DES was no longer effective after aggregation streams had been formed (t_8), thus giving rise to macrocysts. Essentially the same results were obtained using Dm7 cells (data not shown).

When aggregating MF1 cells (t_4) were exposed to 100 μM -DNP, their development was converted from macrocyst to sorocarp formation (data not shown). Such a DNP-effect, however, was never observed with MF1 cells at a later developmental stage (t_8), which was also the case with DES. Potassium propionate (2 mM), was found to shift the development of BK1 cells from

Fig. 1. Photographs showing the effects of DES on the developmental mode of *D. mucoroides* MF1 cells. MF1 cells (t_2 cells) at a density of 1×10^6 cells cm^{-2} were incubated for 30 h at 22 °C on agar (pH 7.0) containing various concentrations of DES, as described in Methods. (a) Control without DES. Large masses of macrocysts were formed. (b) 5 μM -DES. Small masses of macrocysts were formed. (c) 10 μM -DES. Both macrocysts and sorocarps were formed. (d) 15 μM -DES. Only sorocarps were formed. Bars, 200 μm .

macrocyt to sorocarp formation (data not shown). BK1 is a mutant strain that readily forms sorocarps. Macrocyt formation by BK1 cells occurred only under submerged conditions, but never on the agar surface. A developmental shift by propionate was not observed in MF1 and Dm7.

Changes of pH_i in response to addition of DES and DNP

To confirm that DES and DNP actually lowered pH_i , their effects on the pH_i of aggregating MF1 cells, just before the determination of the developmental pathway, were examined using the specifically designed perfusion

Fig. 2. Effective period of DES for sorocarp induction. MF1 cells (1×10^6 cells cm^{-2}) at various developmental stages were transferred to agar containing DES (final concn 15 μ M), followed by incubation for 30 h at 22 °C, as described in Methods. Bars, 200 μ m.

chamber as described in Methods. When the cells were perfused with 20 mM-K/K₂ phosphate buffer (pH 7.0) as control, no significant change in pH_i was observed. Application of 5 μ M-DES caused a transient decrease in pH_i , but the original pH_i was recovered in 20 min. DES at 15 μ M, the most effective concentration for inducing sorocarp formation, caused a marked decrease in pH_i (Fig. 3a). Similarly, 100 μ M-DNP also caused a decrease in pH_i (Fig. 3b).

Relationship between pH_o and pH_i

Effects of pH_o on pH_i were investigated by monitoring the pH_i of MF1 cells shaken for 30 min in 20 mM-MES buffer (pH 5.0 or pH 7.0). In these experiments, dissociated cells from the early aggregation stage were used. During the relatively early developmental period pH_o was expected to affect the choice of developmental modes. There was no significant difference in pH_i between the two different pH_o conditions (Table 1).

Microenvironments of cells developing normally on an agar surface might be somewhat different from those of completely dissociated single cells. In this connection, it has been shown that developing cells release a large amount of NH_3 as a major catabolic product by the degradation of endogenous protein (Gregg *et al.*, 1954;

White & Sussman, 1961; Hames & Ashworth, 1974), and that NH_3 accumulates in the intercellular space of cell masses (Schindler & Sussman, 1977). Therefore, 5 mM- NH_4Cl was added to the incubation media to simulate the microenvironment of normally developing cells. It was found that cells shaken in NH_4Cl -containing buffer at pH 7.0 had a significantly higher pH_i value than cells shaken without NH_4Cl at either pH (Table 1).

Comparison of pH_i in Dm7 cells directed to sorocarp or macrocyst formation

Since Dm7 cells are known to be directed to sorocarp or macrocyst formation by light/dark (Filosa & Dengler, 1972), the pH_i values of cells incubated in the light or dark were compared (Fig. 4). Experiments in which cells were transferred from dark to light showed that the developmental mode of Dm7 cells is determined at the early aggregation stage (t_4 – t_8). The aggregation pattern in the dark was considerably different from that in the light: aggregation streams were formed in the light, whereas such distinct streams were scarcely formed in the dark during 8 h incubation after transfer from the cold (4 °C). No significant difference in pH_i was observed between the two batches of t_4 cells incubated in the light or dark (Fig. 4a, b). It is of interest that there

Fig. 3. (a) Change in pH_i of aggregating MF1 cells in response to DES application. MF1 cells (t_4 cells) were perfused with 20 mM-K/K₂ phosphate buffer (pH 7.0) containing 5 μ M-DES ($\square$) or 15 μ M-DES ($\bullet$) as described in Methods. $\triangle$, Control without DES. The arrow indicates the time point of DES application. (b) Change in pH_i of aggregating MF1 cells in response to DNP application. MF1 cells (t_4 cells) were dissociated and perfused with 20 mM-K/K₂ phosphate buffer (pH 7.0) with ($\bullet$) or without ($\triangle$) 100 μ M-DNP. The arrow indicates the time point of DNP application. A representative pattern of the change in pH_i from one in three experiments is shown in both cases.

was a wide variation of pH_i values among the cells incubated for 8 h in the dark (Fig. 4d), compared with those incubated for 8 h in the light (Fig. 4c), and also that

Table 1. Relationship between pH_o and pH_i

MF1 cells at the early aggregation stage were mechanically dissociated and shaken for 30 min in 20 mM-MES buffer (pH 5.0 or 7.0) with or without 5 mM-NH₄Cl, followed by the measurement of pH_i . Although cells shaken in buffers of pH 5.0 and pH 7.0 had similar pH_i values, a significant difference arose between the two when NH₄Cl was added to simulate the expected microenvironment of the cells during development. The addition of NH₄Cl to the buffer at pH 7.0 gave a significantly higher pH_i value ($P < 0.01$). Values are the mean $\pm$ SD. The number of cells examined is given in parentheses.

Addition	pH_i	
	pH _o 5.0	pH _o 7.0
None	7.09 $\pm$ 0.07 (24)	7.12 $\pm$ 0.06 (18)
5 mM-NH ₄ Cl	7.11 $\pm$ 0.11 (39)	7.27 $\pm$ 0.07 (40)

the cells with pH_i values higher than 7.30 were formed particularly under the dark condition favouring macrocyst formation (Fig. 5d).

Discussion

The results presented here provide additional evidence that pH_i may be important for the choice between the sexual and asexual development in *D. mucoroides*. Chemical agents such as DES and DNP could shift the developmental mode from macrocyst to sorocarp formation. These drugs actually caused a marked decrease in pH_i ; thus the lowered pH_i itself, or something related to decreasing pH_i such as membrane potential (Van Duijn & Vogelzang, 1989) or vacuolar pH (Yamamoto & Takeuchi, 1983; Gross *et al.*, 1988), might be unfavourable to macrocyst formation. It has been shown that the developmental pathways in *D. mucoroides* can be controlled by two chemical substances, cAMP and ethylene; exposure of cells to ethylene gas induces macrocyst formation, while exogenously added cAMP is favourable to sorocarp formation (Amagai, 1984, 1987). Therefore, it is of interest to consider the relationship between pH_i and these chemical regulators. It has been proposed that in higher plant cells the synthesis and release of ethylene may be enhanced by membrane potential generated by plasma-membrane proton ATPase, and that the disappearance of the membrane potential caused by addition of DNP inhibits ethylene synthesis (John, 1983). The membrane potential of *D. discoideum* is mainly generated by an electrogenic proton pump, and the addition of DES depolarizes the

Fig. 4. Comparison of pH_i in Dm7 cell populations developing in the light (sorocarp formation) or in the dark (macrocyt formation). Histograms showing the variation of pH_i : t_4 cells incubated in the light (a) and dark (b), t_8 cells incubated in the light (c) and dark (d).

membrane (Van Duijn & Vogelzang, 1989). It is possible that the proton pump inhibitor, DES, and protonophore, DNP, might counteract the membrane potential of *Dictyostelium* cells, thus blocking ethylene synthesis. Our preliminary experiments showed that the sorocarp-inducing effect of DES is completely nullified by the addition of ethylene gas and that ethylene synthesis by cells is greatly inhibited in the presence of DES (N. Iijima and others, unpublished).

MF1 cells are known to select the developmental mode according to the pH_o (Amagai & Filosa, 1984), and this was confirmed in the present work. It has been claimed previously that pH_i is generally maintained at a constant level, irrespective of experimentally altered pH_o values (Jentoft & Town, 1985), and in the experiments reported here there was no difference in pH_i between the cells shaken in buffers of lower pH_o (5.0) and higher pH_o (7.0). Since the membrane-potential of *D. discoideum* has been shown to be depolarized in response to decreasing pH_o (Van Duijn & Vogelzang, 1989), it is possible that the effect of pH_o might act via the membrane potential. On the other hand, suspensions of completely dissociated cells to which no addition has been made represent a somewhat non-physiological state, because normally

developing cells undoubtedly release metabolites such as NH_3 into the intercellular space. Simulation of the environmental conditions by adding 5 mM- NH_4Cl to cells incubated in buffer of pH 7.0 yielded a significantly higher pH_i ($P < 0.01$) than that produced in the absence of NH_4Cl . Therefore, it is likely that pH_i may change depending on pH_o , possibly through the participation of NH_3 released from the cells. Under low pH_o conditions, ethylene production by cells was considerably inhibited, thus resulting in induction of sorocarp formation (N. Iijima and others, unpublished). All of these results suggest that lowered pH_i or depolarized membrane potential might inhibit the sexual macrocyst development, and that this inhibition may occur through the suppression of ethylene accumulation. Studies are now in progress on the relation of pH_i to another key substance, cAMP.

When pH_i values of Dm7 populations developing toward either macrocysts in the dark or sorocarps in the light were compared, there were no significant differences in the mean values and deviations between the two sets of t_4 cells (Fig. 4a, b). In t_8 cells, however, some differences arose between the two populations: in addition to a larger deviation of pH_i values in the cells

incubated in the dark, conditions favouring macrocyst formation, a few cells with pH_i values above 7.30 were observed (Fig. 4d). The function of these cells is presently unknown. Amagai (1989) recently reported that only a few cells are attributed to the formation of zygotic giant cells. Accordingly, it will be of interest to know if the cells with a higher pH_i function as gametes and thus contribute to giant cell formation.

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