Transient Increase in Intracellular pH during *Dictyostelium* Differentiation

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ABSTRACT The intracellular pH (pHi) of *Dictyostelium discoideum* amebae has been determined using the pH-dependent fluorescence of intracellularly trapped fluorescein (Thomas, J. A., R. N. Buschbaum, A. Zimiak, and E. Racker, *Biochemistry*, 18:2210–2218). The pHi of cells measured 45–60 min after initiation of differentiation was between 6.2 and 6.3. At ~2 h into differentiation cells underwent a transient intracellular alkalinization during which the pHi rose to 7.13 (± 0.3, $n = 4$), after which the pHi returned to approximately the original value (6.2–6.4). Cells that were removed from growth medium but were incubated in differentiation medium containing 3% dextrose did not exhibit this transient increase in pHi. The alkalinization event can also be prevented from occurring by differentiation in Na^+-free solutions or by the addition of amiloride to sodium-containing buffer solutions, suggesting that the alkalinization is sodium dependent. When the alkalinization was prevented by amiloride treatment, cells did not progress normally into differentiation. This increase in pHi was initiated by the cells 2 h after removal from nutrient medium and it could be inhibited by several treatments that had been observed to delay the differentiation program, suggesting that it plays a major role in the initiation of the developmental program of this organism.

Recently it has become clear that a change in intracellular pH (pHi) is an early event in various cellular regulatory processes. Several laboratories have demonstrated an increase in pHi upon treatment of responsive cells with mitogenic factors such as epidermal growth factor, platelet derived growth factor, and serum (1–4), sea urchin egg fertilization (5), and sperm activation (6). These have all been demonstrated to be mediated by an electroneutral Na^+/H^+ exchange mechanism present in the plasma membranes of these cells. Mitogenesis and post-fertilization development are clearly complicated cellular processes that require major changes in cellular organization and metabolism. The importance of the Na^+/H^+ exchange mediated alkalinization to these cellular programs is suggested by the observation that removing sodium from the media or the addition of amiloride, both treatments that prevent intracellular alkalinization from occurring in response to an appropriate "stimulus," also disrupt the initiation of these cellular processes (5–8). It has been suggested that removal of sodium from the starvation medium delays the progression of *Dictyostelium discoideum* amebae through their starvation-induced differentiation program (9). Gross et al. have reported that treatment with the weak base NH_4Cl affects the spore vs. stalk proportioning of differentiating *Dictyostelium* cells and proposed pHi to play a role in this aspect of the cellular differentiation of this organism (10). We undertook the present study to determine if cytoplasmic alkalinization occurred during the differentiation of a synchronous population of cells in which we could initiate differentiation uniformly and under-controlled conditions. Furthermore, the pH optima for a variety of subcellular processes in *D. discoideum* has been determined, but as yet, no direct measurement of pHi in this organism has been obtained. A preliminary report of this work has been presented (11).

MATERIALS AND METHODS

Poly-D-lysine hydrobromide, fluorescein diacetate, monensin, gramicidin, and dimethyl sulfoxide were obtained from Sigma Chemical Company (St. Louis, MO). Amiloride was generously provided by A. W. Alberts of Merck, Sharp and Dohme (Rahway, NJ). All other reagents were reagent grade from local suppliers.

Cell Culture: An axenic strain of *D. discoideum* (A3) was grown in suspension culture in HL-5 medium (12). Stock cultures of *D. discoideum* spores were maintained by serial passage on nutrient agar with *Escherichia coli* B/r serving as the food source. Clones were selected that exhibited normal growth (doubling time 8–9 h in axenic medium), ability to grow on bacteria, and completion of differentiation with appropriate morphogenic properties within ~24 h, essentially as described by Soll and co-workers (13).

Measurement of Intracellular pH (pHi): We undertook to measure the pHi of *Dictyostelium* amebae using the pH-dependent fluorescence of fluorescein as described by Thomas et al. (14). Preliminary work indicated that the pHi of these cells was close to 6.0. Fluorescein was used in our studies because it has a pKa which makes it particularly suitable for use in pH

measurements over the pH range of 5 to 7. The following procedure was used to introduce fluorophore into cells and prepare these cells for analysis. Cells grown in suspension culture where harvested (1–3 × 10^6/ml) from growth medium by centrifugation and washed three times with 17 mM (Na^+/K^+)Pi: 5.7 mM Na^+, 14.2 mM K^+, 17 mM Pi, pH 6.2, at 4°C. Cells were then resuspended in (Na-K)Pi buffer at a concentration of 1 × 10^7 cells/ml and differentiated by gyrating in suspension at 200 rpm for various periods of time at 20°C. 1 ml (10^7 cells) of the differentiating culture was added to 9 ml of (Na-K)Pi buffer containing 125 μM fluorescein diacetate (final concentration) and further differentiated in suspension for 15 min. Cells were then removed from the rotary shaker and 5 ml was pipetted into each of two wells of a six-well plate (Linbro). Each well contained one 1 × 2.5-cm glass slide which had been previously coated with poly-D-lysine (100 μl/slide, 10 mg/ml poly-D-lysine), and then thoroughly rinsed with deionized water. The six-well plate containing two slides plus cells in fluorescent-loading solution was centrifuged for 2–3 min using a microtiter plate holder (Dynatech, Alexandria, VA) in a Sorvall GLC-2 centrifuge at 600 *g* (1,800 rpm) to immobilize the cells on the glass slides. Cells attached to the glass slide were manipulated during subsequent procedures after attachment of a holder which allows insertion of the attached, cell-coated slide into cuvettes. Fluorescently-loaded cells were washed with (Na-K)Pi (four times in 10 ml) and measurements taken every 3–5 min as described below. Fluorescence microscopy of cells loaded with fluorescein by this method revealed the fluorophore to be diffusely localized in the cytoplasm with no concentration of fluorophore in small organelles, vesicles, or nuclei. In fact by this analysis the fluorescence was significantly weaker over organelles, vesicles, and nuclei. Further, the fluorescence remained diffuse and appeared to be excluded from intracellular organelles during the observed alkalinization.

In the measurements presented here fluorescein emission is monitored at 513 nm and the ratio of the emission is determined upon excitation at 491 and 438 nm. Since the local environment can alter the fluorescence characteristics of fluorescein the excitation maxima and emission maxima were determined and found to be identical for intracellular fluorescein and the dye free in solution. The relationship between the ratio value and pH was determined using intracellularly trapped dye and the Na^+/H^+ ionophore monensin (20 μM) to equilibrate pHi and extracellular pH. These measurements were also done in the presence of 0.5 μM gramacidin (so that Na^+ permeability of the cell membrane did not limit equilibration with extracellular pH) and identical results were obtained. The fluorescein-loaded cells were incubated in ionophore containing (Na-K)Pi solution which had been adjusted with H_3PO_4 or NaOH to the desired pH and the cells allowed to equilibrate until a stable 491:438 value was reached (~5 min). The ratio value was then determined in fresh ionophore containing buffer and then the cells were removed from the cuvette and the background fluorescence at 491 and 438 determined to correct for the dye that had leaked from the cells during the measurement. These values were used to correct the intracellular fluorescence and to determine the pH dependence of the ratio (491:438). The extracellular pH was determined using a pH meter. The resulting ratios were fitted by the least squares method to the solid line shown in Fig. 1, and yielded pKa of 5.77 for the fluorescein 491:438 ratio which is similar to the value of 5.9 observed in solution. The use of higher ionophore concentrations did not alter the results indicating that the pHi was equilibrated with extracellular pH. Further, the extracellular pH was measured in the same experiment and found to be the same as the measured pHi. A similar experiment in which monensin and gramacidin were omitted showed that these cells resist following the extracellular pH in a direct fashion (data not shown).

Morphological Assessment of Differentiation: Cells were harvested and differentiated in suspension, as described above. After 0.5, 1.5, 2.5, 3.5, 4.5, or 5.5 h, 10^7 cells (1 ml) were collected by centrifugation and resuspended at 10^8/ml in either 100 μl of (Na-K)Pi buffer alone or buffer containing 200 μM amiloride. 5-μl aliquots were spotted on non-nutrient agar with or without 200 μM amiloride. At 7.5 h post-starvation the extent of differentiation was assessed morphologically for both control and amiloride treated cells.

RESULTS

Having established the relationship of the ratio (491:438) value to pHi for intracellular fluorescein (see Fig. 1) the pHi of *Dictyostelium* amebae could then be determined. Cells that had differentiated for various lengths of time in suspension in (Na-K)Pi were loaded with fluorescent probe and the pHi determined every 3–5 min. The results from an experiment performed on cells soon after initiation of starvation is presented in Fig. 2. The first point demonstrated by this figure is that the pHi of *Dictyostelium* amebae early in differentiation

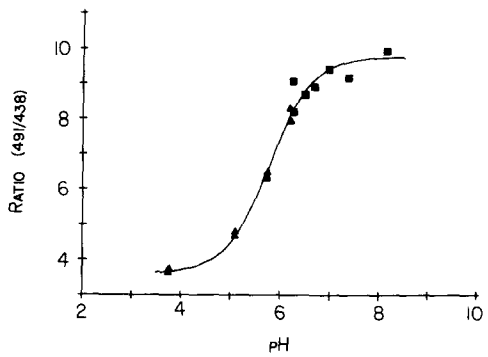

FIGURE 1 The pH dependence of the fluorescence excitation ratio as a function of pHi. *Dictyostelium* cells were cultured, loaded with fluorescein, and attached to glass slides as described in Materials and Methods. The intracellular pH was equilibrated with extracellular pH by the addition of 20 μM monensin and 0.5 μM gramicidin to the (Na-K)Pi buffer. External pH was adjusted to the indicated values by the addition of 1 M H_3PO_4 (▲) or 1 M NaOH (■) and the cells allowed to equilibrate at this pHex until the 491:438 ratio was stable (<5 min). Fresh buffer at the same pH was then added and the 491:438 ratio of intracellular fluorescein was determined as described. Each point in this figure represents the average of three ratio determinations over a 5 min period. This titration was done on three different cultures of cells and the solid line is the least squares best fit for all the data. The individual points in this figure are representative and from one experiment. The inflection point of the solid curve is at pH = 5.77 and compares with an inflection at pH = 5.90 for fluorescein in (Na-K)Pi buffer.

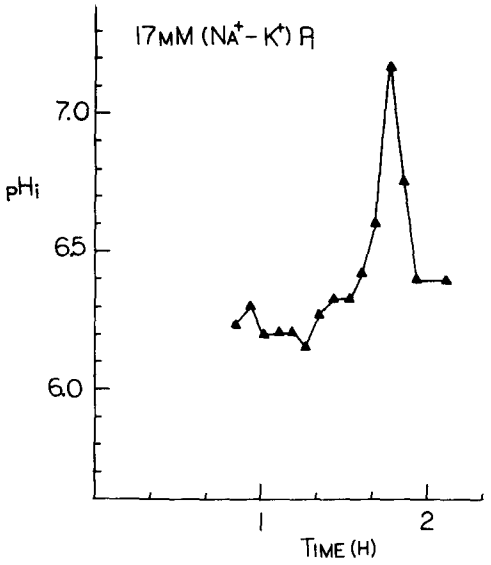

FIGURE 2 pHi of *Dictyostelium* cells during the first 2 h of differentiation. Differentiation was initiated at time 0, the cells loaded, placed on slides, and fluorescence measurements were done as described in Materials and Methods. Intracellular pH was determined from the solid curve in Fig. 1. The time of loading and the beginning of pH measurements was varied from 0–30 min to 60–90 min after the initiation of differentiation without affecting the time or the magnitude of alkalinization. This is a representative experiment, loading was from 15 to 45 min, and each pH represents the average of three ratio determinations done over 5 min. All fluorescence measurements were corrected for dye leakage from cells, which was always <5% of the total fluorescence.

is between 6.2 and 6.3 (6.23 ± 0.1). Secondly, at ~2 h after removal of cells from growth medium a transient intracellular alkalinization (+ 0.9 pH units, ± 0.3) occurs. This transient alkalinization has been observed many times in experiments

and controls and always occurs between 1.5 and 2.5 h after the onset of differentiation.

Incubation of *Dictyostelium* amebae in buffered dextrose solutions has been demonstrated by Marin and Rothman to retard the entrance of cells into their developmental program (9). Soll and co-workers have also used buffered dextrose solutions of higher concentration to induce the de-differentiation of physically dissociated aggregating amebae (13). The presence of dextrose is thought to delay amino acid depletion (13, 15). When cells were incubated, after removal from growth medium, in (Na-K)Pi buffer containing 3% dextrose no transient intracellular alkalinization is observed to occur (Fig. 3, ○). In a parallel experiment, in which cells from the same culture were incubated in (Na-K)Pi buffer alone, the typical transient alkalinization was observed (Fig. 3, ●). The reason for the slight decrease of the pHi of cells differentiating in a dextrose-containing solution is presently unknown. However other experiments in which the pHi of cells was found to be 5.8–5.9 early in differentiation suggest that some variation in pHi reflects differences between batches of growth media and/or the extent of nutrient depletion from the growth medium. These differences may also reflect different points in the cell cycle, since in the slime mold *Physarum polycephalum* (16) and in *Tetrahymena pyriformis* (17) the pHi has been observed to vary by as much as 0.6–0.7 Units during the cell cycle (16).

Several eucaryotes regulate their pHi by means of a Na^+/H^+ exchange mechanism (1–3, 5, 6, 19). If the alkalinization event observed to occur in *Dictyostelium* amebae at ~2 h of differentiation is sodium dependent, incubation of cells in a solution containing amiloride, an inhibitor of Na^+/H^+ exchange in other cells or in Na^+-free medium, may prevent the transient alkalinization. Cells were prepared for analysis as described except that amiloride (200 μM) was present continuously in buffers after the initiation of differentiation. The pHi measured was initially substantially more acidic (pHi 5.2–5.3) than that of cells differentiated and loaded with fluorescent probe in the absence of amiloride (Fig. 4, □). These cells slowly return to a pHi of 6.2–6.3 over the time course of this experiment when continuously incubated in (Na-K)Pi media containing 200 μM amiloride. It appears that intracellular hydrolysis of the fluorescein-diacetate imposes a significant acid load on the cells, decreasing the pHi. In the absence of amiloride (Figs. 2 and 3, for example) the pHi recovery is essentially completed during the washing procedure although it could be observed if washing was done rapidly (not shown). This transient acid load does not impair the ability of cells to complete differentiation with a normal time course or to multiply in nutrient medium. The presence of amiloride inhibits the recovery of cells from an acid load, as has been previously observed for the Na^+/H^+ exchange mediated recovery from acid load in mammalian cells (2, 3). To by-pass this problem a second-type of experiment was performed in which cells were allowed to recover from this acid load and then treatment with amiloride initiated. In this experiment (Fig. 4, ■) the intracellular pH never rises above 6.4 during the experiment. The slight decrease in pHi observed during the later part of this experiment is believed to represent the accumulation of metabolic acid and is more pronounced at higher amiloride concentrations (data not shown).

To test the Na^+-dependence of alkalinization, cells were prepared for analysis as described above except that all procedures were performed in Na^+-free solutions (Na^+ normally present in buffers was replaced with an equivalent amount of K^+, final concentration 19.7 mM K^+). As might be expected the pHi measured for cells prepared in this fashion were substantially more acidic (pHi = 5.3) than that of cells differ-

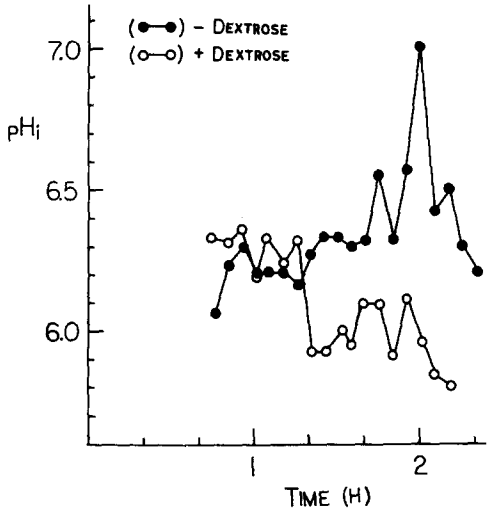

FIGURE 3 The effect of 3% dextrose on pHi during the first 2 h of differentiation. Cells were transferred to (Na-K)Pi (●) or (Na-K)Pi+3% dextrose (○) at time 0. Fluorescein loading and fluorescence measurements were done as previously described and the pH determined from the curve in Fig. 1. Each pH represents 3 fluorescence measurements taken over a 5-min period and corrected for dye leakage from the cells (<5% of the total fluorescence).

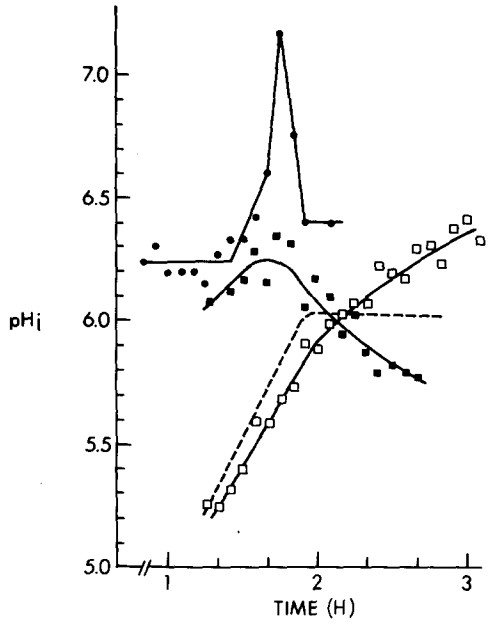

FIGURE 4 The effect of 200 uM amiloride and sodium-free media on pHi during the first 3 h of *Dictyostelium* differentiation. At time 0 the cells were transferred to: (*a*) (Na-K)Pi (● and ■); (*b*) (Na-K)Pi + 200 μM amiloride (□); or (*c*) 17 mM K^+ phosphate buffer (dashed line). The cells were then prepared for fluorescence measurements as previously described. pH determinations were all done in the same buffers except for (□ and ■) where they were done in (Na-K)Pi + 200 μM amiloride as described in Results. The data points in 17 mM K^+ phosphate buffer are not shown individually but are represented by the dashed line. pH determinations were done as described previously. Amiloride (200 μM) had no detectable effect on the emission or excitation spectra, or the total fluorescence.

entiated and loaded with fluorescent probe in the presence of Na^+ (Fig. 4, dashed line). The cells slowly recovered to a pHi of 6.1 and did not display a transient alkalinization over the period of measurement (up to 3 h of differentiation). Hence, it appears that as in the amiloride experiment, Na^+-free media (*a*) inhibits an important mechanism by which amebae regulate their pHi after an acid load; and (*b*) prevents the transient intracellular alkalinization from occurring.

Since amiloride treatment and Na^+-free media prevented the alkalinization from occurring at 2 h and low [Na^+] has been reported to delay differentiation in *Dictyostelium* (9), the effect of 200 μM amiloride on the differentiation program of *Dictyostelium* was examined by adding the drug to differentiating amebae at various times after the onset of differentiation. The results, in Fig. 5, demonstrate that amiloride slows the entry of cells into their developmental program, but only if it is present early in differentiation before the time at which the alkalinization event would be expected to occur. If

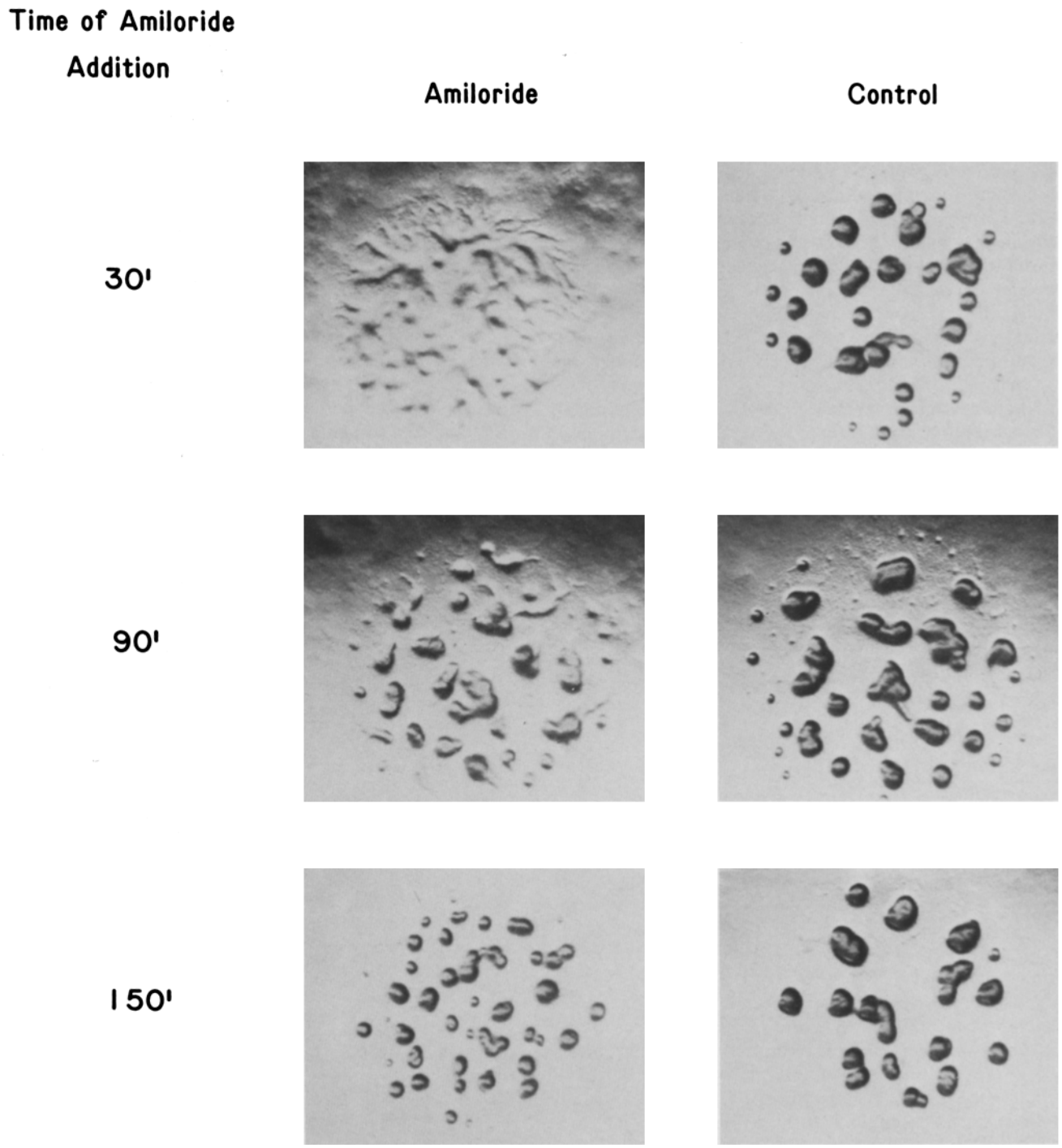

FIGURE 5 Effect of amiloride on the differentiation of *Dictyostelium* cells plated on non-nutrient agar. Differentiation was initiated by transfer to (Na-K)Pi buffer as described in Materials and Methods. At the indicated times the cells were transferred to (Na-K)Pi + 200 μM amiloride and plated onto non-nutrient agar with 200 μM amiloride included. Control cells were transferred to (Na-K)Pi buffer and plated onto non-nutrient agar at the same times. The agar plates were covered and incubated at 20°C. At 7.5 h after initiating differentiation these pictures were taken, thus the elapsed time of differentiation for all six of the plates is the same.

amiloride treatment is begun after the alkalinization event, it has no effect on differentiation. It should be noted that all six photographs in Fig. 5 were obtained at 7.5 h differentiation. Thus it appears that the effect of amiloride on differentiation is restricted to a very early interval before the alkalinization occurs, and because of this it seems that the effect of amiloride is not related to the inhibition of protein synthesis (18) or other toxic effects of this drug.

DISCUSSION

Our observations indicate that in *Dictyostelium* amebae the pHi is lower than that of other eucaryotes that have been studied (2, 3, 14) by these techniques and that there is an increase of pHi that occurs early in differentiation. The large magnitude of the observed pHi increase (ca. 0.9 pH unit) and the observation that fluorescein does not become localized in intracellular organelles during the alkalinization, indicate that this pH change is occurring in the cytoplasm. This does not exclude the possibility that other intracellular compartments may also change pH during the observed alkalinization, but a smaller intracellular compartment could not produce a pHi change of this magnitude without a major redistribution of the fluorescein, which was not observed. The amiloride sensitivity of this alkalinization indicates that its cation specificity may be similar to the Na^+/H^+ exchange processes in mammalian cells (1–4, 7, 19), sea urchins eggs (5), and sperm (6). The only anion present in our differentiation medium is phosphate and hence this phenomenon is probably not related to Cl^-/HCO_3^- exchange (20, 21). The observation that recovery from acid loading, pHi regulation, and the alkalinization at 2 h are all sensitive to Na^+-free or amiloride-containing medium suggests that a cation-dependent proton efflux participates in pHi regulation in these cells before, as well as, during the alkalinization event at 2 h. It should be noted that in our experiments, as in those of Marin and Rothman (9), Na^+ was replaced with K^+ and the increase in K^+ could influence the alkalinization as well as the reduction of Na^+. Thus the molecular basis of the alkalinization remains to be established.

Whatever the mechanism, this alkalinization is clearly the result of a major alteration in the regulation of the pHi of *Dictyostelium* cells, which occurs as part of the differentiation program of the amebae. In other systems changes in cytoplasmic pH have been related to stimulation of DNA transcription, translation, and protein synthesis (5, 7, 8, 22). In addition, in preparations from *Dictyostelium*, raising the pH from 6.0 to 7.0 has been shown to affect chemotactic receptor-cytoskeleton interaction (23), cytoplasmic gelation (24), actomyosin contraction, and actin filament cross-linking (25, 26) and signal transduction by cGMP (27, 28). It may be significant that the alkalinization event described here coincides temporally with stabilization of the developmental program such that cells are required to proceed through a process termed "erasure" to return to the vegetative state (16, 29). Many biochemical reactions are pH-dependent and it is not unreasonable to suggest that this large pHi change could serve to initiate both the metabolic and structural changes in the cell that are necessary for establishing the differentiation program.

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