Cell, Vol. 43, 653–657, December 1985 (Part 2), Copyright © 1985 by MIT 0092–8674/85/130653–05 $02.00/0

Cytoplasmic pH and the Regulation of the Dictyostelium Cell Cycle

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Summary

Cytoplasmic pH (pH_i) was monitored during the cell cycle of synchronous populations of Dictyostelium discoideum by means of a pH "null point" method. There is a cycle of pH_i that closely corresponds to the DNA replication cycle, with a minimum of pH 7.20 in interphase and a peak of pH 7.45 during S phase and mitosis. When DNA replication is blocked by hydroxyurea, pH_i continues to oscillate with a similar period as the uninhibited division cycle. Even when protein synthesis is inhibited by cycloheximide the periodic pH_i cycles persist. Artificially raising pH_i in exponentially growing cells by ~0.1 units causes a severalfold increase in the rates of protein and DNA synthesis. We conclude that an autonomous pH_i oscillator exists in Dictyostelium cells, which operates independently of protein and DNA synthesis and which may have a major role in the timing and regulation of the cell cycle.

Introduction

There is increasing evidence that cytoplasmic pH (pH_i) plays an important role in the regulation of cellular metabolism in general and in the regulation of DNA synthesis and cell proliferation in particular (Busa and Nuccitelli, 1984; Whitaker and Steinhardt, 1982). For example, a rapid and persistent rise in pH_i occurs after fertilization of echinoderm eggs (Whitaker and Steinhardt, 1982) and is commonly observed after mitogenic stimulation of quiescent mammalian cells (Schuldiner and Rozengurt, 1982; Moolenaar et al., 1983). This cytoplasmic alkalinization is a primary signal for the stimulation of protein synthesis after fertilization (Winkler et al., 1980) and is a "permissive" event for the initiation of DNA synthesis in such diverse cell types as sea urchin eggs and mammalian fibroblasts (Whitaker and Steinhardt, 1982; Pouysségur et al., 1985).

Alterations in pH_i during the cell cycle have been observed in a few different cell types. Thus, in the slime mold Physarum polycephalum pH_i rises rapidly just before mitosis (Gerson and Burton, 1977), whereas in the protozoan Tetrahymena there are two alkaline pH_i transients per cell cycle (Gillies and Deamer, 1979). A transient alkalinization has also been observed in synchronous cultures of yeast (Gillies et al., 1981). Taken together, these findings have raised the possibility that pH_i has a regulatory role in the mitotic cycle of eukaryotic cells.

This study was undertaken to determine the dynamic behavior of pH_i during the cell cycle of the cellular slime mold Dictyostelium discoideum and to evaluate the dependence of protein synthesis and DNA replication on pH_i. Dictyostelium has been widely used for studying the mechanisms of cellular differentiation; recent evidence suggests that pH_i may participate in the control of the developmental program of this organism (Gross et al., 1983; Jamieson et al., 1984). However, as yet, no measurements of pH_i during synchronous growth of undifferentiated Dictyostelium cells have been obtained. Here we report a striking alkaline shift in pH_i of about 0.25 units per division cycle which peaks during S phase and mitosis. Surprisingly, when protein synthesis and DNA replication are inhibited, these pH_i oscillations continue. Furthermore, we present evidence suggesting that both protein synthesis and DNA replication in Dictyostelium are extremely sensitive to pH_i. Our results indicate that there is an autonomous pH cycle in the cytoplasm of Dictyostelium cells and they suggest that this pH_i oscillator may function as a regulator of the mitotic cycle.

Results

Measurement of pH_i during the Cell Cycle

Figure 1 shows typical pH recordings from experiments in which addition of digitonin selectively disrupts the plasma membrane of Dictyostelium cells suspended in weakly buffered KH_2PO_4 solutions of different pH. It has been shown that digitonin treatment of Dictyostelium cells leaves the membranes of endoplasmic reticulum and mitochondria essentially intact (Favard-Séréno et al., 1981). Furthermore, we observed that neutral-red-stained vacuoles in Dictyostelium cells do not change their color after digitonin addition (not shown), indicating that digitonin does not disrupt these acidic organelles. Thus, the pH null point, at which the digitonin-induced pH shift is virtually zero, can be considered as a reliable estimate of the average cytoplasmic pH value of the cell population.

Figure 2 shows synchrony data from a typical experiment, in which Dictyostelium cells were synchronized by resuspending stationary phase cells into fresh medium. As illustrated, the cells show regular stepwise increases in cell number, DNA, and protein content. Previous cell cycle analysis (Weijer et al., 1984) has shown that the steps in DNA content are due to nuclear DNA synthesis. The division cycle in Dictyostelium consists primarily of a short S phase and a prolonged G_2 phase, with hardly any G_1 phase (Durston et al., 1984; Weijer et al., 1984). In the following figures DNA and protein data are plotted as fractional increases in DNA and protein content per hour.

Figure 3 illustrates the average pH_i as a function of time during three successive cell cycles of synchronous populations of Dictyostelium. Also illustrated are the cell-cycle-dependent changes in cell number and in the rate of increase in DNA and protein content. It is seen that there is a pronounced variation in pH_i which appears to be correlated with the stage of the cell cycle. The pH_i

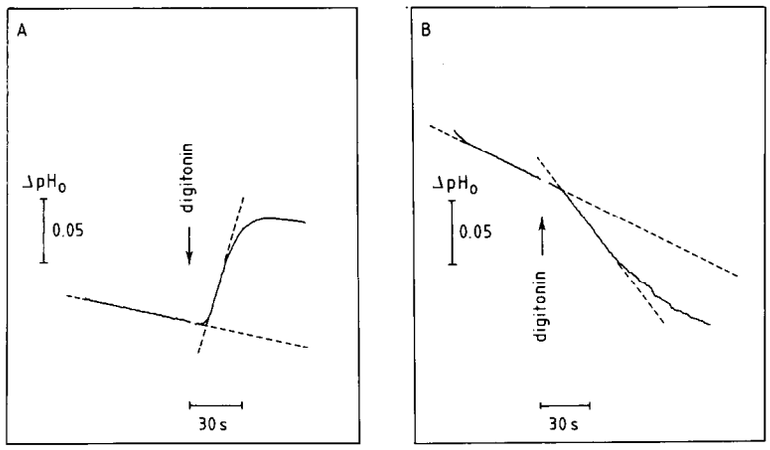

Figure 1. Determination of pH_i by the Digitonin Null Point Method

Changes in extracellular pH induced by addition of digitonin (0.1% w/v) to Dictyostelium cells suspended in weakly buffered media at $pH_0 < pH_i$ (A) and $pH_0 > pH_i$ (B), respectively. The extracellular pH value at which digitonin induces no apparent H^+ movement is taken as an estimate of pH_i. In (A), pH_0 was 7.16 and pH_i was estimated at 7.42. In (B), pH_0 was 7.50 and pH_i was estimated at 7.33.

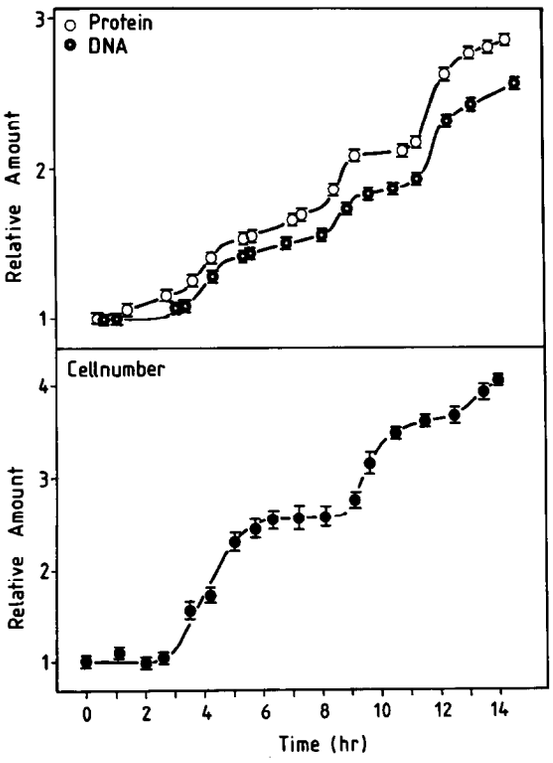

Figure 2. Relative Changes in Cell Number, Total DNA, and Total Protein during Three Successive Cell Cycles

At time zero synchronous cell divisions were induced by reinoculation of stationary phase cells into fresh medium. DNA was determined in quadruplicate, protein in fivefold, and cell numbers in triplicate as described in Experimental Procedures; means ± SE are shown.

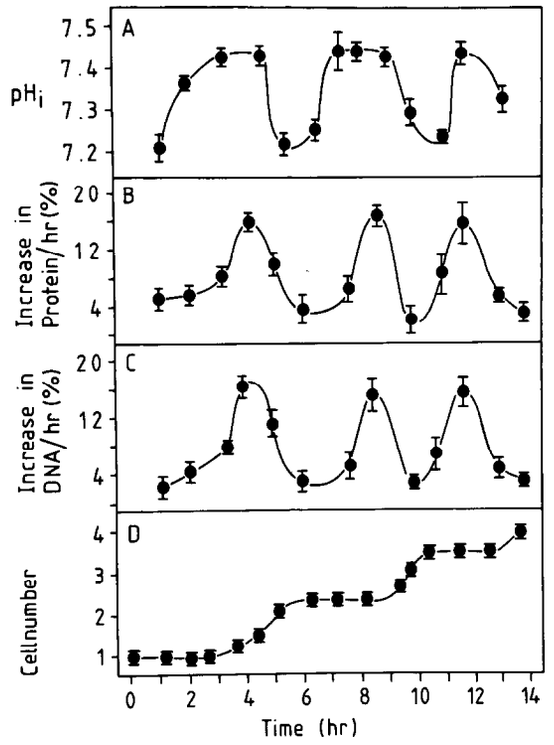

Figure 3. Changes in pH_i, Protein Synthesis, DNA Synthesis, and Cell Number during Three Successive Cell Cycles

For experimental details see text and legend to Figure 2. Data are pooled from three individual synchronization experiments; means ± SE are shown.

(A) pH_i as measured by the digitonin null point method.

(B) Fractional increase in total cellular protein in the same batches of cells as in (A).

(C) Fractional increase in cellular DNA content in the same batches of cells as in (A).

(D) Relative increase in cell number.

rises rapidly by more than 0.2 units just before the onset of protein and DNA synthesis and reaches a plateau near 7.45, which is maintained during S phase and the onset of mitosis. During (or just after) cell division pH_i declines rapidly to its minimum value of about 7.20. Shortly thereafter, a new alkaline pH_i shift is initiated.

We also used the frozen–thawed cell lysate approach for measuring cyclic changes in pH_i. Although the disruption of intracellular organelles and continued metabolic acid production make it impossible to calculate the pH of the bulk cytoplasm by this method, the cell-cycle-dependent intracellular alkalinizations of about 0.2 pH units can still be detected (Figure 4).

Effects of Hydroxyurea and Cycloheximide

After treatment of synchronous Dictyostelium populations with hydroxyurea, a specific inhibitor of DNA replication (Timson, 1975), there is no longer any sign of DNA synthe-

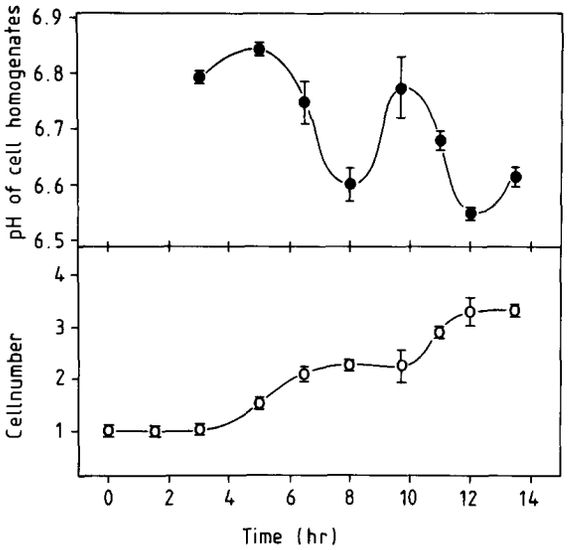

Figure 4. Changes in pH_i during the Cell Cycle as Measured by the Frozen-Thawed Homogenate Method

Cells were synchronized and the pH of frozen-thawed cell pellets was measured in triplicate as described under Experimental Procedures. (Means ± SE are shown). For experimental details see legend to Figure 2.

sis and the rate of protein synthesis is strongly depressed (Figure 5A). However, the periodic pH_i cycles are still observed. As is apparent from Figure 5A, the pH_i oscillations take place at intervals timed closely to the DNA replication cycle in uninhibited cells. It is also clear that in hydroxyurea-arrested cells the mean pH_i exhibits a slow drift upward (~0.03 units/hr), but that the amplitude of the pH_i oscillations is still about 0.25 pH units.

A similar phenomenon is observed when synchronous cells are treated with the protein synthesis inhibitor cycloheximide (Figure 5B). Despite the fact that protein synthesis and DNA replication are fully blocked, the cyclic pH_i variations persist with an amplitude of 0.25 units and a period of 4–5 hr. Again, the mean pH_i shows an alkaline drift of roughly 0.03 pH units per hour, as if the cells somehow accumulate base when the normal cell cycle is interrupted.

Artificially Raising pH_i Stimulates Protein and DNA Synthesis

The finding that pH_i begins to increase just before the onset of protein synthesis and DNA replication during the cell cycle, while the pH_i cycle itself occurs autonomously, suggests that pH_i could function as a primary regulator of protein and DNA synthesis. To test this hypothesis pH_i was artificially changed in exponentially growing, asynchronous populations of Dictyostelium cells and the resulting changes in the rates of protein and DNA synthesis were determined.

The pH_i of Dictyostelium cells was raised by addition of ammonia, while pH_i was lowered by incubation in a buffer containing the metabolically inert weak acid DMO. About 1 hr later we measured pH_i and the rates of protein and DNA synthesis relative to untreated control cultures. Figure 6 summarizes the results. The upper part of Figure 6 shows that pH_i is changed significantly when Dictyostelium cells are exposed to either ammonia or DMO for 45 min. The effects on protein and DNA synthesis are illustrated in the lower part of Figure 6. It is seen that an increase in pH_i from ~7.3 to ~7.4 dramatically increases the rates of protein synthesis and DNA replication. Below

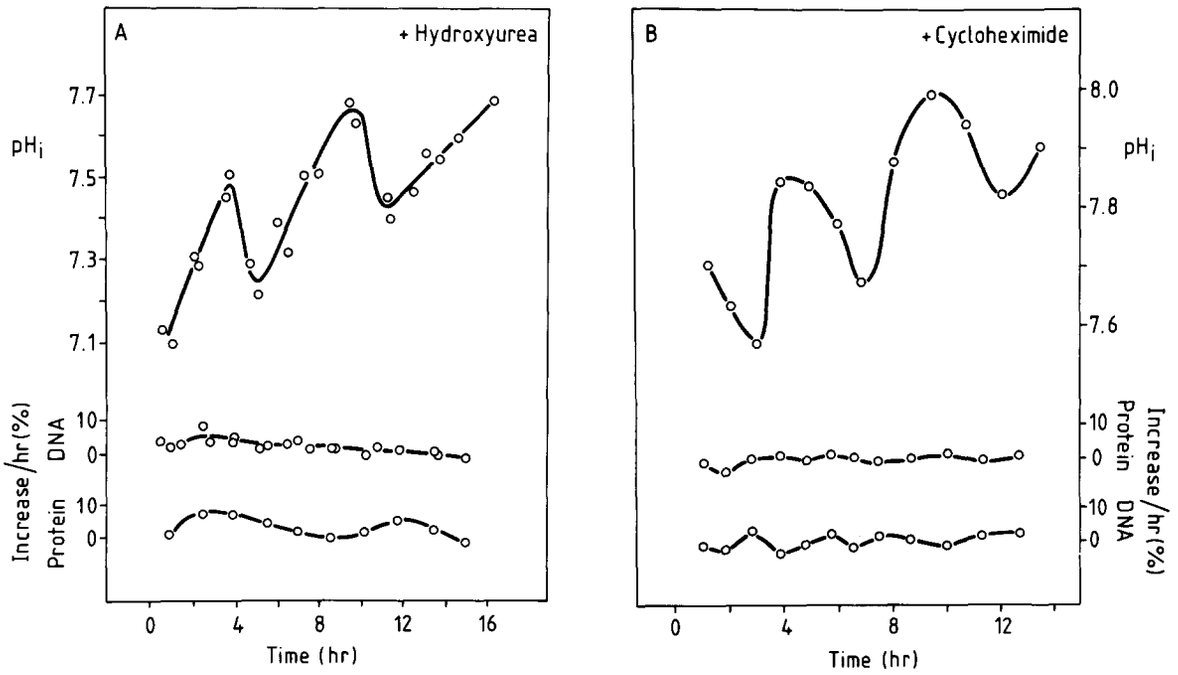

Figure 5. Changes in pH_i of Synchronized Cells with Inhibited Protein and DNA Synthesis

Experimental conditions as in Figure 2 except for the presence of 1 mM hydroxyurea to block DNA synthesis (A) or 0.75 mM cycloheximide to inhibit protein synthesis (B).

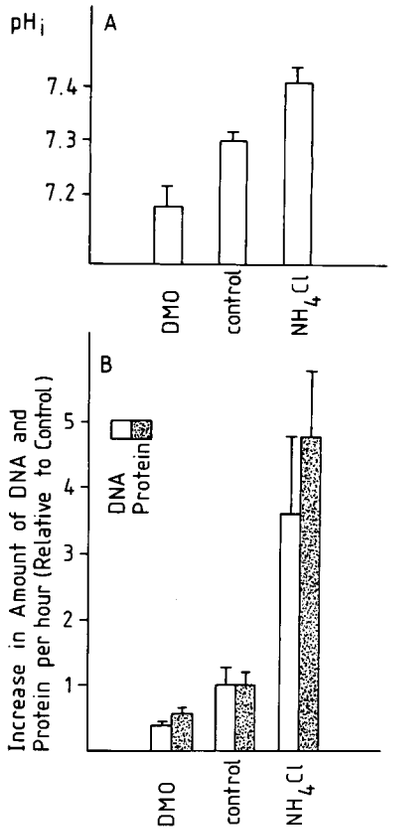

Figure 6. Changes in Protein and DNA Synthesis Induced by Artificial Manipulation of pH_i

(A) Changes in pH_i (measured by the digitonin null point method) after treatment of cells with DMO (10 mM; external pH ~6.6) or NH_4Cl (3 mM; external pH ~7.4) for a period of 45 min. Altering the external pH in itself had no effect on pH_i (not shown).

(B) Increases (induced by NH_4Cl) and decreases (induced by DMO) in the rate of protein and DNA accumulation as compared to untreated control cells.

pH_i 7.3, protein and DNA synthesis are virtually shut off. It seems therefore that the rates of protein and DNA synthesis can be turned on by a relatively small pH_i increase. From these results we conclude that pH_i is a major determinant of the rates of protein and DNA synthesis in Dictyostelium cells.

Discussion

pH_i Oscillations during the Cell Cycle

We have shown that pH_i oscillates during the Dictyostelium cell cycle with the same period as the DNA replication cycle. The rise in pH_i begins just prior to increased protein and DNA synthesis, whereas pH_i rapidly drops during or shortly after cell division. Although the pH_i regulating mechanism in Dictyostelium is not known, there is evidence that the Dictyostelium plasma membrane contains an ATP-dependent proton pump (Poggevon Strandman et al., 1984). It is an attractive hypothesis that the oscillations observed during the Dictyostelium cell cycle are due to periodic alterations in the activity of the plasma membrane H^+ pump.

Autonomy of the pH_i Oscillations

Surprisingly, the cyclic pH_i changes are autonomous in that they continue when protein synthesis and DNA replication are fully blocked. There is some precedent for autonomous oscillations in cellular function when the cell cycle is inhibited. For example, activated Amphibian eggs and enucleated Amphibian zygotes show periodic contraction waves as well as oscillations in maturation promoting factor (MPF). These continue with the same period as the cell cycle in the absence of DNA synthesis (Hara et al., 1980; Newport and Kirschner, 1984). Furthermore, there have been reports of periodic cycles of protein synthesis (Mano, 1970) and Ca^{2+}-ATPase activity (Petzelt, 1976) in nondividing but activated echinoderm eggs. It thus appears that the Dictyostelium pH_i oscillator belongs to a class of basic cell cycle timing mechanisms ("clocks") that reside in the cytoplasm or plasma membrane of certain cell types. As will be discussed below, there is suggestive evidence that the Dictyostelium pH_i clock actually does time the cell cycle, by accelerating protein and DNA synthesis. The only other case where there is any direct evidence for a regulatory function is the MPF clock in Amphibia (Newport and Kirschner, 1984). Unlike the MPF clock, however, the Dictyostelium pH_i clock is independent of protein synthesis.

Dictyostelium is not unique in exhibiting cell-cycle-dependent pH_i variations. Physarum, Tetrahymena, yeast, and mouse lymphocytes also show a fairly close correlation between the timing of a rise in pH_i and the time of increased DNA synthesis and/or mitosis (for review see Busa and Nuccitelli, 1984). However, whether the pH_i oscillations in these cell types also occur independently of protein synthesis and DNA replication is as yet unknown.

Cell Cycle Regulation via pH_i

The results from manipulating the pH_i of Dictyostelium cells with ammonia or DMO (see Figure 6) suggest that a relatively modest shift in pH_i is sufficient to turn the steps leading to increased protein synthesis and to DNA replication on or off. In this respect it is interesting to note that the rise in pH_i occurring after fertilization of sea urchin eggs is sufficient, by itself, to stimulate protein synthesis (Grainger et al., 1979; Whitaker and Steinhardt, 1982), while cytokinesis in this system has an absolute requirement for an elevated pH_i (Dubé et al., 1985).

Taken together, our findings are all consistent with the idea that the autonomous pH_i oscillator has an on–off triggering function in the timing and the regulation of the normal mitotic cycle of Dictyostelium discoideum. There can be little doubt that further investigations on the molecular nature of the pH_i oscillator will lead to a better understanding of the timing and regulation of the cell cycle in eukaryotic cells.

Experimental Procedures

Cell Culture, Synchronization, Protein Determination, and DNA Staining

Cells of D. discoideum (strain Ax-2) were cultured axenically in suspension culture in HL-5 medium at 22°C as described previously (Watts and Ashworth, 1970). Under these conditions the cells grow exponen-

tially up to about 5 × 10^6 cells/ml. Growth then slows down and finally stops at 1–2 × 10^7 cells/ml ("stationary phase"). Synchronous cultures were obtained by resuspending stationary phase cells into fresh medium at a density of about 1 × 10^6 cells/ml (Soll et al., 1976). Optimal synchrony was obtained with cells that had been in stationary phase for a period of 10–20 hr. Cells were counted in a hemocytometer.

The DNA content of the cells was determined fluorimetrically using the DNA-staining dye Hoechst 33258 as described previously (Weijer et al., 1984). Stained cell lysates were excited at 356 nm and fluorescence emission was measured at 458 nm using a Perkin-Elmer model 3000 spectrofluorimeter. The relative rate of increase in DNA content is measured as the increase in the amount of DNA (μg) per hour per μg of DNA.

The total protein content of Dictyostelium cells was determined by homogenizing cell samples in a solution containing Coomassie Brilliant Blue G-250 and measuring the absorbance at 595 nm with bovine serum albumin as a standard. The relative rate of increase in protein content is expressed as the increase in the amount of protein (μg) per hour per μg of cellular protein.

Null Point Determination of Intracellular pH (pH_i)

Initial attempts to measure pH_i in Dictyostelium amebae using bis-(carboxy-ethyl)-carboxyfluorescein as an intracellular pH-sensitive indicator (Rink et al., 1982; Moolenaar et al., 1983) were unsuccessful. We therefore determined pH_i by a null point method using digitonin to permeabilize the plasma membrane (Rink et al., 1982).

Cells were harvested by low speed centrifugation and, after washing, resuspended into 0.35 ml of a weakly buffered potassium phosphate solution (2 mM) at a density of 1 × 10^8 cells/ml. The cell suspension was continuously stirred and its temperature was maintained at 22°C. The external pH(pH_o) was continuously monitored using a mini pH electrode, coupled to a pH meter and a pen recorder. pH_o was adjusted to the required value by aliquots of KOH or H_3PO_4. Digitonin (0.1%, w/v) was then added to the cell suspension to permeabilize the plasma membrane. The pH_o at which digitonin permeabilization induced no apparent shift in pH_o was taken as an estimate of pH_i. A correction for the background acidification rate (mainly due to stirring in of CO_2) was made in all experiments.

pH of Frozen-Thawed Cell Lysates

About 2 × 10^8 cells were rapidly washed twice by low speed centrifugation. The washed pellet was then rapidly frozen in liquid N_2. Frozen cells were thawed out in a 30°C water bath for 2 min and the pH of the resulting cell lysate was measured at 22°C.

Artificial Manipulation of pH_i

Exponentially growing cells were incubated in media containing either DMO (to lower pH_i) or NH_4Cl (to raise pH_i) for a period of 45 min. Cells were then washed twice in DMO- or NH_4-containing potassium phosphate solution and the pH_i was measured immediately thereafter by means of the null point method. It should be noted that Dictyostelium cells, unlike many animal cells (e.g. Moolenaar et al., 1983), do not respond to NH_4^+ washout by an acute fall in pH_i. This suggests that NH_4^+ entry during ammonia treatment is negligible in Dictyostelium cells.

Acknowledgments

We thank Ans Timmermans for excellent technical assistance and S. W. de Laat for reading the manuscript. W. H. M. was supported by a grant from the Netherlands Cancer Foundation (Koningin Wilhelmina Fonds).

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Received July 17, 1985; revised September 20, 1985

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