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Role of Polyamines in Proliferation and Differentiation of *Dictyostelium discoideum* as Ascertained by Difluoromethylornithine Treatment

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R. FERNANDEZ-PINILLA and A. PESTAÑA. *Role of Polyamines in Proliferation and Differentiation of* Dictyostelium discoideum *as Ascertained by Difluoromethylornithine Treatment.* Rev. esp. Fisiol., 43 (4), 439-444, 1987.

The role of polyamines on growth and early development of *D. discoideum* has been investigated following the activity of ornithine decarboxylase (ODC) and the effects of difluoromethylornithine *in vivo*. Pretreatment of growing amoebae with difluoromethylornithine inhibited proliferation and markedly reduced the decarboxylase activity in the first hour after fasting without affecting the subsequent development. These results show the essentiality of ODC for proliferation but are inconsistent with the hypothesis atributing a developmental role to the transient increase in ODC shortly after *D. discoideum* starvation.

Key words: *Dictyostelium discoideum*, Ornithine decarboxylase, Proliferation, Differentiation, Difluoromethylornithine.

The functions of polyamines in eukaryotic cell regulation are not definitely established (13, 19). While there is a general agreement in the essentiality of polyamines for cell growth (7), their role as positive signals in the regulation of cell proliferation seems unlikely (14). More conflicting is the putative role of polyamines in cell differentiation. Although in some instances, polyamines have been shown to play important roles such as in the hormone-dependent differentiation of the mammary gland (7) or in the regulation of calcium fluxes in rapid response to cell-activation signals (8), most of the postulated effects of polyamines in differentiation seems to be secondary to their effects on cell growth (2).

In *Dictyostelium discoideum*, a cellular slime mould which has many advantages for the study of developmental processes (3), the polyamine content is not significantly affected by the mode of growth or during the early developmental cycle (12). Since polyamine levels do not accurately reflect the real status of the amine

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pools *in vivo* (18), the changes in ornithine decarboxylase activity, the rate-limiting step in polyamine biosynthesis, together with polyamine depletion *in vivo* with difluoromethylornithine (11) were used to ascertain the role of polyamines in the life cycle of *D. discoideum*.

Materials and Methods

Dictyostelium discoideum amoebae, Ax2 strain, were axenically grown in HL-5 medium (21). Synchronous cultures were obtained by resuspending stationary phase cells into fresh medium at a density of about 2×10^6 cells/ml (17). Best results were obtained using cells allowed to stay in the stationary phase for 10-20 h (1). For developmental studies, cells were harvested at the density of $2\text{-}4\times10^6$ cells /ml and washed three times in ice-cold differentiation medium (17 mM phosphate buffer, pH 6) and resuspended in the same buffer. Cells were allowed to differentiate in shaken suspensions (4) and in solid support, either submersed (10) or in moisty nitrocellulose filters (4). When indicated, difluoromethylornithine (a gift from Merrell International) was added to cells in the vegetative or developmental phase. Ornithine decarboxylase assay was as previously described (6) using 1-^{14}C ornithine (54-60 μCi/mmol from Amersham). Radioactivity was measured in a Beckman LS 100 liquid scintillation counter. The development of contact sites A was measured by a reaggregation assay in the presence of ethylendiamino tetraacetic acid (EDTA) as described (4). Cells or spores were counted in a hemocytometer after dissociation in phosphate buffer containing 10 mM EDTA. Microphotographs were taken on Plus x-120 film (Kodak) using a Nikon microscope SE with a 10×objective. Protein was assayed by a modification of the Bradford procedure (14) using bovine serum albumin as standard.

Results and Discussion

The axenic strain Ax2 of *Dictyostelium discoideum* grows exponentially with a doubling time of 8-10 h up to cell densities of 1×10^7 cells/ml. Above this density the proliferation rate is drastically reduced and eventually arrested (stationary phase). Concurrently with this transition, the activity ornithine decarboxylase was significantly reduced ($p \leq 0.05$) from 13.71 ± 3.14 units $\times 10^{-7}$ cells/ml) in exponentially growing cells (harvested at $1\text{-}2\times10^6$ cells/ml) to 7.57 ± 0.42 units in stationary cells, harvested at $1\text{-}2\times10^7$ cells/ml.

In order to better define these changes in ornithine decarboxylase activity, stationary phase cells were allowed to proliferate by dilution into fresh medium as described in Methods. Under these conditions, cell division was synchronously reinitiated after a 2 h lag period. There is a 2-3 fold increase in ornithine decarboxylase activity shortly after the completion of amoebae division (fig. 1), in coincidence with the reported peak in RNA and protein accumulation in *D. discoideum* cell cycle (9). A similar increase in

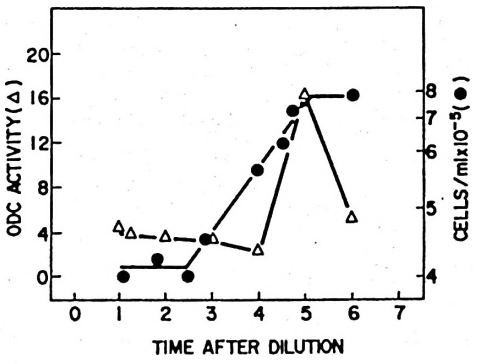

Fig. 1. *Ornithine decarboxylase activity in synchronously growing amoebae.*
Cell density and ornithine decarboxylase activity were measured at the indicated time intervals after diluting stationary phase amoeba (see methods). Result representative of three experiments.

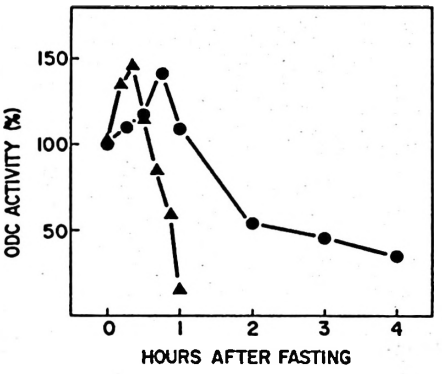

Fig. 2. *Ornithine decarboxylase in early development.*
Development was initiated by quickly washing the cells in cold phosphate buffer pH 6 and resuspending the cells in the same buffer at the concentration of 2×10^7 cells/ml. At the indicated time, aliquots were taken for ornithine decarboxylase assay and protein measurement as indicated in methods. The specific activities found in two experiments were recalculated as percent of the time 0, which were 26 and 11 nmols/hour/mg protein respectively for cells from exponentiallly (●) or synchronously (▲) growing cultures. Zero time correspond to the activity found in aliquots of unwashed cells, lysed and assayed immediately after harvesting in order to avoid proteolytic inactivation.

ornithine decarboxylase activity in the S-phase has been observed in *Physarum polycephalum* (15).

Upon starvation, the amoebae move towards attracting centers by a chemotactic mechanism involving cyclic AMP relay and subsequently they organize into discrete aggregates of 10^5 cells on average from which terminal differentiation to sporocarps occurs (3). Most of the biochemical changes occurring in the preaggregative phase can be reproduced in cells starved in suspension (8). The evolution of ornithine decarboxylase activity during the early developmental phase is characterized by a rapid increase within the first hour after food removal which was followed by a marked decrease in activity expanded over the ensuing 2-4 hours (fig. 2). A similar but faster fluctuation in the decarboxylase activity was observed (triangles in fig. 2) in starved cells harvested from synchronously growing cultures, immediately before the onset of the second wave of cell division. A qualitatively similar pattern of ODC activity during the first hours of development has been reported for cells allowed to develop on solid support (12).

In a previous report (15) it was postulated a developmental significance to these early increase in ODC activity after starvation, which were magnified by a rapid proteolytic inactivation of ODC in homogenates from unwashed growing cells used as time zero control (PINILLA & PESTAÑA, manuscript in preparation). NORTH (12) has argued that these developmental changes in ODC activity could be due simply to the changes in osmolality imposed by the experimental procedure. In order to clarify this point, the developmental effects of difluoromethylornithine (11), which has been widely used in studied aimed to ascertain the functionality of polyamines in the regula-

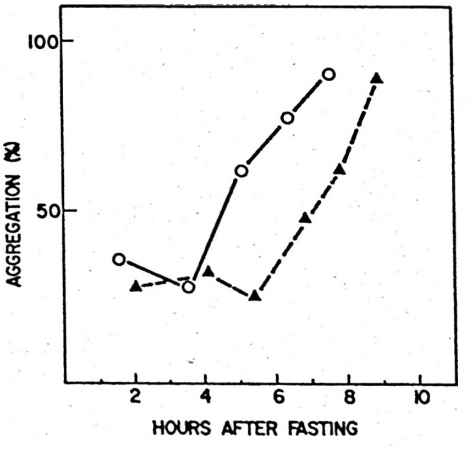

Fig. 3. *Development of aggregation competence in difluoromethylornithine pretreated cells.*
EDTA-resistant aggregation was assayed (see methods) in control (▲) and DFMO pretreated (O) cells at the indicated time intervals after washing and resuspension in differentiating medium.

Table I. *Effect of difluoromethylornithine on* D. discoideum *development.*

DFMO treatment[a]		ODC activity[b]	Aggregation[c]	Sporulation[d]
Growth	*Development*	(units x 10^{-7} cells)	(h)	(%)
−	−	7.36 ± 0.91	8 3/4	100
+	−	0.93 ± 0.05	7 1/2	102
+	+	0.58 ± 0.06	7 1/2	53
−	+	n.d.	8 3/4	70

a) 5 mM difluoromethylornithine (DFMO) was added to the medium of growth or development as indicated.
b) Ornithine decarboxylase (ODC), average activity of five determinations in the interval of 30 to 60 min after the onset of development. $P < 0.001$ for all the differences. n.d., not determined.
c) Time required for the formation of tight cell aggregates.
d) Spore yield after 25 h of development on nitrocellulose filters as percent of controls (average of two experiments in sextuplicate).

tion of cellular processes (13), has been investigated.

In a preliminary experiment directed at establishing the experimental conditions, it was observed that a pretreatment of growing amoebae with 5 mM difluoromethylornithine over a period of 16-18 hours (two doubling times) caused a depletion of the endogenous polyamines enough to arrest cell proliferation. Next it was studied the developmental effects of 5 mM difluoromethylornithine following the experimental design depicted in table I, which also shows the ornithine decarboxylase activity measured in the 30-60 min interval after cell starvation. The marked decrease observed in the decarboxylase activity of difluoromethylornithine treated cells is consistent with the known effects of the drug.

In order to ascertain the developmental effects of difluoromethylornithine it was investigated the acquisition of the aggregative competence, which recapitulates most of the biochemical and cellular differentiation occurring during the early developmental phase, and the production of spores, which represents the most characteristic feature of the terminal differentiation in *D. discoideum* (3). The aggregative phase of the development was studied by microscopic examination of cells allowed to develop on nitrocellulose filters or submersed in small petri dishes. In neither of the approaches we observed any deleterous effect of difluoromethylornithine on the aggregation processes which were even shortened as compared to controls (table I).

Confirming this observation, the developmental expression of contact sites A was also faster in difluoromethylornithine pretreated cells than in the controls (fig. 4). A similar shortening in the preaggregative interfase of *D. discoideum* pretreated with n-bytyrate has been explained on the basis of its cytostatic effects and the known effects of cell cycle phase on the cellular fate during development in *D. discoideum* (4).

Confirming the lack of effect of difluoromethylornithine pretreatment and ornithine decarboxylase inhibition on the developmental cycle in *D. discoideum*, the production of spores was identical in control and pretreated cells (table I). The marked reduction in spore yield observed in cells exposed to DFMO during differentiation (table I) would suggest a developmental significance for the transitory increase in ODC activity reported to occur in the postaggregative phase (12, 20). Wether this increase is directly involved in cellular differentiation or is indirectly associated through the cell division which commits a group of cells to terminal differentiation to spores (16) remains to be ascertained.

On the whole, the results of this study are inconsistent with the hypothesis attributing a developmental role to the transitory increase in ornithine decarboxylase activity after *D. discoideum* starvation.

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Resumen

Se estudia el papel de las poliaminas en la proliferación y diferenciación temprana de *Dictyostelium discoideum*, mediante el estudio de la actividad ornitina decarboxilasa y el efecto del tratamiento con difluorometil ornitina *in vivo*. El pretratamiento de células vegetativas con este inactivador irreversible, inhibe la proliferación celular y suprime la actividad decarboxilasa durante la primera hora tras el ayuno, sin afectar a la diferenciación y morfogénesis. Estos resultados hacen poco verosímil la hipótesis que atribuye protagonismo a la actividad ornitina decarboxilasa y poliaminas en la transición proliferación a diferenciación en *Dictyostelium*.

Palabras clave: *Dictyostelium discoideum*, Ornitina decarboxilasa, Proliferación, Diferenciación, Difluorometil ornitina.

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