

Inhibition of a Nutrient-dependent Pinocytosis in *Dictyostelium discoideum* by the Amino Acid Analogue Hadacidin

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ABSTRACT In the present study we examine the effects of the drug hadacidin (*N*-formyl-*N*-hydroxyglycine) on pinocytosis in the eucaryotic microorganism *Dictyostelium discoideum*. At concentrations of up to ~8 mg/ml, hadacidin inhibited the rate of pinocytosis of fluorescein isothiocyanate (FITC) dextran in cells in growth medium in a concentration-dependent manner but had no effect on cells in starvation medium.

Because hadacidin also inhibits cellular proliferation at this concentration, the relationship between growth rate and pinocytosis was studied further using another drug, cerulenin, to produce growth-arrest. These experiments showed no changes in the rate of pinocytosis even after complete cessation of cellular proliferation.

Other studies showed that the transfer of cells from growth to starvation medium reduced the rate of pinocytosis by ~50%. A reduction of similar magnitude occurred if cells were transferred from growth to starvation medium containing hadacidin. Also, no additional reduction in pinocytosis occurred when cells that had been treated with hadacidin were transferred to starvation medium containing hadacidin. These cells were able to take up [¹⁴C]hadacidin in the starvation medium.

In contrast to the results with hadacidin-treated cells, cells in a cerulenin-induced state of growth-arrest when transferred to starvation medium exhibited the same 50% reduction in pinocytosis observed in cells not previously exposed to either drug. Cells treated with azide, in either growth or starvation medium, exhibited an immediate inhibition of all pinocytotic activity.

After the transfer of log-phase cells to starvation medium supplemented with glucose, the reduction in rate was only ~10–15%. In contrast, a 50% reduction was observed after supplementation of starvation medium with sucrose, KCl, or concanavalin A. Maintaining the cells in growth medium containing hadacidin for as long as 16 h had no effect on the rate at which the cells aggregated.

These results are consistent with the conclusion that *D. discoideum* exhibits two types of pinocytotic activity: one that is nutrient dependent and the other independent of nutrients. This latter activity persists in starvation medium and is unaffected by hadacidin, whereas the nutrient-dependent activity is present in growth medium and is inhibited by hadacidin.

The amino acid analogue hadacidin (*N*-formylhydroxyaminoacetic acid), originally a fungal isolate (1), inhibits growth in a number of systems including bacteria (2), tumor cells (1, 5),

plants (3), and the eucaryotic microorganism *Dictyostelium discoideum* (4). In vitro, hadacidin competes with aspartic acid to inhibit adenylosuccinate synthetase (6), the activity respon-

sible for the condensation of inosine 5'-monophosphate (IMP) and aspartic acid to form adenylosuccinate (AMPS). AMPS synthetase has a role in a number of biological processes including the *de novo* synthesis of AMP (7), recycling of hypoxanthine (7), and the purine nucleotide cycle (8).

Whereas the results of our previous study (4) on the effects of the drug on *D. discoideum* suggested that the inhibition of growth by hadacidin is the result of inhibition of AMPS synthetase, our data also indicated that hadacidin was producing other physiological changes involving cell surface functions as well. For example, we found that the hadacidin-treated cells showed not only an increase in osmotic stability but also a decrease in the rate of nutrient uptake.

In *D. discoideum*, nutrients are taken up by pinocytosis (9, 16), and fluorescein isothiocyanate (FITC) dextran has been shown to be a suitable probe for this process in *D. discoideum* (10) as well as in other cells (11). The present study examines the effects of hadacidin on pinocytosis in *D. discoideum*.

MATERIALS AND METHODS

Materials

Fluorescein isothiocyanate-labeled dextran (FITC-dextran, average mol wt 62,000) and concanavalin A were obtained from Sigma Chemical Co. (St. Louis, Mo.). Sucrose and other inorganic chemicals were purchased from J. T. Baker Chemical Co. (Phillipsburg, N.J.). Growth medium components were obtained from BBL Laboratories (Becton, Dickinson and Co., Cockeysville, Md.). [¹⁴C]Formic acid (4.4 mCi/mmol) was obtained from New England Nuclear (Boston, Mass.). Amberlite IR-120 resin (20–50 mesh H⁺ form) was obtained from Mallinkrodt Chemical Co. (St. Louis, Mo.). Cerulenin was purchased from Calbiochem-Behring Corp. (San Diego, Calif.) and sodium hadacidin was generously supplied by Merck Sharp & Dohme Canada Ltd. (Montreal, Quebec, Canada).

Growth of Organisms and Starvation Conditions

The axenic strain (Ax-3) of *D. discoideum* (parent strain, NC-4 haploid) was grown in HL-5 growth medium as previously described (12). Starvation was initiated by resuspending cells, harvested from the exponential stage of growth, at $1-2 \times 10^7$ cells/ml in starvation medium containing 17 mM potassium phosphate (pH 6.1) and 2 mM MgSO₄.

Pinocytosis Assay

Pinocytotic activity was determined using FITC-dextran and following procedures described (13). Cells, suspended at a density of 5×10^6 cells/ml in growth medium, or 1×10^7 cells/ml in starvation medium were mixed with FITC-dextran at a final concentration of 1 mg/ml. At ~10- or 20-min intervals, 5×10^6 cells were removed and diluted into 10 ml of ice-cold buffer containing 17 mM potassium phosphate (pH 6.1) and 2 mM MgSO₄. Cells were recovered by centrifugation and washed twice with 10 ml of buffer. The final pellet was resuspended in 3 ml of 0.1% Triton X-100 containing 50 mM NaH₂PO₄ (pH 9.6). Cells were incubated at room temperature for 10 min. Fluorescence was determined on a Hitachi Perkin-Elmer (Norwalk, Conn.) fluorescence spectrophotometer (MPF-2A) using excitation and emission wavelengths of 470 and 520 nm, respectively. One fluorometric unit was obtained with 0.65 μg of FITC-dextran. Blanks were prepared with cells treated in the manner described above, except that the FITC-dextran was omitted. The use of FITC-dextran for assay of fluid-phase endocytosis in *D. discoideum* including characterization of the assay has been described (10). The rate of pinocytosis was calculated from a plot of FITC-dextran uptake per unit time.

Preparation of [¹⁴C]Hadacidin

The carbon of the formyl group of hadacidin was radiolabeled by first removing the formyl group from unlabeled hadacidin by hydrolysis in aqueous solution for 6 d at room temperature, after having converted the sodium hadacidin to the free base using IR-120 resin. The *N*-hydroxyl glycine was reacted with ¹⁴C-formic acid and acetic anhydride for 20 min at room temperature and for 15 min at 56°C. The mixture was cooled and the solvent removed at reduced pressure. The semisolid was dissolved in 2 ml of water, and the pH was adjusted

to 6.2–6.5 with 2 M NaOH and 27 ml of ethanol added. Under these conditions, the [¹⁴C]hadacidin crystallized. After filtration and recrystallization, the purity of the product was checked by paper chromatography as described (1).

The specific activity was $\sim 6.64 \times 10^6$ cpm/mg. Studies done to measure the uptake of [¹⁴C]hadacidin were performed as described previously for [¹⁴C]aspartic acid (4).

Initiation of Morphogenesis

The procedures used for initiating morphogenesis on filter pads have been described previously in detail (14). In brief, log-phase cells were washed free of nutrient medium and dispersed on black Whatman no. 29 filters (Whatman, Inc., Clifton, N. J.) that were each supported by two Millipore prefilters (no. AP103700 Millipore Corp., Bedford, Mass.) saturated with a buffered salts solution containing 20 mM KCl, 0.24 mM MgCl₂, 40 mM phosphate buffer (pH 6.4), and 0.34 mM streptomycin sulfate. The cell densities on the filter pads were $\sim 6 \times 10^6$ /cm². Filters were incubated in plastic petri dishes in humidity chambers at 22°C. Development was monitored in a Wild M8 microscope illuminated with glass fiber optics. The time to a morphology represents the time at which > 50% of the morphologies on a development filter were positively identified as that morphology.

RESULTS

Cell Multiplication and Pinocytosis in Axenic Growth Medium

Amoebae of the axenic strain of *D. discoideum*, Ax3, multiplied in suspension in axenic growth medium with a generation time of 12 h (Fig. 1 A). Pinocytosis was assayed in log-phase growth cultures by adding FITC-dextran and monitoring uptake with time (Fig. 1 B). Under standard conditions, cells began taking up dextran immediately after its addition, and continued at a constant rate for at least 40 min (Fig. 1 B). The normal rate of uptake for a cell culture at a concentration of $1-2 \times 10^6$ cells/ml in 1 mg of FITC-dextran/ml was $\sim 1.5 \mu\text{g}/10^7$ cells per 10 min. Uptake was also completely sensitive to 10 mM azide (Table I), indicating that FITC-dextran uptake is an energy-dependent process and not a result of intercellular entrapment during centrifugation.

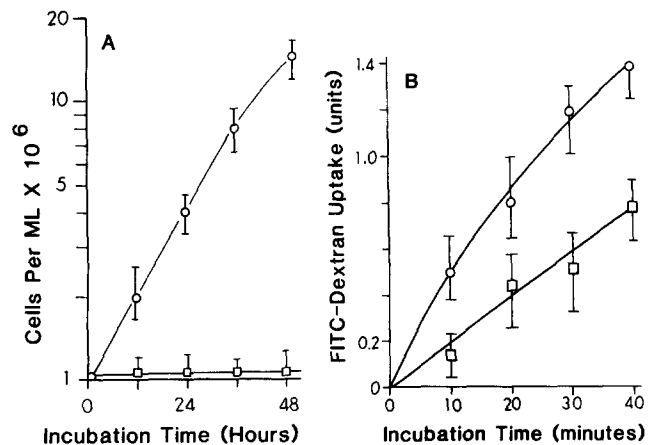


FIGURE 1 Effects of hadacidin on cell multiplication and pinocytosis in growth medium. Cells were grown in 70 ml of HL-5 to 1×10^6 cells/ml in a 500-ml flask. Four 10-ml samples of the cell suspension were removed and transferred to 125-ml flasks. Two of the 10-ml samples received hadacidin at a final concentration of 8 mg/ml and one of these samples together with the one remaining untreated sample received FITC-dextran at a final concentration of 1 mg/ml. (A) Cell multiplication was followed by removing samples, diluting where required, and counting cells directly in a hemocytometer. (B) FITC-dextran uptake was determined on 1-ml samples removed at intervals and treated as described in Materials and Methods. Cell multiplication and pinocytosis with no drug (○) and in the presence of 8 mg/ml hadacidin (□).

TABLE I
Rate of Pinocytosis after Transfer of Cells from Growth Medium to Starvation Medium

Treatments	Rate of pinocytosis*	
	Growth medium %	Starvation medium %
None	100	53
Hadacidin in GM	50	58
Cerulenin in GM	100	55
Azide (10 mM)	0	0

For rate determinations in growth medium (GM), cells were grown to 2×10^6 cells/ml and either hadacidin or cerulenin was added at 8 mg/ml and 10 μ g/ml, respectively. For rate determinations in starvation medium, cells were grown to 2×10^6 cells/ml, harvested, and resuspended in starvation medium containing drugs at concentrations described above. Rate of pinocytosis determined with FITC-dextran as described in Materials and Methods. * Rate expressed as a percentage of maximum.

Effects of Hadacidin on Cell Multiplication and Pinocytosis in Growth Medium

When the amino acid analogue hadacidin was added, at a final concentration of 8 mg/ml, with the FITC-dextran to a log-phase culture, subsequent growth was completely inhibited (Fig. 1 A) and the rate of FITC-dextran uptake was reduced by ~50% (Fig. 1 B). At concentrations of hadacidin below 8 mg/ml, the magnitudes of the reduction in the rate of growth and in the rate of pinocytosis were proportional to the concentration of the drug (Fig. 2). At concentrations > 8 mg/ml, there was no further reduction in the rate of pinocytosis, which remained 50% of the maximum rate, even though cell multiplication was completely inhibited (Fig. 2).

Effects of Cerulenin on Cell Multiplication and Pinocytosis in Growth Medium

To test whether the 50% reduction in the rate of pinocytosis is simply the result of the cessation of cell multiplication, the rate of pinocytosis was measured in log-phase cultures after the addition of the growth inhibitor cerulenin (15). Concentrations of cerulenin that completely inhibited cell multiplication (10 mg and greater) had no effect on the rate of pinocytosis (Table II). These results indicate that it is not the cessation of cell multiplication alone, but rather the drug hadacidin, that causes the 50% reduction in the rate of pinocytosis.

Effects of Hadacidin on Pinocytosis in Starved Cell Cultures

The developmental program of *D. discoideum* can be initiated by washing amoebae free of nutrients and resuspending them in starvation medium consisting of a buffered salt solution. When this was done, the rate of pinocytosis decreased to half the rate observed in growth medium (Fig. 3). The addition of hadacidin to these latter cultures had no effect on the subsequent rate of pinocytosis (Fig. 3). This is in direct contrast to the results obtained with growing cells, and indicates that the portion of pinocytotic activity lost by the removal of nutrients was that portion which was completely sensitive to hadacidin.

One explanation for the resistance of pinocytosis to hadacidin in starvation medium is that the cells were no longer permeable to the drug. To test this possibility, [14 C]hadacidin was prepared (see Materials and Methods) and used to monitor

the rate of drug internalization by cells in growth medium and in starvation medium. The results of this experiment are presented in Fig. 4. It is clear that cells placed in starvation medium continue to take up the drug at a significant rate for 4 h. In addition, cells pretreated with hadacidin in growth medium for 1 h before dilution into starvation medium containing hadacidin accumulated the same amount of FITC-dextran as cells not pretreated before dilution into starvation medium (Table I). These results reinforce the interpretation that pinocytosis in starvation medium is completely insensitive to the drug hadacidin.

Finally, if cells that have been pretreated with cerulenin for 1 h and pinocytose at 100% of the growing cell rate are diluted into starvation medium, they will pinocytose at 50% of the rate of growing cells (Table I), indicating that it is not simply the cessation of cell multiplication but the starvation condition which halves the rate of pinocytosis.

Addition of Glucose to Starvation Medium Inhibits the Reduction in Pinocytosis

When log-phase cells were added to starvation medium supplemented with 100 mM KCl or 100 mM sucrose, a 50%

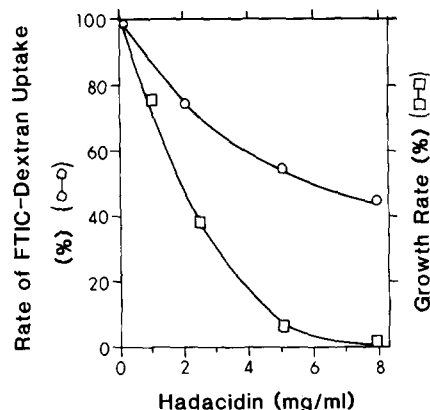


FIGURE 2 Cell multiplication and pinocytosis as a function of hadacidin concentration. Cells were grown to 1×10^6 cells/ml and 10-ml samples transferred to 125-ml flask containing various concentrations of hadacidin or hadacidin together with 1 mg/ml FITC-dextran. Cell multiplication was followed for an additional 48 h whereas the amount of FITC-dextran taken up was determined at 10-min intervals for 1 h. Rates were obtained from plots of uptake vs. time or cell number vs. time. Data are presented as percent of value obtained in the absence of any hadacidin. Rate of cell multiplication (\square) and FITC-dextran uptake (\circ).

TABLE II
Effects of Cerulenin on Rate of Pinocytosis and Cellular Proliferation

Concentration μ g/ml	Growth $\times 10^4$, cells/ml/h	Pinocytosis %
0	8.0	100
5	5.0	110
10	1.6	98
20	0.8	105

Cells were grown, in HL-5 medium, to a titer of 1×10^6 cells/ml. At this concentration, FITC-dextran was added to a final concentration of 1 mg/ml, together with cerulenin at the indicated concentration. Samples were removed at 10-min intervals for determination of rate of pinocytosis, and prepared for fluorescence measurements as described in Materials and Methods. For growth measurements, the incubation was continued for an additional 48 h, samples were removed and the cell concentration was determined with an hemocytometer.

reduction in the rate of pinocytosis still occurred (Table III). In addition, cells added to starvation medium containing the 0.1 mg/ml of the lectin concanavalin A still showed a 50% reduction in the rate of pinocytosis (Table III). However, when log-phase cells were added to starvation medium supplemented with 100 mM glucose, only a 10% reduction in the rate of pinocytosis occurred; in other words, cells continued at the rate of pinocytosis observed in full growth medium (Table III).

Effect of Growth Inhibition by Hadacidin on Subsequent Morphogenesis

Morphogenesis is initiated in *Dictyostelium* by the depletion or removal of nutrients. This is accompanied by a significant reduction or cessation of cell multiplication (17–19). Under the growth conditions used in this investigation, cells that enter stationary phase in growth medium progress 2–3 h into the developmental program which is reflected by the earlier timing of the stages of aggregation when compared to log-phase cells (21, 22; also Table IV). Because hadacidin inhibits cell multi-

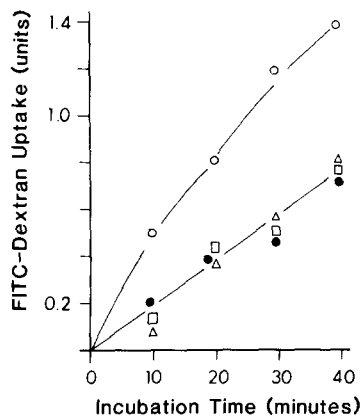


FIGURE 3 Effect of hadacidin on FITC-dextran uptake under different incubation conditions. Cells were grown to 1×10^6 cells/ml in 70 ml of HL-5 in a 500-ml flask. Cells from this flask were harvested and resuspended in 10 ml of starvation medium in 125-ml flasks at a concentration of 1×10^7 cells/ml. Two of these flasks were prepared: one received hadacidin at 5 mg/ml, the other did not. Two other 125-ml flasks were filled with 10 ml of growth medium containing cells at 1×10^6 cells/ml. One of these flasks also received 5 mg/ml hadacidin. All four 125-ml flasks received 1 mg/ml FITC-dextran and all four flasks were incubated for an additional 60 min. At intervals, samples were removed and the amount of FITC-dextran taken up was determined as described above. FITC-dextran uptake in growing cells without hadacidin (○); growing cells with hadacidin (□); starved cells without hadacidin (△); starved cells with hadacidin (●).

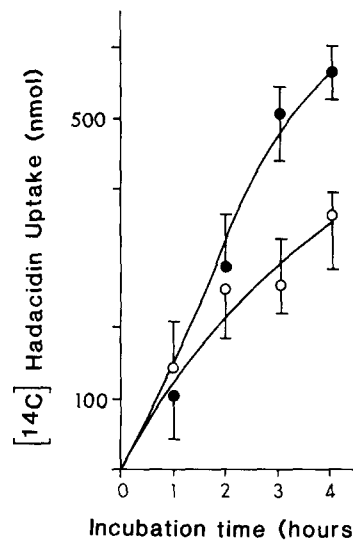


FIGURE 4 Uptake of [14 C]hadacidin by cells incubating in starvation medium. Cells were grown to 1×10^6 cells/ml in 70 ml of HL-5. The cells from one flask were washed and resuspended in starvation medium at a final concentration of 1×10^7 cells/ml. 5 mg/ml [14 C]hadacidin ($\sim 1 \times 10^6$ cpm) was added and 10-ml samples were removed over a 4-h period, the cells were collected by filtration, and the amount of radioactivity was determined by counting the filter. Another flask received 5 mg/ml hadacidin and the incubation in growth medium continued. After ~ 16 h, the cells from this flask were harvested and resuspended in starvation medium containing 5 mg/ml [14 C]hadacidin and the incubation was continued for an additional 4 h and the amount of radioactive hadacidin taken up was determined as described above. Uptake by cells pre-exposed for 16 h to unlabeled hadacidin in growth medium (●), and uptake by cells not pre-exposed (○).

TABLE III
Rate of Pinocytosis after Transfer of Cells from Growth Medium to Starvation Medium Containing Various Supplements

Supplement	Reduction in rate of pinocytosis %
None	50
Glucose (100 mM)	10
Sucrose (100 mM)	56
KCl (100 mM)	48
Concanavalin A (0.1 mg/ml)	52

Cells were resuspended in starvation medium (at 1×10^7 cells/ml) alone, or in starvation medium containing a supplement at the indicated concentration and FITC-dextran (1 mg/ml). Samples (0.5 ml) were removed at 10-min intervals over the next 60-min incubation period. FITC-dextran uptake was determined as described in Materials and Methods.

TABLE IV
Effect of Growth Inhibition by Hadacidin on the Timing of Subsequent Morphogenesis

Condition	Time (h)				
	Ripple	Loose aggregate	Tight aggregate	Finger	Early culmination I
Log phase, no hadacidin	6.25	8.0	11.0	12.25	13.5
Stationary phase, no hadacidin	4.5	6.25	8.5	11.75	13.0
Log phase, treated with hadacidin for 16 h	7.0	9.0	11.0	12.75	13.5

Log-phase cells were incubated in growth medium containing 5 mg/ml hadacidin for 16 h. They were then washed free of growth medium and dispersed on pads saturated with buffered salts solution to induce morphogenesis. The timing was compared to that of parallel cultures derived from log-phase cells and 24-h stationary-phase cells. The standard deviations for all stages never exceed 5% of the mean (14).

plication, we tested whether the subsequent timing of inhibited cells was similar to that of stationary-phase cells or whether log-phase cell timing was retained. Log-phase cells were incubated in growth medium containing 5 mg/ml hadacidin, which completely blocked further cell multiplication. After 16 h, these cells were washed free of growth medium plus hadacidin and dispersed on filters saturated with nonnutrient, buffered salts solution. The hadacidin-treated cells developed with the timing of log-phase rather than stationary-phase cells (Table IV), even though they had not multiplied for 16 h.

DISCUSSION

These results indicate that ~50% of the pinocytotic activity exhibited by *D. discoideum* cells in growth medium can be inhibited by hadacidin. This inhibition does not appear to be the general consequence of the inhibition of cellular proliferation because the inhibition of growth by cerulenin has no effect on pinocytosis.

In contrast to its effect on cells in growth medium, hadacidin has no effect on the pinocytotic activity of cells in starvation medium. In addition to this difference in sensitivity, the pinocytotic activity of cells in starvation medium is ~50% of that exhibited by cells in growth medium, and this decrease occurs shortly, if not immediately, after the transfer of the cells to the starvation medium.

The finding that proliferating cells, in which the pinocytotic activity had been reduced by addition of hadacidin to the growth medium, exhibited no additional reduction in the rate of pinocytosis after their transfer to starvation medium supports the conclusion that hadacidin inhibits only the nutrient-dependent pinocytotic activity. Because hadacidin is able to inhibit the nutrient-dependent activity while the cells are still in a complete nutrient medium, this suggests that the drug induces a state of nutrient-deprivation within the cell similar to that obtained after the transfer of the cells to a starvation medium. It is not unreasonable to conclude that the cessation of cellular proliferation observed with hadacidin is a consequence of this exclusion of nutrients.

Although these data do not provide the exact mechanism by which hadacidin inhibits pinocytosis, they do suggest that if adenylosuccinate synthetase is the target for the drug (6), it could be involved with pinocytosis. One explanation derived from recent studies identified an AMPS synthetase activity in muscle and showed that the enzyme binds to F-actin specifically (20). Whether or not these results can be used to infer a role for actin in the pinocytotic activity of *D. discoideum*, as suggested by studies with other systems (24), remains to be shown.

We have also demonstrated in this report that even though hadacidin inhibits cell multiplication, it does not initiate expression of the stationary-phase phenotype, at least from the standpoint of subsequent morphogenesis (21): stationary-phase cells appear to progress ~2–3 h into the development program and therefore aggregate ~2–3 h earlier than log-phase cells

(21, 22). However, cells blocked from multiplying for 16 h by hadacidin exhibited the exact timing of log-phase cells, suggesting that hadacidin blocks them in some form of a log-phase phenotype comparable to a state of "suspended animation." In this regard, it will be interesting to test whether hadacidin-inhibited cells have accumulated in G₂ as have cells in stationary phase (23) and development (18), or whether they are heterogeneously distributed throughout the cell cycle.

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